BIODIVERSITY DYNAMICS AND MONITORING DURING RAPID ENVIRONMENTAL CHANGE

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To my family

without whose love, care, and patience,

I could not complete the journey.

I am forever grateful.

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ABSTRACT

Understanding how biodiversity is altered by rapid environmental change contributes to fundamental biological knowledge and can inform applied management of biological resources and threats. Here, I conduct empirical experiments on natural communities of bacteria to observe how taxonomic and genetic diversities change after abruptly experiencing severe environmental shifts. Community recovery after disturbance is relevant to both ecosystem and human health, and predicting such responses requires a mechanistic understanding of community assembly processes. Human skin piercings may be a useful model since the practice involves environmental disturbance (local sterilization) and the introduction of a novel ecological niche (physical epidermal penetration and occlusion caused by the introduction of a foreign metal object). Humans have been piercing their skin for thousands of years across many diverse cultures, yet how piercings affect local skin bacteria is unknown. In my exploration of the human piercing microbiome, I demonstrate that ear piercings induce significant ecological shifts that yield more diverse, complex, and deterministic communities.

Within communities, individual species are also capable of responding to rapid environmental stress through evolutionary adaptation and genetic drift. These processes of species sorting and microevolution are neither mutually exclusive nor independent, and historical exposure to stressors may pre-adapt communities to greater stress in the future. By manipulating pre-exposure of natural lake bacterial communities to severe acidification, I present evidence that species composition transforms in tandem with significant changes in allele frequencies, giving pre-exposed communities a ‘head start’ in their evolutionary trajectory.

My experiments with human skin piercing microbiomes and natural lake bacterial communities contribute novel insights into fundamental ecological and evolutionary processes,

but this type of biodiversity science also has practical applications beyond basic research. Crucial to our ability to apply such basic knowledge to real world problems facing biodiversity is accurate and sensitive monitoring of biological communities. An increasing number of species and transgenes are now used in commercially available genetically modified (GM) animals. Intentional release and unintentional escape of GM animals may have significant ecological and evolutionary impacts for natural populations through over competition and hybridization, highlighting the need for effective detection methods that can distinguish them from their natural counterparts. I provide proof-of-concept that artificial transgenes can be extracted and sequenced from non-invasively collected aquatic and terrestrial environmental DNA (eDNA) of GM vertebrates and invertebrates, which may serve as an essential tracking tool for GM animals in the future. Together, this research provides insight into the intrinsic and extrinsic factors affecting biodiversity at community and genetic levels during rapid environmental change, and it demonstrates a powerful new method for monitoring the impacts of GM animals on biological communities via eDNA.

RÉSUMÉ

Comprendre comment la biodiversité est altérée par des changements environnementaux rapides contribue aux connaissances fondamentales et à la gestion appliquée des ressources et des menaces biologiques. Dans la présente thèse, je décris des expériences empiriques sur des communautés naturelles de bactéries visant à observer comment la diversité taxonomique et la diversité génétique changent après avoir subi des changements environnementaux rapides et forts. Le rétablissement des communautés après une perturbation est important à la fois pour la santé des écosystèmes et pour la santé humaine, et la prévision de ces réponses nécessite une compréhension des mécanismes par lesquelles les communautés s'assemblent. Les piercings sur la peau humaine peuvent ainsi fournir un modèle utile, car cette pratique implique une perturbation de l'environnement (stérilisation locale) et l'introduction d'une nouvelle niche écologique (pénétration physique de l'épiderme et occlusion causée par l'introduction d'un objet métallique étranger). Les êtres humains de diverses cultures se percent la peau depuis des milliers d'années, mais on ne sait pas comment les piercings affectent les bactéries cutanées locales. Dans mon exploration du microbiome des piercings humains, je démontre que les piercings d'oreille induisent des changements écologiques significatifs qui donnent lieu à des communautés plus diverses, plus complexes et plus déterministes.

Au sein d'une communauté, les espèces individuelles sont également capables de répondre à un stress environnemental rapide par l'adaptation évolutive et la dérive génétique. Ces processus de tri des espèces et de microévolution ne sont ni mutuellement exclusifs ni indépendants, et l'exposition historique à des facteurs de stress peut préadapter les communautés à un stress plus important à l'avenir. En manipulant la pré-exposition de communautés bactériennes de lacs naturels à une acidification sévère, je présente des preuves que la

composition des espèces se transforme en tandem avec les fréquences alléliques, donnant aux communautés pré-exposées une « longueur d'avance » dans leur trajectoire évolutive.

Mes expériences sur les microbiomes des piercings humains et sur les communautés bactériennes des lacs naturels apportent de nouvelles connaissances sur les processus écologiques et évolutifs de base, mais elles suggèrent aussi des applications pratiques qui vont au-delà de la recherche fondamentale. Pour appliquer de telles connaissances aux problèmes concrets concernant la biodiversité, il est crucial de surveiller les communautés biologiques avec précision et finesse. Un nombre croissant d'espèces et de transgènes sont désormais utilisés dans le commerce d'animaux génétiquement modifiés. La dissémination intentionnelle et la fuite involontaire de ces animaux peuvent avoir des incidences écologiques et évolutives importantes sur les populations naturelles en raison de la compétition et de l'hybridation, ce qui souligne la nécessité de disposer de méthodes de détection efficaces permettant de les distinguer de leurs homologues naturels. J'apporte la preuve de concept que des transgènes artificiels peuvent être extraits et séquencés à partir de l'ADN environnemental (ADNe), aquatique et terrestre, de vertébrés et d'invertébrés génétiquement modifiés, collecté de manière non invasive, ce qui pourrait constituer un outil de suivi essentiel pour les animaux génétiquement modifiés à l'avenir. Ainsi, mes travaux dans leur ensemble permettent de mieux comprendre les facteurs intrinsèques et extrinsèques qui affectent la biodiversité au niveau des communautés et des gènes lors d'un changement environnemental rapide, et apportent la démonstration d'une nouvelle méthode pour surveiller les impacts des animaux génétiquement modifiés sur les communautés biologiques par le biais de l'ADN environnemental.

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PREFACE

Contribution to original knowledge

In this thesis, I investigated biodiversity dynamics in response to environmental disturbances. In doing so, I propose a novel study system and monitoring method. Here, original contributions to scientific knowledge of each chapter are highlighted.

Chapter 1.

I characterized the unexplored human piercing microbiome for the first time by recruiting volunteers from a Montreal tattoo parlor. I discovered that piercings are novel microhabitats that alter the community composition of skin microbiomes, thereby representing a form of ecosystem engineering on the human body. I show that despite the sudden disturbance of local skin sterilization prior to piercings, piercing microbiome communities become increasingly diverse, ecologically complex, and deterministic over time. Thus, I demonstrate the utility of human skin piercings as a model system for studying community assembly processes after rapid environmental change. This work is currently under peer review for publication in *Proceedings of the Royal Society B.*

Chapter 2.

I investigated the impact of pre-exposure to sublethal pulse acidification on the response of natural lake bacteria communities to severe press acidification at an ecological scale using replicated aquatic mesocosms. I found that pre-exposure can improve resistance but not resilience to changes in community composition. Using cutting-edge metagenomic methods and deep whole-genome shotgun (WGS) sequencing, I also show that pre-exposure can protect

against declines in genetic diversity across species and result in independent evolutionary trajectories, thereby serving as a potential driver for pre-adaptation. This work suggests that both ecological species sorting and evolutionary adaptation serve important mechanistic roles in how pre-exposure can protect biodiversity as well as towards community responses to environmental stress in general. This work is currently being prepared for submission to *Proceedings of the National Academy of Sciences*.

Chapter 3.

I provide proof-of-concept for using environmental DNA (eDNA) methods to track genetically modified (GM) animals by detecting shed transgenes in their environment. I demonstrate the utility of this approach using a variety of terrestrial and aquatic eDNA samples collected from invertebrate and vertebrate systems. The production of GM animals is expected to increase globally along with the risk of negative consequences from both intentional and unintentional releases into the wild. I present eDNA as an accurate, sensitive, and cost-effective monitoring method for GM animals and their transgenes, which may help mitigate the potential ecological and evolutionary impact of GM animals on natural biodiversity. This work was published in *PLOS ONE* on August 26, 2021 (DOI: 10.1371/journal.pone.0249439).

Contribution of authors

All chapters of this thesis represent original research that I have led. Each chapter has been or will be submitted for publication as a first-author manuscript in peer-reviewed scientific journals. Contributor Role Taxonomy (CRediT) of co-authors are detailed below by chapter (Brand et al. 2015).

Chapter 1.

Charles C.Y. Xu: conceptualization, methodology, validation, formal analysis, investigation, data curation, writing – original draft, writing – review & editing, visualization, supervision, project administration

Juliette Lemoine: validation, investigation, data curation

Avery Albert: methodology, validation, data curation

Élise Mac Whirter: methodology, validation, investigation, resources, supervision

Rowan D.H. Barrett: conceptualization, methodology, resources, data curation, writing – review & editing, supervision, project administration, funding acquisition

Chapter 2.

Charles C.Y. Xu: conceptualization, methodology, validation, formal analysis, investigation, data curation, writing – original draft, writing – review & editing, visualization, supervision, project administration, funding acquisition

Vincent Fugère: conceptualization, methodology, validation, investigation, resources, data curation, supervision, project administration, funding acquisition

Naíla Barbosa da Costa: conceptualization, methodology, validation, investigation, resources, data curation, supervision, project administration

Beatrix Beisner: conceptualization, methodology, supervision, project administration, funding acquisition

Graham Bell: conceptualization, methodology, validation, data curation, supervision, project administration, funding acquisition

Melania E. Cristescu: conceptualization, methodology, resources, supervision, project administration, funding acquisition

Gregor F. Fussmann: conceptualization, methodology, resources, supervision, project administration, funding acquisition

Andrew Gonzalez: conceptualization, methodology, resources, supervision, project administration, funding acquisition

Jesse B. Shapiro: conceptualization, methodology, validation, resources, supervision, project management, funding acquisition

Rowan D.H. Barrett: conceptualization, methodology, validation, resources, writing – review & editing, supervision, project administration, funding acquisition

Chapter 3.

Charles C.Y. Xu: conceptualization, methodology, validation, formal analysis, investigation, data curation, writing – original draft, writing – review & editing, visualization, project administration

Claire Ramsay: methodology, validation, resources, writing – review & editing

Mitra Cowan: conceptualization, methodology, validation, resources, writing – review & editing, project administration

Mehrnoush Dehghani: visualization

Paul Lasko: methodology, resources, writing – review & editing, supervision, project administration

Rowan D.H. Barrett: conceptualization, methodology, resources, writing – review & editing, supervision, project administration, funding acquisition

Figures and tables

Introduction

Chapter 1

Chapter 2

Abbreviations

ABI	Applied Biosystems
AFLP	Amplified fragment length polymorphism
ahcY	Adenosylhomocysteinase
ANI	Average nucleotide identity
ASV	Amplicon sequence variant
attB	Attachment site in the bacterial genome
attP	Attachment site in the phage genome
BLAST	Basic Local Alignment Search Tool
βNRI	Beta net relatedness index
βMNTD	Beta mean nearest taxon distance
bp	Base pair
C	Celsius
CBD	Convention on Biological Diversity
COG	Clusters of Orthologous Genes
COI	Cytochrome c oxidase subunit I
CRediT	Contributor role taxonomy
CRISPR	Clustered regularly interspaced short palindromic repeats
ctaD	Cytochrome c oxidase subunit I
cynS	Cyanase
cytB	Cytochrome b
ddPCR	Digital droplet PCR
DNA	Deoxyribonucleic acid
dnaE	DNA polymerase III subunit alpha
dN/dS	Non-synonymous to synonymous substitution
EDI	Equity, diversity, and inclusion
eDNA	Environmental DNA
eGFP	Enhanced green fluorescent protein
FtsK	Filament temperature sensitive mutant K
g	Gram
Gb	Gigabase
FQRNT	Fond Québécois de la Recherche – Nature et Technologies
F_{ST}	Fixation index
GenBank	Genetic Sequence Data Bank
GM	Genetically modified
GMO	Genetically modified organism
GRIL	Groupe de Recherche Interuniversitaire en Limnologie
GTDB	Genome Taxonomy Database
HGP	Human Genome Project
HGT	Horizontal gene transfer
HMP	Human Microbiome Project
HPC	High performance computing
HTS	High-throughput
IDH	Intermediate disturbance hypothesis

iCAMP	Phylogenetic-bin-based null model analysis
iNAP	Integrated Network Analysis Pipeline
ISC	Inclusive science communication
ITS	Internal transcribed spacer
JAX	The Jackson Laboratory
Kb	Kilobase
KEGG	Kyoto Encyclopedia of Genes and Genomes
KOFAC	Korea Foundation for the Advancement of Science & Creativity
LEAP	Large Experimental Array of Ponds
MAG	Metagenome-assembled genome
Mb	Megabase
MENA	Molecular Ecological Network Analysis Pipeline
µg	Microgram
MICAM	McGill Integrated Core for Animal Modeling
µL	Microliter
mL	Milliliter
µM	Micromolar or micrometer
mM	Millimolar
NCBI	National Center for Biotechnology Information
ng	Nanogram
NGS	Next-generation sequencing
NSERC	Natural Sciences and Engineering Research Council of Canada
PacBio	Pacific Biosciences
PCoA	Principal coordinates analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational analysis of variance
pH	Potential of hydrogen
π	Nucleotide diversity
P/N	Positive-to-negative
pN/pS	Non-synonymous to synonymous rates of polymorphism
pNST	Phylogenetic normalized stochasticity ratio
prpB	Methylisocitrate lyase
QCBS	Quebec Centre for Biodiversity Science
qPCR	Quantitative PCR
RAC	Resource Allocation Competition
RAPD	Random amplified polymorphic DNA
R-C	Raup-Crick
REB	Research ethics board
RFLP	Restriction fragment length polymorphisms
rho	Transcription termination factor Rho
RMT	Random matrix theory
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RSV	Respiratory syncytial virus
SCG	Single-copy core gene

sdhA	Succinate dehydrogenase flavoprotein subunit
SNP	Single nucleotide polymorphism
speE	Polyamine aminopropyltransferase
SSR	Simple sequence repeat
STR	Short tandem repeat
Taq	*Thermus aquaticus*
TLR	Toll-like receptor
TSS	Total sum scaling
VNTR	Variable number tandem repeat
WGS	Whole-genome shotgun

INTRODUCTION

"In the end we will conserve only what we love,
we will love only what we understand,
and we will understand only what we are taught."

– Baba Dioum, 1968

Biodiversity and dynamics

The neologism "biodiversity" is a portmanteau of biology and diversity; first coined by Walter G. Rosen in 1986 through preparations for the National Forum on BioDiversity conference in Washington D.C. (Pietarinen and Oksanen 2004). Two years later, Eduard O. Wilson's influential book titled *Biodiversity* based on the proceedings of that conference cemented the term within the field and eventually into the common vernacular (Wilson et al. 1988). Since then, biodiversity science has emerged as a major nexus within the biological sciences; giving rise to new research fields like conservation biology, bridging scientific objectives and evidence with environmental stewardship, and catalyzing policy changes focused on sustainability (Ehrlich and Wilson 1991, Fjeldsaå and Lovett 1997, Meine et al. 2006, Morar et al. 2015).

Biodiversity refers to the totality of biological variation that exists across a spectrum of size and organization – regional landscape, community-ecosystem, population-species, and genetic – and encompasses three major attributes: composition, structure, and function (Noss 1990, Maclaurin and Sterelny 2008). Legally, biodiversity has been defined by the Convention on Biological Diversity (CBD) as "the variability among living organisms from all sources including, *inter alia*, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity within species, between species and of ecosystems" (United Nations Environment Programme 1992).

Biodiversity dynamics describe the interactions within and between levels of biodiversity, their change across different timescales, as well as their causes and effects, which ultimately characterize ecological and evolutionary processes (McKinney and Drake 2001). These dynamics exist along a stochastic-deterministic spectrum and range from genetic mutations within an individual organism to intraspecific competition between populations; from fluctuating predator-prey cycles to community turnover during ecosystem succession (Levin 2013). Interactions of these processes can form emergent properties such as the synergism of community diversity and stability, carrying capacities limited by stable equilibria, dependency effects of frequency, density, and diversity, as well as the nature of adaptive peaks, among others (Wright 1932, Barbault 1995, Rosenzweig 1995, Storch et al. 2022, Hu et al. 2022).

The importance of biodiversity and its dynamics have long been recognized, and their ecological, economic, and cultural roles continue to be active areas of research (Council et al. 1999, Evers et al. 2018, Paul et al. 2020, Manfredo et al. 2021). Despite these valuable contributions, the world faces unprecedented rates of biodiversity loss, which has been mainly attributed to the frequency and intensity of anthropogenic changes to the natural environment (Butchart et al. 2010, Dirzo et al. 2014, Newbold et al. 2015). Understanding how biodiversity is shaped and maintained is thus not just an endeavor for fundamental biological knowledge, but it also serves to improve our ability to mitigate the effects of environmental change on biodiversity goods and services (Martin et al. 2019, Weiskopf et al. 2020, Heino et al. 2021, Greenop et al. 2021).

Biodiversity value

Humanity directly relies on the living world for survival through demand for food, medicine, and materials. Biological communities also provide essential ecosystem services such as conversion of carbon dioxide into oxygen, serving as sinks for key elements (like carbon, nitrogen, and phosphorus), pollination, flood regulation, erosion prevention, and providing recreational spaces (Costanza et al. 1997). Specific mechanisms through which biodiversity improves ecosystem stability have been elucidated (Yachi and Loreau 1999, Loreau et al. 2003, 2021). These are only known benefits from known biodiversity. Novel instrumental values are discovered regularly (*e.g.*, biopharmaceuticals and biomaterials) and much of the biological world remains undescribed (Stork 1993).

A fundamental utilitarian argument for the conservation of biodiversity is that variety begets opportunity as often repeated by the traditional adage of "hedging your bet". In fact, this was explicitly acknowledged in the 1980 World Conservation Strategy that served as a landmark document guiding international sustainable development where the preservation of biodiversity (specifically genetic diversity) was considered "both a matter of insurance and investment… to keep open future options, as a buffer against harmful environmental change" (International Union for Conservation of Nature and Natural Resources et al. 1980). Change is the unwavering constant: changing environmental conditions will create new challenges, future human societies will have novel demands, and technological progress will enable greater capacity for resource exploitation. With such uncertainty surrounding which gene or species will become useful in the future, minimizing biodiversity loss will ultimately help maximize overall benefit, particularly for future generations (Randall 1991). In a now famous analogy, the loss of biodiversity has been compared to the losing of rivets from an airplane (Ehrlich and Ehrlich 1981). Although the loss

of any single rivet is unlikely to cause critical failure, exactly how many and which rivets are keeping the plane in the air is uncertain. Similarly, the loss of any unit of biodiversity, from a single mutation to a whole biome, is unlikely to render Earth uninhabitable. However, because we don't know exactly what we can afford to lose, we can choose to invest now in the insurance of maximizing our biodiversity options.

Other than the utilitarian argument, other perspectives on why we should care about biodiversity emphasize its aesthetic values and moral obligation as a first principle. The aesthetic value of biodiversity relates to human pleasure derived from the existence of or interaction with the natural environment and other lifeforms (Tribot et al. 2018). Aesthetic perception of beauty is influenced by the cultural background of the perceiver and can have ties to intimate and powerful emotions that drive responses to certain forms of biodiversity over others (*e.g.*, intact over fragmented or colorful over dull) (Vercelloni et al. 2018, Senior et al. 2022). Moral value of biodiversity can manifest in two main ways: the moral responsibility of present generations to maintain biological resources for future generations and the idea that life is inherently precious and protection of it is an ultimate, non-reducible principle (Oksanen 1997). While the former is related to future utilitarian value as discussed previously, the latter invokes thinking beyond anthropocentrism towards "an awareness of the equal right (of all things) to live and blossom" (Naess 1973). This "deep ecology" perspective cultivates an ecological consciousness through self-realization of a mutual identity with nature and the belief in biocentric equality echoed by the Bodhisattva, "No one is saved until we are all saved" (Leopold 1966, Devall and Sessions 1985, Luke 2002, Gregory and Sabra 2008).

Environmental change

Ever since its origins, life has had to survive within its environment. Changes in environmental conditions have been the primary driver of biological diversification as evidenced by the prevalence of allopatric speciation and geographic correlatives of biodiversity patterns like latitudinal gradients (Cracraft 1985, Hillebrand 2004). Ecological opportunity through niche availability and discordance fosters adaptive diversification of species and their phenotypes by enabling persistence and divergent natural selection (Wellborn and Langerhans 2015). Ecological niches are composed of biotic and abiotic factors along with their interdependencies and can have a combination of natural and anthropogenic sources (Fath 2014). The influence of biotic factors stems from ecological interactions (*e.g.*, predation, competition, mutualism, parasitism) that affect birth and death rates, which can be modulated by abiotic factors (*e.g.*, photoperiod, soil moisture, eutrophication) and vice versa (Holt 2009).

The process of natural selection can be defined as the impact of environmental change on the survival and reproduction of species leading to heritable shifts in genotypes and phenotypes, and eventually to speciation after sufficient divergence and reproductive isolation (Darwin 1859, Fisher 1930, Dobzhansky 1937, Mayr 1963, Hood et al. 2020). Environmental change may not always impose direct selection on genes, traits, or species but can also influence other processes like migration (*e.g.*, by reducing dispersal and thus gene flow between populations through habitat fragmentation) and drift (*e.g.*, through sharp indiscriminate demographic decline that increases the likelihood of random loss of variation over time) (Hubbell 2001). The unified neutral theory of biodiversity considers all species within a trophic level equally with identical birth and death rates (Hubbell 2001). From this perspective, community composition and structure are primarily explained by neutral drift of species abundances through replacement

from the local or metacommunity parameterized by dispersal limitation (Rosindell et al. 2011). There is also evidence of direct causal links between environmental factors and mutation rates through regulation of mutator genes (Metzgar and Wills 2000, Liu and Zhang 2019).

Many characteristics of environmental change are significant to its influence on biodiversity. The magnitude or intensity of disturbance, such as the number of degrees a tidepool increases during the day or the speed of wind and amount of rain in a storm, push individuals, species, and communities beyond their normal tolerable ranges leading to decreased fitness, diversity, and functioning (Willig and Presley 2018). Duration, rate, and frequency of change matter in similar ways and combinations of environmental stress from multiple sources can have emergent properties (Walker 2011). Specific characteristics of change can yield predictable patterns such as the intermediate disturbance hypothesis (IDH). IDH suggests that species diversity, biomass, or some function of productivity is maximized when disturbance is intermediately frequent/intense or spatially patchy due to the balance between competitive exclusion by few dominant members when disturbance is weak and the demographic/diversity costs of strong disturbance (Grime 1973, Connell 1978).

Environmental disturbance can manifest through various modes including pulse, press, cycle, and ramp (Lake 2000, Arens and West 2008, Jentsch and White 2019, Burton et al. 2020). These modes differ in the previously mentioned characteristics (*e.g.* pulses are shorter in duration than presses, cycles are more frequent than pulses/presses, and ramps are slower in rate than pulses/presses) as well as in their predictability (Glasby and Underwood 1996, Smith et al. 2009, Villnäs et al. 2013). Repeated exposure to environmental stressors can confer protection against future stress by acting on phenotypes to induce homeostasis, which is known generally as priming and has been well documented in bacteria, plants and fungi (Foster and Hall 1991,

Hilker et al. 2016, Lämke and Bäurle 2017, Chang et al. 2020). Molecular mechanisms of how memory of prior stress is stored and used to anticipate/predict future environmental disturbance typically invoke epigenetic regulation of adaptive genes and are understood to represent a cost-saving strategy as compared to constitutive expression (Zangerl 2003, Tagkopoulos et al. 2008, Mitchell et al. 2009).

Ecology and evolution

The impact of environmental stress can be observed beyond phenotypic responses of organisms and populations to the level of species across communities and generations. Ecological responses of communities can be characterized by their resistance and resilience to change, which respectively refer to the capacities to minimize biodiversity loss and to recover after loss has occurred (Lake 2013). The nature of these responses shape ecological or alternative stable states of relatively persistent system parameters caused by positive and negative feedback mechanisms (Holling 1973, Beisner et al. 2003). Periods of stability can be punctuated by rapid, transformative, and potentially irreversible change once critical thresholds are exceeded at ecological tipping points where even small incremental environmental disturbances can lead to outsized biodiversity effects (Lenton 2013, Dakos et al. 2019). The ecological effects of environmental change can be observed through shifts in community composition, species distributions and interactions, and altered ecosystem functions (Walker 2011).

Complex evolutionary effects of environmental change often involve frequency-dependent processes related to both the range of viable traits as well as the magnitude and direction of selection pressures (Heino et al. 1998). For example, evolutionary traps describe situations where adaptation does not lead to fitness optima despite their existence and can even

lead to local extinction termed evolutionary suicide (Schlaepfer et al. 2002, Rankin and López-Sepulcre 2005). This may happen when the demographic costs of strong adaptive selection lead to significant increased risk of stochastic extinction (Dieckmann and Ferrière 2004). However, such runaway processes can be mediated by genetic and ecological constraints like correlated traits and lack of standing variation that slow or stop evolutionarily trapped adaptation (Matsuda and Abrams 1994). More often, adaptation is thought of as a process that improves fitness; environmental change can also lead to persistence at otherwise lethal levels of stress through evolutionary rescue (Carlson et al. 2014). This extends to community rescue when eco-evolutionary mechanisms enable community persistence despite previously lethal stress through the spread of rare, better adapted types thereby maintaining key ecological interactions preventing community collapse (Low-Décarie et al. 2015).

Diversity indices

Biodiversity is measured primarily in terms of diversity indices, of which there are many. Diversity indices differ mostly in terms of how rare occurrences are treated and can be generally categorized into alpha, beta, and gamma diversities (Simpson 1949, Whittaker 1972, Peet 1974). Alpha diversity is calculated from species richness and evenness, which is used to describe variability within a single community, space, or time (Hill 1973). Direct comparisons of alpha diversity are especially vulnerable to sampling effort so standardization through rarefaction is generally recommended (Gotelli and Colwell 2001). Beta diversity describes how communities differ in their taxonomic composition and is derived from community turnover or the numbers of shared and unique species between communities (Whittaker 1960, Wilson and Shmida 1984). Cumulative alpha diversities across habitats at the regional or landscape level refers to gamma

diversity (Whittaker 1960). Other levels of diversities have also been proposed within this hierarchical framework like delta and epsilon diversity that respectively describe diversity among and within regions, but these have been less prominently used (Whittaker et al. 2001, Tuomisto 2010).

Genetic methods

Regardless how biodiversity is quantified, measuring the impact of environmental change relies on the effectiveness of monitoring methods, which aim to capture biological data accurately and precisely so that estimated abundances and distributions honestly reflect biological reality. Traditionally, investigations of biological variation have been limited to that observable to the naked eye such as species composition of communities and phenotypic trait distributions across populations. Similar to how the invention of the microscope enabled the field of microbiology by exposing the previously unexplored realms of microorganisms, the advent of modern genetics and especially accessible DNA sequencing has been revolutionary for the study of biodiversity (Karp et al. 1997).

Genetic tools within biodiversity science have experienced rapid turnover driven by the constant development of novel technologies throughout the past century (Jorde et al. 2005). A major breakthrough was the discovery of a method to determine the nucleotide sequence of deoxyribonucleic acid (DNA) via chain elongation inhibition known today as Sanger sequencing, which continues to be widely utilized (Sanger et al. 1977). That same year, the technique was modified with radioactive phosphorus and applied to bacterial cultures to generate the sequences of 16S and 18S small subunit ribonucleic acid (RNA) (Woese and Fox 1977, Wolfe 2014). Comparison of these DNA sequences led not only to the discovery of Archaea, a new domain of

life, but also the birth of molecular systematics whereby genetic sequences are used to infer evolutionary relationships (Woese 1987, Koonin 2014).

DNA fingerprinting soon followed where variation at multiple genetic loci is simultaneously assessed, whether through restriction digest enzymes for amplified fragment length polymorphisms (AFLPs) or restriction fragment length polymorphisms (RFLPs), random polymerase chain reaction (PCR) for random amplified polymorphic DNA (RAPD), or determining the number of repeats for a variable number tandem repeat (VNTR) also known as short tandem repeats (STRs), simple sequence repeats (SSRs), or microsatellites/minisatellites (Botstein et al. 1980, Tanksley et al. 1989, Welsh and McClelland 1990, Williams et al. 1990, Hadrys et al. 1992, Vos et al. 1995, Karp et al. 1998, Mueller and Wolfenbarger 1999). These multi-locus approaches are less affected by sampling biases caused by marker choice and have been used to delineate species and populations, estimate genetic relatedness and divergences, measure rates of gene flow, calculate heterozygosity and inbreeding coefficients, construct pedigrees, and build phylogenies across diverse biological systems (Jeffreys et al. 1985, Burke and Bruford 1987, Smith et al. 1997, Ciofi et al. 1998, Eldar et al. 1999, Viaud et al. 2000, Arif and Khan 2009, Lazzi et al. 2009). While useful for answering questions that only require an assessment of population genetic structure at a coarse scale, a major weakness of these approaches is the lack of resolution and thus statistical power when marker density is limited in comparison to the sizes of the genomes being interrogated (Parker et al. 1998, Allendorf and Seeb 2000, Chambers et al. 2014). This can be especially problematic for capturing adaptive genetic variation and detecting signatures of natural selection (Sunnucks 2000, Moss et al. 2003, Schlötterer 2004, Stapley et al. 2010).

By the turn of the millennium, the completion of the Human Genome Project (HGP) heralded the genomic era, marked by a drastic drop in sequencing costs along with a massive increase in sequencing capacity (Guttmacher and Collins 2003). Today, a number of different high-throughput (HTS) or next-generation sequencing (NGS) platforms like Illumina, Ion Torrent, Pacific Biosciences (PacBio), and Oxford Nanopore are able to generate sequencing data on the order of thousands to billions of reads per sample (Reuter et al. 2015). Single nucleotide polymorphism (SNP) microarrays are a separate but related technology that is popular for rapid genotyping of known genome-wide variation (Reuter et al. 2015). Because of these technological advancements, biodiversity researchers have been able to increase marker density and sample sizes, determine the genetic basis for adaptation, and uncover whole communities at the same time directly from their environment (Tringe and Rubin 2005, Stapley et al. 2010, Ekblom and Galindo 2011, Schlötterer et al. 2015, Porter and Hajibabaei 2018).

Microbial communities

The application of sequencing directly from environmental samples was pioneered through characterizing the microbial communities associated with hydrothermal vents and a hot spring at Yellowstone National Park using autoradiography and high-resolution gel electrophoresis of 5S rRNA (Stahl et al. 1984, 1985). This work was significant because traditionally, microbes could only be catalogued or assigned taxonomies after culturing, and it is known that most microbes remain unculturable (Colwell 1997). Research focus soon moved on to the more phylogenetically useful 16S rRNA, which has become the most dominant method for genetic surveys of microbial community structure (Lane et al. 1985, Tringe and Hugenholtz 2008). Amplicon sequencing via HTS/NGS of 16S rRNA along with 18S rRNA, the internal transcribed

spacer (ITS), and cytochrome b (cytB) among others have transformed microbial ecology through discovery of microbial biodiversity (bacteria, archaea, fungi, algae, and protists) in their natural habitats across a wide range of environments including air, built environments, biofilms, sediment, soil, wastewater/sludge, fresh/marine water, and on/within host organisms (Caporaso et al. 2011, Thompson et al. 2017, Gonzalez et al. 2018).

The accumulation of these studies has caused a paradigm shift where it is now generally recognized that not only are microorganisms ubiquitous, but there are characteristic microbial communities found in reasonably well-defined habitats occupying specific ecological niches that are relevant to the function and health of their ecosystems including their hosts (Berg et al. 2020). These microbial communities have been termed "microbiome" and the recent emergence and mainstreaming of the term is evidenced in the rapid growth in literature volume within just the last decade (Fig. 1). The idea of the human microbiome was first coined "to signify the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space and have been but all ignored as determinants of health and disease" (Lederberg and McCray 2001). Early studies on human microbiomes quickly expanded the catalog of known species present in and on the human body including the gut, mouth, vagina, and skin (Hyman et al. 2005, Eckburg et al. 2005, Gao et al. 2007, Faveri et al. 2008). Such findings spurred the Human Microbiome Project (HMP), a major international collaboration to explore the biodiversity of human microbiomes (Turnbaugh et al. 2007, The NIH HMP Working Group et al. 2009, Human Microbiome Project Consortium 2012). Beyond descriptive work, longitudinal microbiome studies have also been especially useful for addressing how environmental change impacts microbial biodiversity and functioning over time and space such as how the human gut microbiome forms after birth and its relevance to host factors such as

obesity (Ley et al. 2006, Palmer et al. 2007). Through such quantitative measurements of both the size and structure of community assemblages, species abundance distributions can shed light on the drivers of diversity patterns (Magurran 2021).

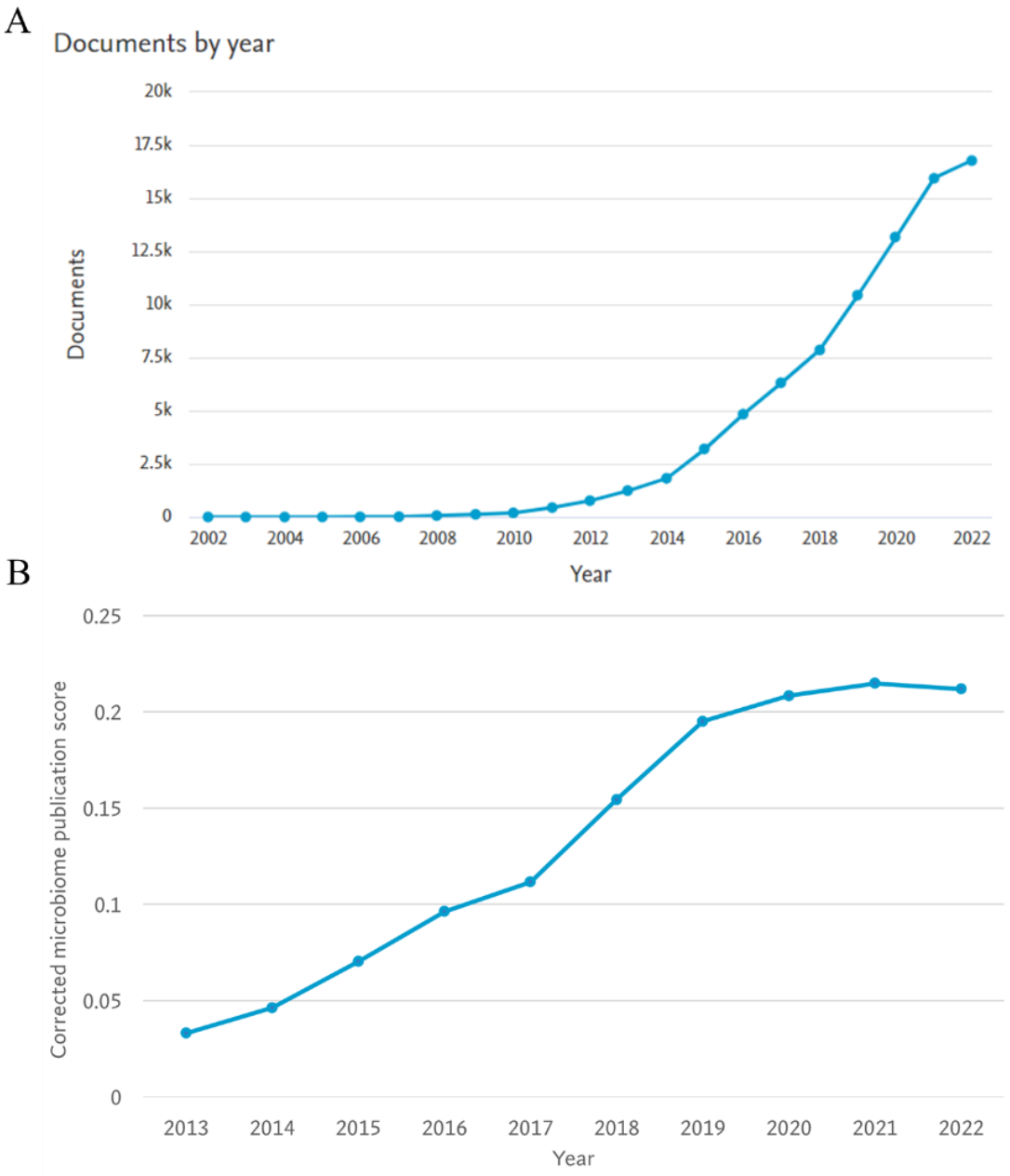

Fig. 1. Growth of microbiome literature. A) Number of documents published between 2002-2022 that mention "microbiome" in the article title, abstract, or keywords within the Scopus database (Search field: TITLE-ABS-KEY, Search date: August 12, 2023). B) Corrected

publication score between 2013-2022 to control for publication effort (number of "microbiome" publications divided by the total number of publications from the top 10 journals: *Frontiers in Microbiology*, *Scientific Reports*, *Nutrients*, *PLOS ONE*, *International Journal of Molecular Sciences*, *Frontiers in Immunology*, *Microbiome*, *Frontiers in Cellular and Infection Microbiology*, *Gut Microbes*, and *Microorganisms*)

Metagenomics

There is some confusion in the literature regarding what constitutes "metagenomics". Some include 16S rRNA amplicon sequencing and other marker-based community analyses as metagenomics based on their application to heterogeneous samples of DNA from multiple species within a community (Petrosino et al. 2009, Hamady and Knight 2009, Kim et al. 2013). The literature also refers to such methodology as "metabarcoding", which may partially explain the confusion given the identical prefixes (Bacci et al. 2015, Beckers et al. 2016, Bird et al. 2017, Orwin et al. 2018, Corse et al. 2019, Bukin et al. 2019, Casero et al. 2019, Bahram et al. 2019, Santos et al. 2020). The application of metabarcoding to microbial communities is somewhat of a misnomer because DNA metabarcoding only emerged in 2012 having evolved from DNA barcoding and was originally intended to describe HTS of environmental DNA or bulk samples of macro-organisms (Coissac et al. 2012, Taberlet et al. 2012b, 2012a, Yu et al. 2012).

While the term metagenomics continues to be used in multiple ways, generally, it describes the untargeted, culture-independent, direct whole-genome shotgun (WGS) sequencing of total DNA from environmental samples, typically of microscopic origins due to the more manageable genome sizes of prokaryotes and archaea (Hugenholtz 2002, Hugenholtz and Tyson 2008, Wooley et al. 2010). Metagenomics goes beyond community surveys based on amplicon

sequencing as genome-wide sequencing data can reveal not just taxonomic identities of what are present but can also be useful to uncover functional diversity at the level of mutational frequencies, gene content, and predicted proteins (Harrington et al. 2007, Godzik 2011). The reconstruction of full or partial metagenomes can be used to estimate gene gain/loss, calculate linkages, and infer metabolic pathways (Gianoulis et al. 2009, Qin et al. 2012, Teeling and Glockner 2012). As with taxonomic identification, functional profiling of microbial communities has historically been limited to culturable microbes, and functional assays are often biased towards testing activities of particular enzymes or responses to specific antimicrobial agents (Torsvik and Øvreås 2002). Because genes and gene networks can be bioinformatically annotated through automated comparisons with publicly available reference databases, metagenomics has enabled connections between microbial genotype, phenotype, and fitness on an unprecedented scale (Gray et al. 2015, Alberdi et al. 2016). Metagenomics can be used to simultaneously investigate contemporary evolution due to environmental change across multiple co-occurring species and are thus especially powerful in understanding eco-evolutionary dynamics of microbial communities (Bengtsson-Palme et al. 2014, Hand et al. 2015, Bendall et al. 2016, Macke et al. 2017, Le Roux and Blokesch 2018, Rainey and Quistad 2020). Unlike other targeted methods that can be biased towards pre-conceived notions of relative ecological and evolutionary importance, WGS sequencing indiscriminately produces sequences and can be used to generate data-driven hypotheses from a bottom-up approach (Garza and Dutilh 2015).

Environmental DNA

Environmental DNA (eDNA) evolved from microbial ecology methods when it was realized that DNA of all organisms, not just microbes, could be detected and sequenced directly from

environmental samples (Ficetola et al. 2008). eDNA methods have been demonstrated to be more accurate, sensitive, and cost-effective compared to traditional monitoring methods, and the non-invasive nature of eDNA lends well to study of sensitive or threatened species (Taberlet 2018). Due to these advantages, eDNA studies can be conducted in locations that have historically been understudied due to practical concerns and has quickly become a popular method to study biodiversity across the tree of life for academic research, as well as a boon for natural resource management and the environmental consulting industry (Rees et al. 2014a, Rodgers et al. 2017, Ruppert et al. 2019, Wang et al. 2019, Ji et al. 2022, Lim et al. 2022). However, eDNA is often prone to contamination issues and can be sensitive to parameters of sample collection, data processing, and bioinformatic analyses. As such, best practices for applications to specific systems and environmental contexts continue to be an active area of research (Turner et al. 2014a, 2015, Barnes and Turner 2016, Somervuo et al. 2017, Axtner et al. 2019)

Despite known limitations, eDNA has successfully been used in a variety of systems to assess the impact of anthropogenic environmental change on the biodiversity of aquatic protists/metazoans/phytoplankton/macroinvertebrates, birds, coral reefs, fish, and terrestrial plants/vertebrates, spurring novel applications in other fields like ecotoxicology, trophic ecology, and wildlife forensics (Bakker et al. 2017, Ushio et al. 2018, Li et al. 2018, Zhang 2019, Bourret et al. 2020, DiBattista et al. 2020, D'Alessandro and Mariani 2021, Ibrahim et al. 2021, Johnson et al. 2021, Brantschen et al. 2021, Lynggaard et al. 2022). Conversely, eDNA can be used to assess the biotic drivers of environmental change such as in the monitoring of introduced/invasive species (Nathan et al. 2015, Sepulveda et al. 2020). When the source of eDNA is from the ancient past, it can be described as "ancient DNA", which can be recovered

from preserved environmental samples such as permafrost and lake sediments (Pedersen et al. 2015). Such longitudinal data can be exceptionally valuable for studying rare historical disturbances and the effects of long-term incremental climate change on biodiversity (Bremond et al. 2017, Bálint et al. 2018). Population-level inferences regarding evolutionary processes are also possible through studying the genetic diversity of recovered DNA, which have yielded estimates of haplotype frequencies and phylogeographic structure (Sigsgaard et al. 2020).

Rationale and objectives

Environmental change is a primary driver of biodiversity patterns and processes. Human-induced environmental impacts such as pollution, habitat loss, and climate and land-use change are expected to continue increasing at ever-faster rates due to resource consumption demands of global economic development (United Nations Environment Programme and International Resource Panel 2011). The significance of human activity towards the natural world has marked what has been termed the Anthropocene (Lewis and Maslin 2015, Steffen et al. 2020). The fundamentally unsustainable consumption of finite resources in this new epoch is nowhere else more evident than in the unprecedented rates of biological extinction that have been estimated to be 1,000-10,000 fold greater than expected without human influence (Heywood and Dowdeswell 1995, Pimm et al. 2014, Tilman et al. 2017, International Union for Conservation of Nature and Natural Resources 2022). This “biodiversity crisis” underscores the need to better understand how rapid environmental change, like those induced by humans, affect all levels of biodiversity and their dynamics (Singh 2002, Storch et al. 2022).

Elucidation of the ecological and evolutionary mechanisms underlying biodiversity response to environmental disturbance may lead to greater predictive power and evidence-based

management strategies with potential to mitigate or reverse the negative consequences of human impacts. The complexity of biodiversity calls for an integrated approach to synthesize knowledge and methods across basic and applied disciplines such as through 1) exploration of novel model systems under natural conditions, 2) longitudinal studies of specific and quantifiable environmental stress, and 3) development of new monitoring approaches for characterizing biological variation and dynamics. In this thesis, I take such a holistic perspective to address hypotheses regarding the community assembly of skin microbiomes in response to human piercings in Chapter 1, the protective effects of acidification pre-exposure on resistance and resilience of natural lake bacteria communities in Chapter 2, and the potential for eDNA to be used in the detection of transgenes from genetically modified animals to track them in case of release in Chapter 3.

CHAPTER 1. COMMUNITY ASSEMBLY OF THE HUMAN PIERCING MICROBIOME

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1.1 Abstract

Predicting how biological communities respond to disturbance requires understanding the forces that govern their assembly. We propose using human skin piercings as a model system for studying community assembly after rapid environmental change. Local skin sterilization provides a 'clean slate' within the novel ecological niche created by the piercing. Stochastic assembly processes can dominate skin microbiomes due to the influence of environmental exposure on local dispersal, but deterministic processes might play a greater role within occluded skin piercings if piercing habitats impose strong selection pressures on colonizing species. Here we explore the human ear-piercing microbiome and demonstrate that community assembly is predominantly stochastic but becomes significantly more deterministic with time, producing increasingly diverse and ecologically complex communities. We also observed changes in two dominant and medically relevant antagonists (*Cutibacterium acnes* and *Staphylococcus epidermidis*), consistent with competitive exclusion induced by a transition from sebaceous to moist environments. By exploiting this common yet uniquely human practice, we show that skin piercings are not just culturally significant but also represent ecosystem engineering on the human body. The novel habitats and communities that skin piercing produce may provide general insights into biological responses to environmental disturbance with implications for both ecosystem and human health.

1.2 Introduction

How communities of coexisting species originate and are maintained is known as community assembly, and these processes determine which species thrive and which perish (Fukami 2009, Nemergut et al. 2013). Similar ecological conditions across environments might result in community convergence because deterministic niche selection can promote analogous community profiles (Clements 1936). Community divergence may be driven by changing ecological pressures, but stochastic processes such as the order and timing of migration and random extirpation of populations can also play significant roles (Fukami 2015). Initial colonizers may exert priority effects where the arrival of one species affects the subsequent colonization and/or establishment of a different species and produces historical contingency, in which chance effects can have long lasting consequences for community structure (Fukami 2015, Debray et al. 2022). These priority effects can be pronounced during ecological succession as communities shift to a stable state after perturbation (Zhou et al. 2014, Chang and HilleRisLambers 2016). Understanding the mechanisms that underlie community assembly will ultimately lead to better predictions of community as well as individual species responses to environmental change.

Community assembly processes of human microbiomes have gained recent interest due to an increasing awareness of their ecological and medical significance (Trivedi et al. 2020, Debray et al. 2022, Martino et al. 2022). Human microbiomes refer to the collective microorganisms and genes found within or on human beings (Marchesi and Ravel 2015). Such microbiota consist largely of bacteria but also viruses, archaea, and microscopic eukaryotes like protists and fungi, and different communities inhabit various parts of the human body (Marchesi and Ravel 2015, Gilbert et al. 2018). As the largest organ, the skin represents a diverse

ecosystem of habitats for microbes that are constantly exposed to changing external conditions (Grice and Segre 2011). Although a dearth of nutrients results in the skin containing relatively low biodiversity (Byrd et al. 2018), distinct core taxa can be found on ecologically dissimilar parts of the skin (Grice et al. 2009, Costello et al. 2009). Two of the most common and abundant genera present on human skin include *Cutibacterium* (formerly *Propionibacterium*) and *Staphylococcus*, which dominate at sebaceous and moist sites, respectively (Kong and Segre 2012, Byrd et al. 2018). Functionally, the human skin microbiome plays an important role in maintaining cutaneous health (Schommer and Gallo 2013). Pathogenic species such as *Staphylococcus pyogenes*, *Staphylococcus aureus*, and group A *Streptococcus* are known to cause skin infections while commensal species like *Cutibacterium acnes* and *Staphylococcus epidermidis* can protect from pathogens through regulation of the immune system (Sanford and Gallo 2013). Shifts in the human skin microbiome have been associated with many disease states like atopic dermatitis (Nakatsuji and Gallo 2019), psoriasis (Tett et al. 2017), acne vulgaris (Xu and Li 2019), and chronic wounds (Tomic-Canic et al. 2020), but studies on the community dynamics of healthy skin microbiomes, especially after extrinsic perturbation, are less common (but see Meadow et al. 2013, Lax et al. 2014, Brandwein et al. 2018, Moskovicz et al. 2020). These studies show that many common activities such as using cosmetics and topical creams, sun exposure, direct contact sport, mineral bathing, and moving homes constitute rapid environmental disturbances for the human skin microbiome. These activities have potentially significant and unintentional impacts on microbial community assembly with differential ecological and evolutionary responses according to scale and functional contexts of specific taxonomic groups. Thus, the human skin microbiome is highly dynamic and the underlying assembly processes can have important health implications, such as in understanding ecological

succession of microbial communities during wound recovery or dermatological disorders (Musthaq et al. 2018, White and Grice 2023). Community assembly processes of human skin microbiomes are also readily apparent due to relatively simple taxonomic compositions and short bacterial generation times.

One uniquely human activity that might affect the skin microbiome is skin piercing. Skin piercing has been present in human societies at least as far back as Ötzi the Iceman, who lived nearly 5000 years ago and was found to have pierced earlobes (Rauf 2019). Skin piercings have been used to express both individual and group identities and served significant roles in traditional customs and rites of passage in various cultures around the world (Rush 2005). In addition to its anthropological and sociological importance, here we propose human skin piercings as a model for studying biological community assembly processes after rapid environmental change. Piercing practices typically begin with surface sterilization of the skin, which we hypothesize functions as a major environmental disturbance to the local skin microbiome. The piercing of the skin then reshapes the skin's physical topology, which is immediately followed by insertion of surgical stainless-steel studs for usually at least two weeks. This is expected to produce a novel ecological niche that differs from the previously unpierced skin in many ways such as surface area, temperature, acidity, humidity, and environmental exposure. This drastic environmental shift should fundamentally alter the ecological and evolutionary forces acting on the piercing microbiome.

Here, we hypothesize that human ear-piercing microbiomes (1) become more diverse and ecologically complex because the novel piercing environment offers increased protection, stability, and nutrients, (2) will exhibit less historical contingency because ecological succession will result in the deterministic assembly of an equilibrium community structure, and (3) reflect a

transition of the skin environment from sebaceous to moist through increased moisture retention, resulting in a reduction of *Cutibacterium* and an increase in *Staphylococcus*. We tested these hypotheses by sampling the microbiomes of human ear-piercings over a two-week time period. Longitudinal samples of skin microbiomes from adjacent unsterilized and unpierced skin were collected simultaneously as controls for temporal variation. Other than previous clinical investigations of piercing infections, to our knowledge, this study represents the first investigation of the human piercing microbiome.

1.3 Methods

1.3.1 Human research ethics approval

Protocols for study participant recruitment, data security, sample collection, and associated procedures were approved by the McGill University Research Ethics Board Office (REB-1 no. 70-0617).

1.3.2 Sample collection

From October 2019 to March 2020, we recruited 28 individuals who were receiving earlobe piercings at Tattoo Lounge MTL in Montreal, Quebec, Canada and received their written, informed consent to participate in the study (electronic supplementary material, Fig. S3). Following standard ear-piercing protocols, we sterilized the earlobe skin area to be pierced with a benzalkonium chloride antiseptic towelette (Jedmon Products) immediately before piercing. We pierced earlobes using a sterilized beveled hollow needle (Ruthless/Precision) and then inserted a 5/16” surgical steel grade (316L) piercing labret stud composed of chromium, nickel, and molybdenum. Both needle and stud were dipped in a water-based lubricant jelly (Personelle,

Jean Coutu) to minimize friction and then cleaned off afterwards using a cotton-tipped swab. We collected skin swab samples using the DNA/RNA Shield Collection Tube w/Swab – DX (Zymo Research), which was used to preserve nucleic acids within samples at ambient temperatures. The piercer collected samples from the earlobe to be pierced and an adjacent unsterilized part of the ear farther up the ear but still part of the earlobe skin to serve as a temporal control. Samples were collected both before and after the piercing event (defined as a three-part process that includes (A) skin sterilization followed by (B) skin piercing and then (C) insertion of the metal stud). Study participants were then instructed to self-sample both the piercing and the adjacent skin control while wearing gloves over the following two weeks at specified timepoints: 12 h, 1 day, 3 days, one week, and two weeks. Additionally, environmental controls were collected by the piercer before the piercing and by the participant at the one- and two-week timepoints by waving a swab in the air for 30 s. In total, we collected 17 samples from each participant.

1.3.3 DNA extraction and amplicon sequencing

We extracted DNA from swabs using the DNeasy PowerSoil kit (QIAGEN) and then purified using the OneStep PCR Inhibitor Removal kit (Zymo Research). Skin swab samples and environmental controls were processed with a DNA extraction negative control included within each batch of 24 extractions. This work was carried out in a laboratory facility designed to handle low-copy and highly degraded environmental DNA samples through mitigation of contamination factors (e.g. no exposure to PCR products, regular deep cleaning, and strict usage protocols limited to trained personnel). The V1-V3 region of the 16S rRNA gene was PCR amplified using the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 518R (5'-ATTACCGCGGCTGCTGG-3') (Meisel et al. 2016). Library preparation, quality control, and

high throughput sequencing with Illumina MiSeq v2/v3 were conducted at Génome Québec and the McGill Genome Centre (Montreal, Quebec, Canada).

1.3.4 Data processing

We processed raw sequences using the QIIME2 bioinformatics pipeline (Bolyen et al. 2019). We trimmed primer sequences using cutadapt before generating amplicon sequence variants (ASVs) using DADA2 (Callahan et al. 2016). We identified contaminant ASVs using environmental and DNA extraction negative controls for each sequencing batch with the prevalence-based method at a classification threshold of $p^* = 0.5$ within decontam (Davis et al. 2018). We considered the unpierced control of each individual to be experimentally valid only if it exhibited no significant differences from the microbiome of the skin to be pierced prior to piercing. Thus, we defined statistical outlier individuals as having an absolute difference in ASV richness between sample and control prior to piercing that was greater than 1.5 times the interquartile range across all individuals (Rousseeuw and Hubert 2011). We removed a total of 1047 contaminant ASVs and two statistical outlier individuals resulting in 10 915 ASVs across 392 samples with a mean sequencing depth of 27 817 reads per sample. We aligned ASVs using MAFFT and built phylogenetic trees using FastTree 2 based on Jukes-Cantor distances. For taxonomic assignment, we used the 27F/518R 16S rRNA primers to *in silico* extract the target V1-V3 amplicon from the SILVA 132 database (Yilmaz et al. 2014). We trained a naïve Bayes classifier using scikit-learn on the extracted database and then used it to taxonomically assign ASVs from domain down to species. We accepted assignments if classification confidence was at least 0.7 (Ziemski et al. 2021).

1.3.5 Statistical analyses

We normalized ASV counts via total sum scaling (TSS), and calculated biodiversity indices, principal coordinates analysis (PCoA), and PERMANOVA (999 permutations) using the R 'phyloseq' and 'vegan' packages implemented within MicrobiomeAnalyst 2.0 (McMurdie and Holmes 2013, Chong et al. 2020, Oksanen et al. 2022). We did not rarefy data to maximize the amount of data analyzed and the number of participants included in the study (McMurdie and Holmes 2014). We measured alpha and beta diversities using Chao1 and Bray–Curtis dissimilarity, respectively. We calculated betadisper separately using the R 'vegan' package version 2.6-2 and used 'ggstatsplot' version 0.10.0 for plotting within RStudio Desktop version 2022.12.0 + 353 and R version 4.2.2 (Posit team 2022, R Core Team 2022, Oksanen et al. 2022). We built ASV co-occurrence networks using random matrix theory (RMT)-based Spearman's rank correlation through the Molecular Ecological Network Analysis (MENA) pipeline implemented within the Integrated Network Analysis Pipeline (iNAP) (Feng et al. 2022). We first filtered data by retaining only ASVs present in greater than 15% of samples and then log transformed the filtered data before calculating similarity matrices allowing a single timepoint lag for time-dependent interactions. We visualized co-occurrence networks using Cytoscape version 3.9.1 keeping only nodes with valid genus-level taxonomic assignments and edges with a p-value < 0.05. We used the 'iCAMP' R package v. 1.5.12 (Ning et al. 2020) to calculate pNST (Ning et al. 2019) and infer community assembly mechanisms by phylogenetic bin-based null model analysis. We used bootstrapping tests with a resampling size of 1000 to assess significant pairwise differences between timepoints. We classified core microbiome community taxa based on a minimum of 5% relative abundance across at least 50% of all samples.

1.4 Results and discussion

1.4.1 Human piercings increase diversity and ecological complexity

Sudden events that cause drastic environmental change for human skin can lead to fundamental shifts in skin microbiomes. For example, human birth involves moving from an environment that is liquid and mostly sterile to one exposed to air and microbial colonization, which contributes to increased skin microbiome diversity and differentiation for human infants through their first year of life (Capone et al. 2011). Analogously, we found that skin piercings were strongly associated with a significant increase in ASV richness (i.e. number of unique DNA sequences) at the piercing site over two weeks (Fig. 1A). By contrast, ASV richness of the unpierced controls remained stable over the same time period (Fig. 1B). Skin piercings likely represent the creation of hospitable niches for certain bacteria that thrive in areas of greater occlusion, moisture, and nutrient retention. Occlusion increases skin pH and produces moisture through transepidermal water loss, which supports bacterial growth and survival (Aly et al. 1978). Piercing studs may physically trap and accumulate debris including sweat, sebum, and pieces of stratum corneum that serve as primary nutrient sources for most human skin microbiome members (Scharschmidt and Fischbach 2013). Undisturbed occluded skin microbiomes also exhibit the greatest longitudinal stability, presumably due to physical protection from external perturbation (Grice et al. 2009).

Piercings were also associated with a significant increase in dispersion of beta diversity (i.e. increased dissimilarity in community composition between piercing microbiomes) by two weeks after piercing (Fig. 1C) whereas beta diversity did not change between timepoints in the unpierced controls (Fig. 1D). Thus, the piercing environment may have caused greater variance in community structure over time, which is consistent with the increase in alpha diversity and

supports the idea that piercings produce novel ecological niches. The greatest source of temporal variation in beta diversity of both piercing microbiomes and unpierced controls, however, was differences between individuals (electronic supplementary material, Appendix, Fig. S1). Metadata of study participants collected through questionnaire surveys revealed no significant relationships between the skin piercing microbiome and host factors and behaviors including hygiene, travel, and physical activity (electronic supplementary material, Appendix, Methods). However, some behavioral differences across individuals, such as heterogeneity during self-sampling (e.g. pressure applied to swabs), may not have been captured by our questionnaire and these effects may have masked the importance of more nuanced factors on the skin piercing microbiome.

Because beta diversity did not change significantly until the two-week timepoint, the two-week sampling period of this study may have been insufficient to fully capture the ecological succession process. Human skin microbiome communities can be surprisingly stable even at highly exposed and perturbed body sites like the face and palm as well as in the long-term for up to two years (Oh et al. 2016, Hillebrand et al. 2021). Other recent longitudinal studies on chronic and acute skin disturbances such as diabetic foot ulcers (MacDonald et al. 2019), burn wounds (Lima et al. 2021), and chlorhexidine disinfectants (Mougeot et al. 2022) have demonstrated that post-disturbance community structure remains quite stable from 3 to 56 days later despite other significant ecological impacts.

Network analyses revealed co-occurrence and exclusion patterns of human skin microbiomes driven largely by body sites representing distinct microbial habitats (Faust et al. 2012). Environmental factors (e.g. elevation (Li et al. 2019) and urban living (Kim et al. 2018)), skin physiology (e.g. aging (Kim et al. 2019) and skin sensitivity (Keum et al. 2020)), and skin

products (e.g. lotion (Murphy et al. 2022)) can also affect various properties of skin microbiome network topology. Correlational analyses have been widely used to infer real-world biotic interactions from amplicon sequencing data but suffer from producing spurious and indirect associations, especially for rare ASVs in zero-inflated data typical of skin microbiomes (Layeghifard et al. 2017, Hirano and Takemoto 2019, Carr et al. 2019). Time-series experiments can help address these issues by making it possible to infer directionality and time-dependency of interactions, which are often asymmetrical (Ai et al. 2019). To examine how piercings may have impacted the ecological interactions within skin microbiomes, we constructed co-occurrence networks via the MENA pipeline. Although there was little difference in the absolute number of nodes between piercing and unpierced networks, the number of edges was consistently higher in the piercing network, suggesting a greater number of biotic interactions among microbiome members (Fig. 2A-B). This increase in ecological complexity is potentially associated with more available resources (Guo et al. 2020), which we predicted to occur due to accumulation of nutrients in the occluded piercing environment. The relationship between ecological complexity and resilience to environmental disturbance can be either positive (Santolini and Barabási 2018) or negative (Wang et al. 2016) depending on the interdependency of interactions, and complexity can have significant implications for microbial ecosystem functioning (Wagg et al. 2019). Ecological complexity is also sensitive to shifting selection pressures (Xiong et al. 2021), which is further evidence suggesting that the piercing environment represents a novel ecological niche.

1.4.2 Stochasticity and determinism during community assembly in piercing microbiomes

To directly assess the relative contribution of deterministic and stochastic processes in the

community assembly of the piercing microbiome, we used community assembly mechanisms by phylogenetic-bin-based null model analysis (iCAMP). iCAMP employs the beta net relatedness index (βNRI) and taxonomic beta diversities with the modified Raup–Crick (RC) metric to partition deterministic processes into either heterogeneous selection or homogenous selection, and stochastic processes into homogenizing dispersal, dispersal limitation, or drift (Ning et al. 2020). In contrast to heterogeneous selection, homogenous selection occurs when environmental conditions are stable and consistently exerting similar selection pressures over space and/or time (Stegen et al. 2015). Homogeneous selection typically leads to greater phylogenetic relatedness because related communities are often ecologically similar whereas heterogeneous selection produces greater phylogenetic dissimilarity. The iCAMP analysis indicated that stochasticity was dominant in both piercing microbiomes and unpierced controls (Fig. 3A), specifically through dispersal limitation (relative importance of 73.1% in piercing, 76.8% in unpierced) with minimal contributions from drift (0.25% in piercing, 0.43% in unpierced) and none from homogenizing dispersal (Fig. 3B). Deterministic assembly processes were largely accounted for by homogenous selection (24.3% in piercing, 19.8% in unpierced) with minor contributions from heterogeneous selection (2.3% in piercing, 2.9% in unpierced) (Fig. 3B). We found that two weeks after piercing, the relative contribution of dispersal limitation decreases while homogenous selection increases (Fig. 3B). Stochasticity between the to-be pierced and unpierced control skin differed, with high variation observed in both. This may suggest substantial variation in the proportion of stochastic versus deterministic assembly processes even at very short distances between adjacent skin of the same body part. However, alpha diversity between pierced samples and unpierced controls does exhibit strong correspondence, providing support for the validity of using adjacent unpierced skin as controls (electronic supplementary material,

Appendix, Fig. S1 *A* and *C*). The dominance of stochasticity and its decrease over time in piercing microbiomes was also supported by phylogenetic normalized stochasticity ratio (pNST) analyses (electronic supplementary material, Appendix, Fig. S2), which is based on beta mean nearest taxon distance (βMNTD) (Ning et al. 2019). These results suggest that community assembly of the piercing microbiome becomes more deterministic with time, consistent with the hypothesis that piercings produce a novel yet consistent and stable microhabitat that leads to homogenous selection pressures.

To better understand the difference in temporal dynamics between piercing microbiomes and unpierced controls, we next explored time-lagged correlations within ecological networks, which can be indicative of time-dependent interactions such as priority effects. Both piercing microbiomes and unpierced controls were comprised of significantly more time-dependent interactions, with 69% in the piercing (one-sided one-proportion Z test, $p = 9.66 \times 10^{-6}$) and 79% in the unpierced (one-sided one-proportion Z test, $p = 2.11 \times 10^{-8}$) networks (Fig. 2A-B). The lower proportion of time-dependent interactions in the piercing network could be caused by an increase in deterministic selection forces (e.g. environmental filtering) of the newly created environmental niches within piercings. An increase in determinism reduces the relative importance of stochastic processes like historically contingent dispersal (Zhou and Ning 2017), although the difference in proportions between piercing and unpierced networks was insignificant (one-sided two-proportion Z test, $Z = -1.54$, $p = 0.062$, 95% CI [-1, 0.004]). Another potential and non-mutually exclusive explanation could be that environmental disturbance from piercings compress the spatio-temporal niche of the microbiome by increasing species abundances which leads to greater species interactions that are not time-lagged. Evidence

for this mechanism was recently discovered when anthropogenic landscape modification was found to increase co-occurrence of wildlife species (Gilbert et al. 2022).

Network correlations can be either positive or negative reflecting the nature of potential ecological interactions. The positive-to-negative links (P/N) ratio has been proposed as a marker for differentiating healthy and diseased microbiome networks by detecting shifts in the balance between facilitative and inhibitive interactions (Ma 2018). Here, the P/N ratio was able to distinguish piercing (P/N = 1.42) from unpierced (P/N = 0.88) networks (Fig. 2A-B), with more positive than negative edges in the piercing network, whereas the opposite was true of the unpierced network. The proportion of positive edges was significantly greater in the piercing than unpierced network (one-sided two-proportion Z test, $p = 0.0418$, 95% CI [0.007, 1]). Positive network associations may represent facultative and obligatory commensalisms or mutualisms between taxa, but they can also reflect the co-occurrence of taxa with high niche overlap that are ecologically or functionally similar (i.e. environmental filtering) (Hernandez et al. 2021). There is evidence that, during secondary succession (i.e. post-disturbance recolonization), a general shift to positive interactions may help a community respond to environmental stress through neighborhood habitat amelioration, where one species changes the environment in a way that facilitates the growth and survival of another species (Bertness and Callaway 1994). Positive biotic interactions and environmental filtering are not mutually exclusive because positively interacting taxa that share similar niches would increase positive network associations through both mechanisms. Regardless, both are deterministic processes (Chase and Myers 2011). Thus, contrasting P/N ratios of piercing and unpierced networks suggests that piercings are strongly associated with a deterministic ecological shift for the local skin microbiome.

1.4.3 Piercings cause a shift towards moist skin microbiomes

While piercing infections are common medical complications (Stirn 2003) and a variety of specific pathologies have been identified (Tweeten and Rickman 1998, Messahel and Musgrove 2009), the community composition and temporal dynamics of uninfected human piercing microbiomes have yet to be characterized. Because piercings can potentially trap moisture by mitigating evaporation, we predicted that the piercing microbiome should develop to resemble skin microbiomes found in moist areas such as the nose, armpit, or groin. We found that the two-week phylum-level community composition of the piercing microbiome was dominated by Actinobacteriota (Actinomycetota) and Firmicutes (Bacillota), followed by Proteobacteria (Pseudomonadota) with relatively few Bacteroidota (Bacteroidetes). Actinobacteriota was largely represented by the families Propionibacteriaceae and Corynebacteriaceae, specifically the genera *Cutibacterium* and *Corynebacterium*, respectively. Firmicutes was mainly comprised of Staphylococcaceae and Streptococcaceae at the family level and *Staphylococcus* and *Streptococcus* at the genus level, respectively. Although we could not assign a species identity to a large proportion of ASVs, just two species, namely *Cutibacterium acnes* and *Staphylococcus epidermidis*, emerged as core taxa of the piercing microbiome given their relative abundance and wide prevalence (Fig. 4A). These two species encompassed more than half of the community in 58% of our samples, with an average of 44% *C. acnes* and 8.6% *S. epidermidis*. *Corynebacterium* was the third most dominant genus at 6.6%, but the most prominent *Corynebacterium* ASV could not be classified to species-level and all genus-level *Corynebacterium* ASVs failed to meet the core taxa criteria. Following *C. acnes* and *S. epidermidis* across time confirms that they experience dramatic longitudinal shifts in the

expected directions: a significant decrease in the relative abundance of *C. acnes* and a significant increase in relative abundance of *S. epidermidis* (Fig. 4B-C). These findings are consistent with a moist piercing environment because *Cutibacterium* species are known to be dominant members of sebaceous skin microbiomes, including specifically the external auditory canal, while *Staphylococcus* is mainly associated with moist skin (Fournière et al. 2020). These significant longitudinal changes in *C. acnes* and *S. epidermidis* were not observed in the unpierced controls (Fig. 4D-E).

Beyond associations with distinct skin ecologies, the two core taxa, *C. acnes* and *S. epidermidis*, are well-known commensals and opportunistic pathogens of human skin as well as direct antagonists. Both *C. acnes* and *S. epidermidis* are common members of skin microbiomes that help maintain skin homeostasis through competitive exclusion of potential pathogens, production of antibacterial bacteriocins, and priming of the skin's innate Toll-like receptor (TLR) immune system (Cogen et al. 2008, Sanford and Gallo 2013). Against each other, however, they compete using a variety of methods including the production of antimicrobial short chain fatty acids (Nakamura et al. 2020), bacteriocins and polymorphic toxins (Christensen et al. 2016), and electricity (Marito et al. 2021). The strong antagonism between *C. acnes* and *S. epidermidis* may help explain the observed change in the piercing microbiome. If the novel piercing environment directly affects a single species, through either greater selection against *C. acnes* or increased relative fitness of *S. epidermidis*, it should induce an opposite trajectory of the corresponding species. Numerous skin diseases like acnes, dermatitis, rosacea, and psoriasis have been associated with lower relative abundance and/or a loss in taxonomic diversity of *Cutibacterium* (Rozas et al. 2021). However, over-colonization of *C. acnes* can lead to microcomedone formation and acne if *S. epidermidis* fails to control its proliferation (Claudel et

al. 2019). An imbalance between *C. acnes* and *S. epidermidis* has also been shown to activate skin inflammation through the production of cytokines (Dagnelie et al. 2022). While both species are known opportunistic pathogens given the right environmental context (Niazi et al. 2010), *S. epidermidis* represents the most common source of infections on indwelling medical devices such as central venous catheters and joint prostheses (Uçkay et al. 2009). A major similarity between internal medical implants and external skin piercings is the insertion of foreign metal objects into the human body, which involves direct contact with the microbiota living in and on human skin. We hypothesize that the novel piercing ecological niche is more advantageous to *S. epidermidis* due to increased moisture, decreased sebum, and/or the new metal surface area that may support biofilm growth. The growing population of *S. epidermidis* can then reduce *C. acnes* abundance through antagonistic interactions, but further studies will be needed to confirm this hypothesis. Such ecological relationships between specific dominant species could potentially be exploited to inform pre- and probiotic treatments to prevent and control skin infections through competition or direct antagonisms with pathogenic microbiota (Maguire and Maguire 2017). Thus, skin piercings may serve as a model for understanding environmental disturbances by shedding light on the ecological dynamics of specific, medically relevant species.

1.4.4 Piercings as a model for studying biological responses to environmental change

Our study provides the first glimpse into the bacterial communities inhabiting human ear-piercings. We show that the piercing process–skin sterilization, piercing of the skin, and insertion of a metal stud–has a demonstrable impact on the ecology of the local skin microbiome. Despite sterilization serving as a major environmental disturbance that kills many resident species, we

found that, over time, the new piercing environment was significantly associated with greater biodiversity and ecological complexity, with fundamental differences in the nature of biotic interactions compared to exposed earlobe skin. The assembly of piercing microbiomes, however, remained dominated by stochastic dispersal typical of other skin microbiomes. Piercing microbiomes did not converge towards a single community structure but rather composition varied widely across individuals. Despite this, deterministic homogeneous selection did become more important with time, indicating some level of environmental filtering in the piercing environment. Piercing microbiomes showed less historical contingency than unpierced controls, consistent with greater contemporary selection. Similar to the microbiome of belly buttons (Hulcr et al. 2012), piercing microbiome communities are diverse but contain a few predictably dominant taxa. Specifically, we identified two major species, *C. acnes* and *S. epidermidis*, that show a change consistent with their known competitive antagonism and habitat associations, suggesting that piercings are moist environments. Studying how these two medically significant core species respond to rapidly changing environmental conditions within their natural communities may provide novel avenues to understanding their pathogenicity, of which interspecies interactions are known to play a major role (Sabaté Brescó et al. 2017, Ramasamy et al. 2019). Ecological disturbance experiments in natural ecosystems have traditionally been labor-intensive and difficult to replicate (Simberloff and Wilson 1970). By significantly altering the composition and ecology of the resident human microbiome, skin piercings could serve as a model for insights into the response of microbiomes to environmental disturbance as well as community assembly processes more generally. As human beings, we have practised the art of skin piercing for cultural, religious, and personal expression across diverse societies for

thousands of years. Here we reveal that skin piercings also represent a form of ecosystem self-engineering of the ecological landscape that is the human skin.

1.5 Data accessibility

Data and materials availability: raw sequencing data are available on NCBI Sequence Read Archive under BioProject PRJNA956301. Data and code are available on Zenodo: https://doi.org/10.5281/zenodo.7832626

1.6 Authors' contributions

C.C.Y.X.: conceptualization, methodology, validation, formal analysis, investigation, data curation, writing – original draft, writing – review and editing, visualization, supervision, project administration; J.L.: validation, investigation, data curation; A.A.: methodology, validation, data curation; É.M.W.: methodology, validation, investigation, resources, supervision; R.D.H.B.: conceptualization, methodology, resources, data curation, writing – original draft, writing – review and editing, supervision, project administration, funding acquisition.

1.7 Conflict of interest declaration

The authors declare that they have no competing interests.

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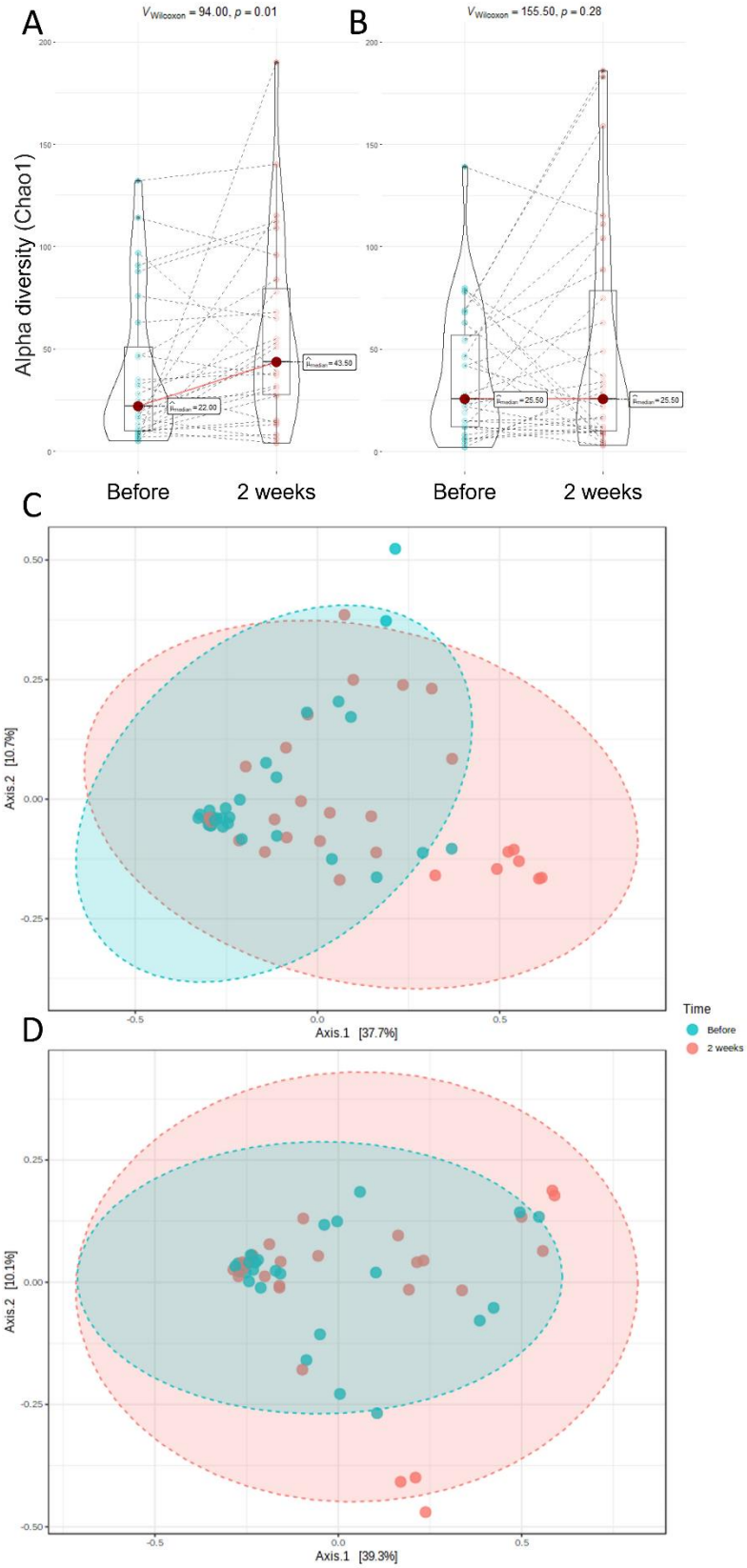
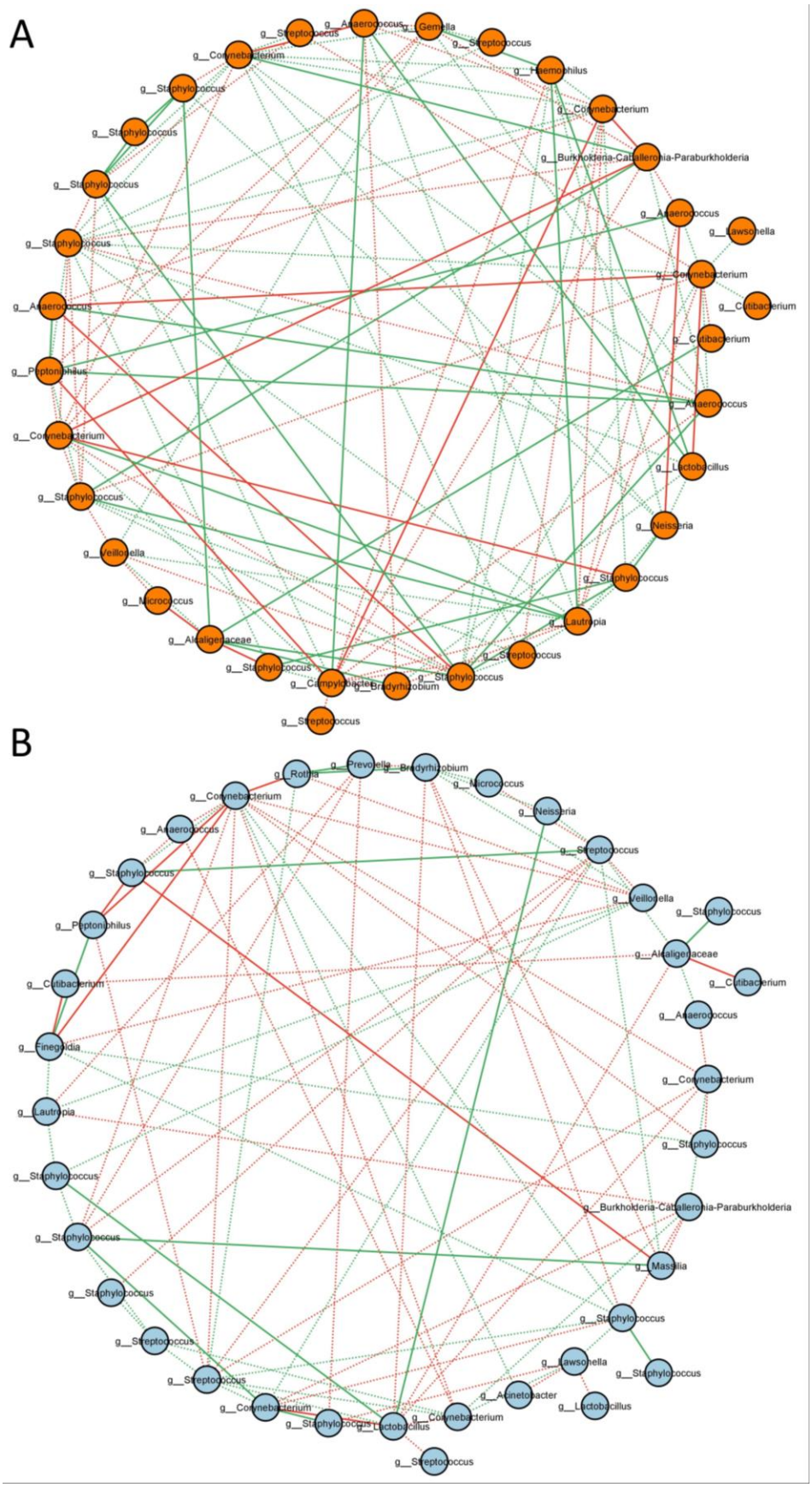
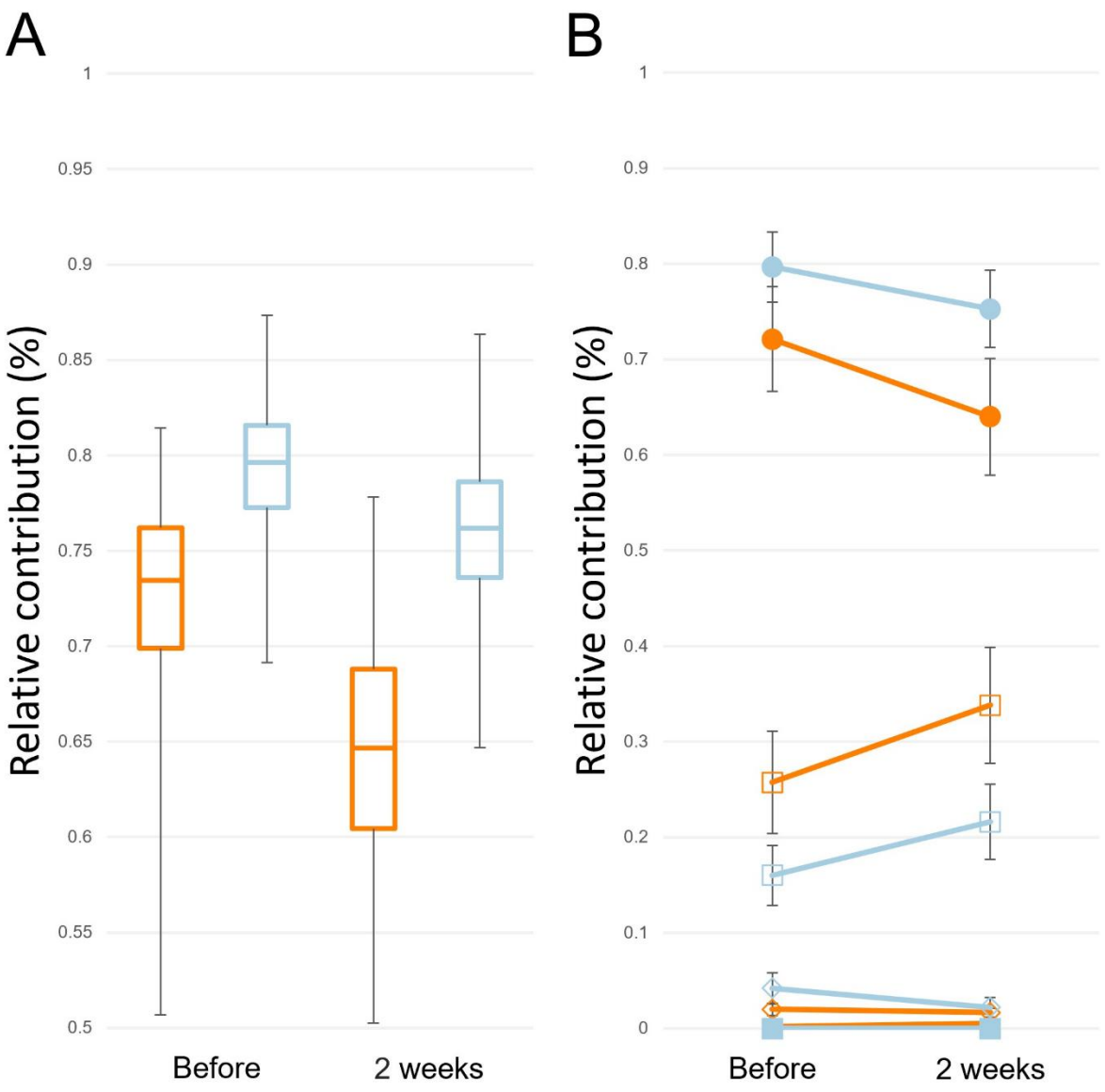
1.11 Figures


A
$V_{Wilcoxon} = 94.00, p = 0.01$
B
$V_{Wilcoxon} = 155.50, p = 0.28$
Alpha diversity (Chao1)
Before
2 weeks
Before
2 weeks
C
Axis.2 [10.7%]
Axis.1 [37.7%]
D
Axis.2 [10.1%]
Axis.1 [39.3%]
Time
Before
2 weeks

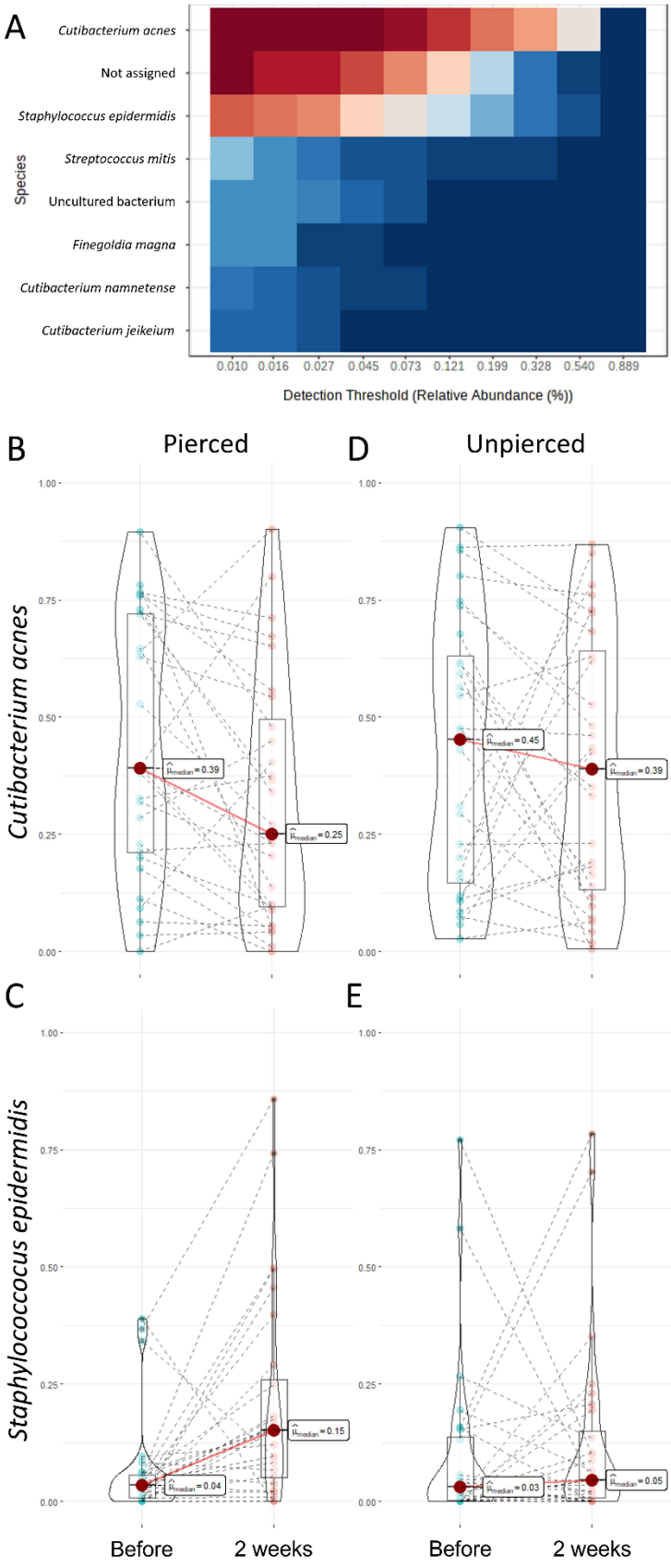
1.11.1 Fig. 1. Human piercings affect skin microbiome diversities. Alpha diversity of the (A) piercing microbiome increased significantly by two weeks after piercing, but (B) unpierced controls did not. Large red dots indicate medians, box and whiskers show the minimum, maximum, median, and 25^{th} and 75^{th} percentiles, and violin plots represent probability densities. Pairwise Wilcoxon signed-rank test V statistics and *p*-values are shown above each plot. (C) PCoA of Bray–Curtis dissimilarities before piercing (teal) and two weeks after (rose) show a significant increase in dispersion over time (betadisper, ANOVA, $F = 4.9053$, $p = 0.03101$). (D) No significant changes were observed in the unpierced controls (betadisper, ANOVA, $F = 0.0189$, $p = 0.8911$; PERMANOVA, $F = 1.0692$, $p = 0.321$).


A
g__Anaerococcus
g__Gemella
g__Streptococcus
g__Streptococcus
g__Corynebacterium
g__Haemophilus
g__Staphylococcus
g__Corynebacterium
g__Staphylococcus
g__Burkholderia-Caballeronia-Paraburkholderia
g__Staphylococcus
g__Anaerococcus
g__Lawsonella
g__Staphylococcus
g__Corynebacterium
g__Anaerococcus
g__Cutibacterium
g__Cutibacterium
g__Peptoniphilus
g__Anaerococcus
g__Corynebacterium
g__Lactobacillus
g__Staphylococcus
g__Neisseria
g__Veillonella
g__Staphylococcus
g__Micrococcus
g__Lautropia
g__Alkaligenaceae
g__Streptococcus
g__Staphylococcus
g__Staphylococcus
g__Campylobacter
g__Bradyrhizobium
g__Streptococcus
B
g__Rothia
g__Prevotella
g__Bradyrhizobium
g__Micrococcus
g__Corynebacterium
g__Neisseria
g__Anaerococcus
g__Streptococcus
g__Staphylococcus
g__Veillonella
g__Staphylococcus
g__Peptoniphilus
g__Alkaligenaceae
g__Cutibacterium
g__Cutibacterium
g__Anaerococcus
g__Finegoldia
g__Corynebacterium
g__Lautropia
g__Staphylococcus
g__Staphylococcus
g__Burkholderia-Caballeronia-Paraburkholderia
g__Staphylococcus
g__Massilia
g__Staphylococcus
g__Staphylococcus
g__Streptococcus
g__Lawsonella
g__Staphylococcus
g__Streptococcus
g__Acinetobacter
g__Lactobacillus
g__Corynebacterium
g__Corynebacterium
g__Staphylococcus
g__Lactobacillus
g__Streptococcus

1.11.2 Fig. 2. Piercing microbiomes exhibit a greater proportion of positive and direct ecological interactions. Molecular ecological networks of the (A) piercing microbiome (orange) contained 35 nodes and 121 edges, and (B) unpierced controls (blue) contained 37 nodes and 90 edges. Each node represents an individual ASV labelled by its genus identity (only nodes with genus identities shown), and edges represent correlation-inferred interactions. Concurrent interactions are shown as solid lines and time-dependent interactions are shown as dotted lines. The piercing and unpierced networks contain 69% and 79% time-dependent interactions, respectively. Positive interactions are coloured in green and negative interactions are coloured in red. The positive-to-negative links (P/N) ratio is 1.42 for the piercing network and 0.88 for the unpierced network.


A
B
Relative contribution (%)
1
0.95
0.9
0.85
0.8
0.75
0.7
0.65
0.6
0.55
0.5
Before
2 weeks
Relative contribution (%)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Before
2 weeks

1.11.3 Fig. 3. Community assembly of piercing microbiomes became more deterministic. (A) Relative contribution of stochastic assembly processes between piercing microbiomes (orange) and unpierced controls (blue). Stochasticity significantly decreased by two weeks after piercing (pairwise bootstrap, $p = 0.0352$). Box and whiskers show the minimum, maximum, median, and 25^{th} and 75^{th} percentiles. (B) Relative contribution of deterministic (open markers) and stochastic (closed markers) processes to community assembly in piercing microbiomes (orange) and unpierced controls (blue). Deterministic processes include homogeneous selection (open square) and heterogeneous selection (open diamond), and stochastic processes include dispersal limitation (solid circle), homogenizing dispersal (solid square), and drift (solid triangle). Error bars indicate standard deviations around the mean.


A
Cutibacterium acnes
Not assigned
Staphylococcus epidermidis
Streptococcus mitis
Uncultured bacterium
Finegoldia magna
Cutibacterium namnetense
Cutibacterium jeikeium
Species
0.010 0.016 0.027 0.045 0.073 0.121 0.199 0.328 0.540 0.889
Detection Threshold (Relative Abundance (%))
B
Pierced
D
Unpierced
Cutibacterium acnes
1.00
0.75
0.50
0.25
0.00
$\hat{\mu}_{median} = 0.39$
$\hat{\mu}_{median} = 0.25$
$\hat{\mu}_{median} = 0.45$
$\hat{\mu}_{median} = 0.39$
C
E
Staphylococcus epidermidis
$\hat{\mu}_{median} = 0.04$
$\hat{\mu}_{median} = 0.15$
$\hat{\mu}_{median} = 0.03$
$\hat{\mu}_{median} = 0.05$
Before
2 weeks
Before
2 weeks

1.11.4 Fig. 4. Two species, *Cutibacterium acnes* and *Staphylococcus epidermidis* dominate the piercing microbiome and shift in opposite directions over time in terms of prevalence and relative abundance. (A) Species-level heat maps of piercing microbiomes at the two-week timepoint. Teal and rose respectively represent more and less prevalence at increasing relative abundance thresholds. (B) *C. acnes* decreased significantly in relative abundance (Wilcoxon signed-rank test, $V = 309$, $p = 0.02$), and (C) *S. epidermidis* increased significantly in relative abundance (Wilcoxon signed-ranked test, $V = 66$, $p = 1.88 \times 10^{-3}$) in the piercing microbiome two weeks after piercing. No significant changes in relative abundance were observed for (D) *C. acnes* (Wilcoxon signed-rank test, $V = 234$, $p = 0.49$) or (E) *S.* epidermidis (Wilcoxon signed-rank test, $V = 159$, $p = 0.48$) in unpierced controls by the two-week timepoint. Large red dots indicate medians, box and whiskers show the minimum, maximum, median, and 25th and 75th percentiles, and violin plots represent probability densities.

1.12 Supplementary material

Community assembly of the human piercing microbiome

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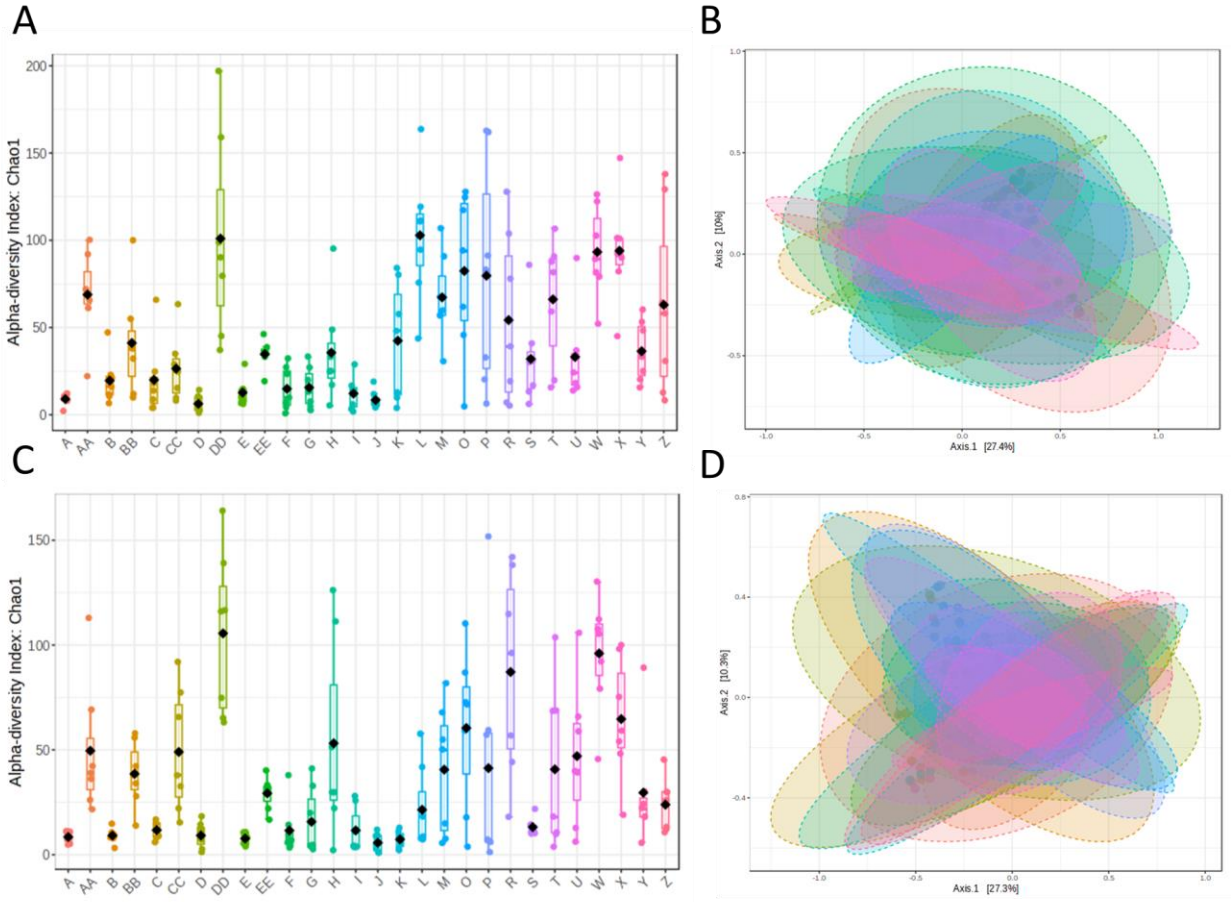
1.12.1 Methods

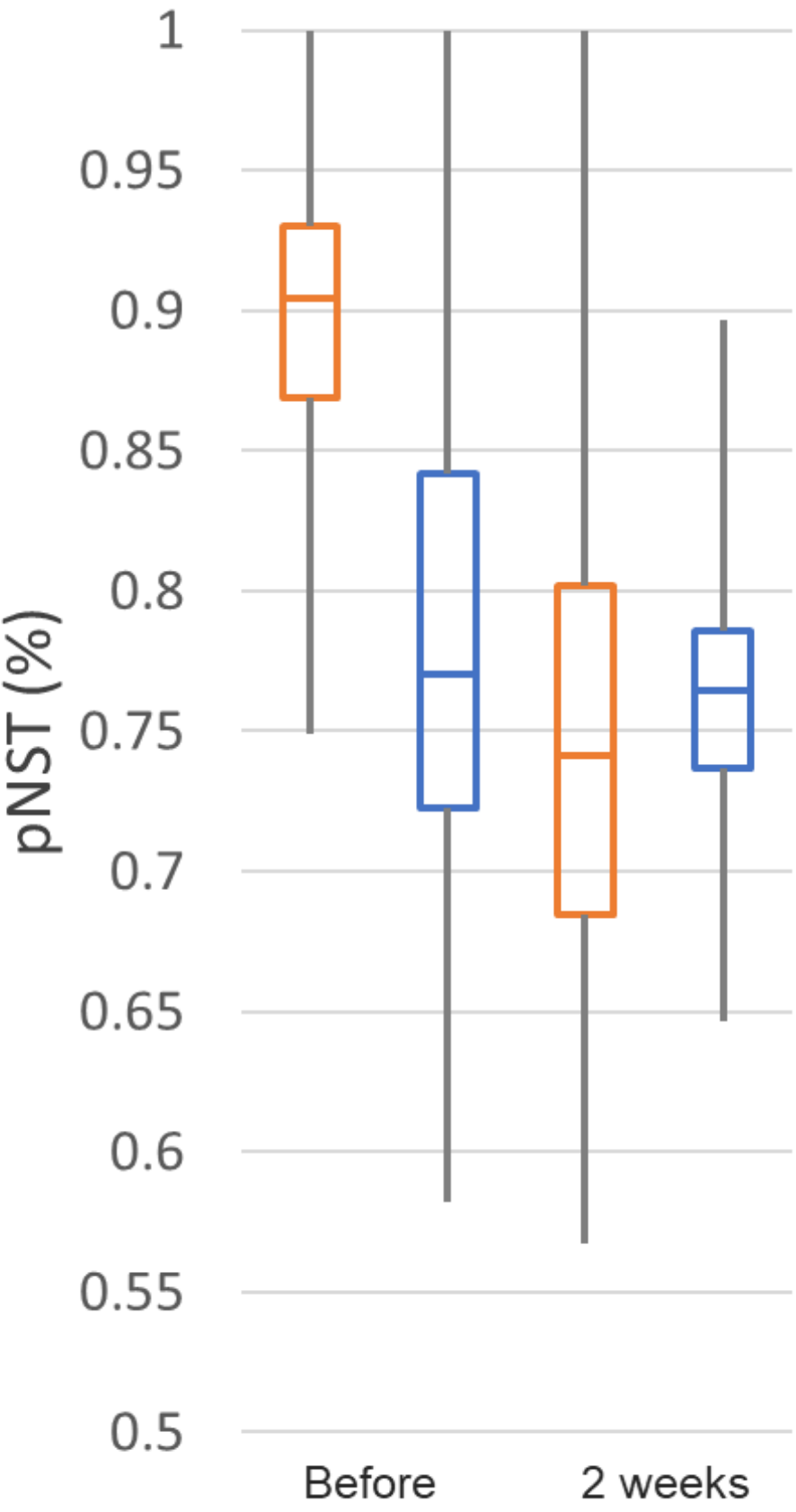
1.12.1.1 Metadata collection and analysis. Anonymized metadata of individual characteristics and behaviors known to possibly affect human skin microbiomes were collected via questionnaires taken before the piercing and 2 weeks later. Prior to being pierced, study participants were required to complete an initial questionnaire created in Google Forms available in both English and French. The initial questionnaire included questions regarding sex, age, ethnicity, height, weight, occupation, community setting (rural/urban/suburban), number/type of previous piercings, diabetes, and physical activity. At the end of the 2-week sampling period, study participants were instructed to complete a follow-up questionnaire that included questions regarding piercing discomfort, personal hygiene, travel history, and use of antibiotics and topical skin cream during the 2-week sampling period. Questionnaires were designed to collect individual metadata known to be potentially relevant to human skin microbiomes. Analyses of the collected metadata revealed no significant relationships with alpha and beta diversities of the piercing microbiome using univariate and multivariate statistical tests. Likewise, linear mixed effect models revealed no significant relationships with ASV abundances or relative abundances of *Cutibacterium acnes* or *Staphylococcus epidermidis*.

1.12.1.2 Assay validation experiment. The 27F/5118R primers used to amplify the V1-V3 region of the 16S rRNA gene in this study was validated in vitro using the ATCC© 20 Strain Even Mix Genomic Material (MSA-1002) as a mock community. Of the 20 bacteria species, 15 were successfully identified to species-level including specifically *C. acnes* and *S. epidermidis*. Mock community species that were not detected at species-level include *Acinetobacter baumannii*, *Bacillus pacificus*, *Bifidobacterium adolescentis*, *Escherichia coli*, and *Pseudomonas*

aeruginosa. Two species, *Enterococcus bacterium Te65R* and *Pseudomonas indoloxydans*, were detected but are not found in the mock community. These false positives could explain the false negatives of *E. coli* and *P. aeruginosa* since they are within the same family and genus respectively.


A
B
C
D
Alpha-diversity Index: Chao1
Axis.1 [27.4%]
Axis.2 [10%]
Axis.1 [27.3%]
Axis.2 [10.3%]

1.12.2 Fig. S1. The greatest source of variation in biodiversity metrics is among individuals. (A) Alpha diversities of pierced samples (Chao1, ANOVA, F=7.0752, p=1.46E-16), (B) beta diversities of pierced samples (PCoA of Bray-Curtis, PERMANOVA, F=2.65, p=0.001), (C) alpha diversities of unpierced samples (Chao1, ANOVA, F=7.757, p=3.66E-18), and (D) beta diversities of unpierced samples (PCoA of Bray-Curtis, PERMANOVA, F=2.4325, p=0.001) differed significantly across individuals. Samples from each individual are differentiated by color.

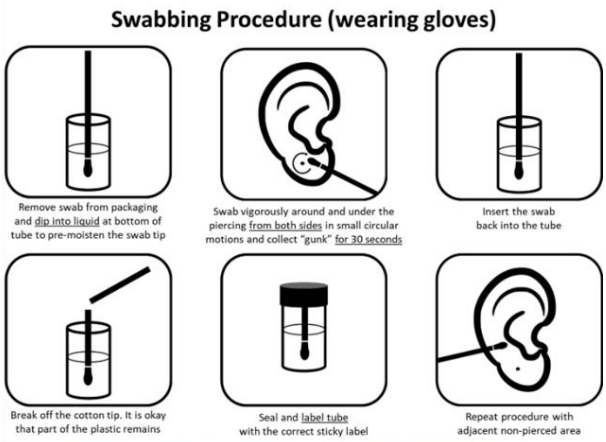

1
0.95
0.9
0.85
0.8
0.75
0.7
0.65
0.6
0.55
0.5
pNST (%)
Before
2 weeks

1.12.3 Fig. S2. Stochasticity decreased significantly in piercing microbiomes. Phylogenetic normalized stochasticity ratio (pNST) significantly decreased after 2 weeks in piercing microbiomes (orange; pairwise bootstrap, p=0.0425), but not in unpierced controls (blue; pairwise bootstrap, p=0.4602. Box and whiskers show the minimum, maximum, median, and 25th and 75th percentiles.

A


Want to find out what's living on your skin?
Your new ear piercing could contribute to cutting-edge research.*
Consider participating in our study on the bacteria that make their home on the surface of your skin!
Why do we want to study your skin?
We are studying how the complex microbes on your skin, collectively called the skin microbiome, react to extreme environmental events: for example, the sterilization of skin before it is pierced.
This study may seem small, but it has a big impact. The effects of extreme events on biological communities on a microscopic scale can help us to understand similar events, such as extreme weather events triggered by climate change, on a much larger scale.

B


Swabbing Procedure (wearing gloves)
Remove swab from packaging and dip into liquid at bottom of tube to pre-moisten the swab tip
Swab vigorously around and under the piercing from both sides in small circular motions and collect "gunk" for 30 seconds
Insert the swab back into the tube
Break off the cotton tip. It is okay that part of the plastic remains
Seal and label tube with the correct sticky label
Repeat procedure with adjacent non-pierced area

C

1.12.4 Fig. S3. Recruitment material and sampling procedure. (A) Front side of the informational card used to recruit study participants. (B) Infographic of swabbing procedure that was included with each self-sampling kit. (C) Photo of piercing microbiome sampling at Tattoo Lounge MTL, Montreal.

1.13 Bridging Chapter 1 – Chapter 2: From ecology to evolution

Environmental change can be a major driver of biodiversity dynamics by altering species composition and ecological interactions of communities. The rate of such change can influence community responses and their underlying processes (Pinek et al. 2020, Synodinos et al. 2023). Rapid change like local sterilization immediately followed by the introduction of a novel niche as observed during skin piercings in Chapter 1 is expected to have more pronounced effects due to stronger selection pressures so long as there exists sufficient phenotypic diversity for selection to act on (Ratajczak et al. 2017, Chaparro-Pedraza 2021). In contrast, slower environmental change facilitates evolutionary rescue, enabling greater community resistance and resilience (Lindsey et al. 2013). In Chapter 2, I investigate how pre-exposure to an environmental stressor through pulse treatments of acidification over the course of seven weeks had a protective effect on natural lake bacterial communities against severe acidification in the future.

The response of biological communities to environmental disturbance is multifaceted and extends beyond changes in species composition and interactions. Community-level impacts as explored in Chapter 1 are ultimately driven by evolutionary processes acting on populations of individual species (Hairston et al. 2005, Ellner et al. 2011, Urban et al. 2020). Populations have the potential to evolve over generations to disturbed conditions through selection of genetic variants with higher relative fitness (Lenski et al. 1991). To demonstrate the role of adaptation in community recovery after environmental stress, I also utilized a metagenomics approach to characterize genetic and functional variation across multiple species in Chapter 2 (Wooley et al. 2010). Rapid evolution can affect community ecology and such ecological changes can then serve as drivers for evolution in what is known as eco-evolutionary dynamics (Hendry 2017). Thus, understanding the mechanisms of biodiversity dynamics after environmental stress must

consider the simultaneous roles of ecological and evolutionary processes within and among co-existing species as well as their interactions.

Unfortunately, experimental evidence of evolutionary adaptation during species sorting as a response to disturbance is limited. Highly replicated experiments where environmental stressors can be quantitatively and precisely manipulated can be powerful in understanding the contributions of these processes during community recovery (Stewart et al. 2013). In addition to focusing on community dynamics in lieu of population genetics, one of the limitations of Chapter 1 was that it was observational, relying on participant volunteers whose demographic and behavioral traits could not be controlled. Multiple factors known to affect the diversity and ecology of human skin microbiomes, such as sex and age, exhibited little variation, providing low explanatory power for discrimination or correlation. Additionally, although skin piercings did significantly affect local microbiomes, the environmental change was qualitative because it was not feasible to measure ecological variables within the novel piercing environment, and the separate contributions of selection through skin sterilization versus environmental filtering of the piercing niche remain unknown. These limitations were addressed in Chapter 2 by conducting the experiment at the Large Experimental Array of Ponds (LEAP) facility where replicate aquatic mesocosms containing diverse and natural lake communities were stressed with known levels of pulse and press acidification. However, Chapter 1 remains complementary to Chapter 2 since it proposes a novel and convenient model system within the human environment, providing a new perspective on typical ecological disturbance studies that are often laborious, of which Chapter 2 was not exempt from. By combining Chapters 1 and 2, this thesis is able to holistically address the ecological and evolutionary processes driving biodiversity dynamics after rapid

environmental change, and its findings are widely relevant from biomedical science and human health to natural resource management and environmental conservation.

CHAPTER 2. PRE-EXPOSURE PROTECTS AGAINST SEVERE ENVIRONMENTAL STRESS IN COMPLEX NATURAL COMMUNITIES

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2.1 Abstract

Biological communities must contend with stress from changing environmental conditions or else face local extinction. Understanding the drivers and mechanisms that contribute to community resistance and resilience is crucial to biodiversity management. Pre-exposure to stress is known to help maintain community diversity and function against more severe stress in the future. Induced plasticity and ecological species sorting have been demonstrated to be important in this process. However, the role of evolutionary adaptation in community response to stress after pre-exposure is unclear, especially in complex natural communities. Here we show that pre-exposure to acidification protects lake bacterial communities against severe acidification through both species sorting and genetic adaptation. We found that communities pre-exposed to pulse treatments of acidification increased resistance but not resilience to shifts in community composition after severe press acidification even though all communities ultimately converged. Communities experiencing severe acidification were dominated by an acidophilic bacterium, *Acidiphilium rubrum*, which contained greater genetic diversity in pre-exposed relative to naïve communities. *A. rubrum* in pre-exposed communities exhibited significant genome-wide changes in allele frequencies and followed distinct evolutionary trajectories from populations in naïve communities. We observed similar patterns across other minor acidophilic species, providing independent parallel evidence for the impact of pre-exposure. Our results suggest that exposure history is a significant driver of lake bacterial community response to acidification, and that evolutionary adaptation plays a mechanistic role in this process.

2.2 Introduction

Environmental change that reduces fitness is considered stress, and such stress is common for biological communities (Hoffmann and Hercus 2000). Largely due to increasing anthropogenic impacts, environmental stress is expected to intensify on a global scale (Díaz et al. 2019). Severe stress can rapidly cause significant biodiversity decline and fundamental shifts in community composition and functioning (Sousa 1984). Community responses to stress are characterized by resistance (the capacity of a system to withstand disturbance) and resilience (the ability to recover after disturbance) (Holling 1973, Allison 2004). If community resistance and resilience are insufficient and stress is not alleviated, local biological collapse may occur. Elucidating the ecological and evolutionary mechanisms of community recovery after severe environmental stress is crucial for understanding biodiversity dynamics and improving its management and conservation.

Various modes of environmental stress exist, such as pulse and press perturbations. Pulse perturbations are relatively short in duration and allow environmental conditions to return to their pre-disturbed equilibrium, whereas disturbances that cause press perturbations are sustained over time (Bender et al. 1984). Pulse and press perturbations can elicit different community responses resulting in distinct eco-evolutionary consequences (Harris et al. 2018). One key difference lies in exposure history since pulse disturbances involve periods of stability and recovery when resistant types may increase in relative frequency and thereby contribute towards community resistance and resilience (Shade et al. 2012). In the same way that vaccination with a sub-lethal dose of a virus can prepare an individual's immune system for a potentially deadly future infection, pre-exposure to low or moderate levels of abiotic stressors can protect ecological communities from future greater disturbance. Pre-exposure has been demonstrated to

mitigate the impact of severe environmental stress on community structure in a variety of systems (Bouskill et al. 2013, Backhaus et al. 2014, Bell et al. 2019, O'Connor et al. 2020), even when the specific stressors are different in each exposure period (Bressan et al. 2008). This protective effect can extend to community and ecosystem functioning as well (Sjöstedt et al. 2018, Chen et al. 2021, van Moorsel et al. 2021).

The mechanisms through which historical exposure to stress stabilizes biological communities are complex and can involve combinations of plasticity, species sorting, and adaptation. Phenotypic plasticity may enable individual organisms to exhibit induced tolerance later in life after exposure to sublethal levels of stress (Diamond and Martin 2016). Community tolerance may also arise from environmental filtering of susceptible species during pre-exposure, shifting community composition towards more robust taxa. This species sorting process can maintain essential ecological interactions and functioning, and prevent community collapse when confronted with initially lethal levels of stress (Low-Décarie et al. 2015, Fugère et al. 2020). While the contributions of phenotypic plasticity and species sorting toward the effects of pre-exposure to stress have been well documented, much less is known about the role of evolution in this process, especially in complex natural communities. Historical exposure to environmental stress is expected to select for genotypes with higher relative fitness under severe stress, provided that adaptive alleles are present within standing variation or appear via mutation and have sufficiently positive selection coefficients to counter the effects of drift (Bell 2013a, Martin et al. 2013, Orr and Unckless 2014). Mean absolute fitness should increase with the relative frequency of adaptive genotypes. Following initial selection, population sizes of adaptive genotypes will increase, thus permitting additional, mostly neutral, mutational input and thereby maintaining greater genetic diversity (Amos and Harwood 1998, Banks et al. 2013). This process

of evolutionary rescue has been documented across many systems, but experiments investigating the mechanisms and drivers of evolutionary rescue have been typically limited to individual species in laboratory environments (Gomulkiewicz and Shaw 2013, Carlson et al. 2014, Bell 2017).

Acidification is well-known to be a major environmental stressor for aquatic ecosystems, and its detrimental effects on biodiversity has been a considerable challenge for management and conservation (Dillon et al. 1984, 1987, Baker and Christensen 1991, Camargo and Alonso 2006). Several whole ecosystem studies have been conducted on acidification of freshwater lakes, revealing declines in species diversity and disruptions to primary production and nutrient cycling (Kwiatkowski and Roff 1976, Rudd et al. 1988, Schindler 1990). Despite continuing relevance of acidification to ecological and human health (Lacoul et al. 2011, Falkenberg et al. 2020), understanding of the evolutionary processes underlying community responses in aquatic ecosystems remains limited. Unfortunately, it is inherently difficult to disentangle evolutionary adaptation from ecological species sorting because their effects on community composition can be indistinguishable using standard community profiling techniques. As such, demonstrating the dual roles of ecology and evolution in recovery of natural communities has been challenging.

To address this gap, we experimentally manipulated aquatic mesocosms containing complex microbial communities from a natural lake to test the impact of pre-exposure on community resistance and resilience after severe acidification. In addition to monitoring taxonomic composition, we employed whole-genome shotgun (WGS) sequencing to investigate the potential role of evolutionary adaptation within species. We hypothesized that (1) pre-exposure to pulse treatments of acidification will improve community resistance and resilience to severe and sustained acidification through species sorting for acidophiles, and (2) surviving

species will evolve along distinct trajectories in pre-exposed and naïve communities. We found that pre-exposure to pulse treatments of acidification reduced communities to several primarily acidophilic genera, but these simpler communities were more resistant to severe acidification and suffered lower losses in taxonomic richness and evenness compared to naïve communities. This resulted in higher absolute taxonomic diversity for the pre-exposed communities. Pre-exposure caused significant divergence in community composition between pre-exposed and naïve communities following pulse treatments, indicating distinct species sorting processes, but ultimately communities converged again after approximately eight weeks of severe acidification. We found evidence that pre-exposure lessened genome-wide changes in allele frequencies across multiple acidophilic species when faced with severe stress. In addition, we observed independent evolutionary trajectories in pre-exposed and naïve communities for the overwhelmingly dominant species under severe acidification, *Acidiphilium rubrum*, which also maintained greater genetic diversity in pre-exposed communities than it did in naïve communities. Thus, we demonstrate how pre-exposure can protect communities from the effects of environmental stress through the simultaneous mechanisms of species sorting and evolutionary adaptation. More broadly, these results provide further insight into the ecological and evolutionary drivers and mechanisms of resistance and resilience in complex natural communities.

2.3 Methods

2.3.1 Study site

We conducted the experiment at the Large Experimental Array of Ponds (LEAP) facility located at the Gault Nature Reserve in Mont-Saint-Hilaire, Quebec, Canada. We created replicate 1,000 L mesocosms on May 23/24, 2017 by sourcing water from the nearby glacial-eroded Lake Hertel

(45°32’ N, 73°09’ W), which is protected under UNESCO as part of the Mont Saint Hilaire Biosphere Reserve. The naturally mesotrophic lake has a maximum depth of 8.2 m and a natural pH of 7.5-9.5 (Goswami 1971, Thibodeau et al. 2015). We used a coarse sieve to filter water from Lake Hertel before it entered the mesocosms, which prevented introduction of fish and most invertebrates leaving a complex community of naturally co-occurring zooplankton, phytoplankton, bacteria, and viruses.

2.3.2 Experimental design

We designed the biphasic pulse-press experiment to test the isolated and interacting effects of several levels of acidification pre-exposure and dispersal regimes on community response to severe acidification, which has been fully described in a previous study (Bell et al. 2019). Here, we focused on only the 16 mesocosms pre-exposed to the strongest acidification treatment of pH 4 as well as the 16 naïve mesocosms that were left untreated and remained at their natural acidity of approximately pH 8.5 (Fig. 1A). Briefly, in phase I of the experiment, we maintained pre-exposure to pH 4 through weekly pulse titration with 10N HCl for seven weeks, from June 7 – July 26. Pre-exposed mesocosms exhibited a sharp decrease in pH buffering capacity with each acidification pulse in the first weeks of phase I (Fig. 1B). Half of pre-exposed and naïve mesocosms were also under a global dispersal regime where we mixed 1% of water from each metacommunity of four mesocosms in a pool and then redistributed on a weekly basis allowing for migration within metacommunities. We initiated phase II on August 2 when all mesocosms were acidified to pH 3 in a sustained press treatment and dispersal regimes were terminated. Phase II lasted for approximately eight weeks until the end of the experiment on September 25.

We also established four isolated control mesocosms subjected to neither phase I nor phase II treatments.

2.3.3 Sample collection

We monitored mesocosms weekly for water pH (Fig. 1B). We used integrated water samplers made from 35 cm long, 2.5 cm diameter PVC tubing to sample water biweekly from the top 35 cm of the water column at five random locations within each mesocosm until a total of 2 L of water was collected. We used independent samplers for each mesocosm to minimize cross-mesocosm contamination. We subsequently stored water samples in dark, triple-washed Nalgene bottles at 4°C before filtration later that same day. For each sample, we filtered 500 mL of water at an on-site lab on using 0.22 µm pore size, 47 mm diameter Millipore hydrophilic polyethersulfone membranes (Sigma-Aldrich). We then transported filters to the laboratory on dry ice and stored them at -80 ºC prior to DNA extraction.

2.3.4 DNA extractions

We extracted DNA from samples collected across four time points at the beginning and end of phase I (June 7, July 26) and phase II (August 9, September 25) hereafter referred to as “Start”, “Phase I”, “Phase II”, and “End” (Fig. 1B). In total, there were 128 samples and 12 controls. We extracted and purified total genomic DNA from half filter papers using the DNeasy PowerWater kit (QIAGEN) following QIAGEN guidelines including a 5-minute vortex of the filter with beads and an additional incubation of 30 minutes at 37°C with 1 µL RNase (Thermo Scientific) after cell lysis and before the first supernatant transfer to remove RNA contamination (Barbosa Da Costa et al. 2021).

2.3.5 16S rRNA sequencing

We profiled bacterial community composition using 16S rRNA amplicon sequencing. Specifically, we used the primers U515_F (5'-ACACGACGCTCTTCCGATCTYRYRGTGCCAGCMGCCGCGGTAA-3') and E786_R (5'-CGGCATTCCTGCTGAACCGCTCTTCCGATCTGGACTACHVGGGTWTCTAAT-3') to target an approximately 200 bp amplicon of the V4 region of the 16S rRNA gene as described previously (Preheim *et al.* 2013). We treated samples that initially failed to PCR amplify with sodium acetate and then ethanol precipitated with GenElute LPA Linear Polyacrylamide (Sigma-Aldrich) to increase DNA concentration (Bartram et al. 2009). Genomic DNA quality control, sequencing library preparation, two-step PCR (Barbosa Da Costa et al. 2021), and amplicon sequencing via Illumina MiSeq v2 PE250 was conducted at the McGill Genome Centre.

2.3.6 Amplicon analysis

We processed raw 16S rRNA amplicon sequences using the QIIME2 bioinformatics pipeline (Bolyen et al. 2019). We first removed primer sequences using cutadapt followed by identification of amplicon sequence variants (ASVs) using DADA2 (Martin 2011, Callahan et al. 2016). We aligned ASVs using MAFFT and constructed phylogenetic trees using FastTree 2 based on Jukes-Cantor distances (Price et al. 2010, Katoh and Standley 2013). We created a custom reference database by using the U515_F/E786_R primers to *in silico* extract the target 16S rRNA V4 region from the SILVA 138 database (Yilmaz et al. 2014). We generated taxonomic bespoke weights according to occurrence records in freshwater habitats using redbiom and Qiita by limiting sample type to "fresh water" or "freshwater" and context to

"Deblur_2021.09-Illumina-16S-V4-90nt-dd6875" (Gonzalez et al. 2018, McDonald et al. 2019, Kaehler et al. 2019). We then assigned taxonomies to ASVs with a naïve bayes classifier trained using scikit-learn on the extracted SILVA 138 database that was modulated by the freshwater taxonomic weights (Pedregosa et al. 2011). We accepted taxonomic assignments if classification confidence was at least 0.7 (Ziemski et al. 2021). We assessed alpha diversity using Shannon's index computed after we rarified ASVs of each sample to a depth of 1,000 based on saturation of rarefaction curves (Shannon 1948). We compared alpha diversity between pre-exposed and naïve communities at each time point using Kruskal-Wallis tests (Kruskal and Wallis 1952). We also assessed longitudinal differences in alpha diversity using Wilcoxon signed-rank and Mann-Whitney U tests and statistical significance via Benjamini & Hochberg corrected q-values (Wilcoxon 1945, Mann and Whitney 1947, Benjamini and Hochberg 1995, Bokulich et al. 2018).

2.3.7 Whole-genome shotgun sequencing

We selected samples from all isolated (*i.e.*, no dispersal in phase I) pre-exposed and naïve mesocosms except for one mesocosm from each phase I treatment for further metagenomic analysis along with control mesocosms. In total, we subjected 68 samples across the four time points to deep WGS sequencing at an average of 220 million reads per sample. We focused sequencing on phase I samples (~330 million reads/sample) compared to phase II samples (~110 million reads/sample) to maximize the probability of detecting dominant phase II species that were initially at low abundances in phase I. Quality control, library prep, and sequencing on Illumina NovaSeq 6000 PE150 were conducted at the McGill Genome Centre.

2.3.8 Metagenomic analysis

We processed and analyzed WGS sequences within the anvi'o framework (Eren et al. 2021). We first removed Illumina TruSeq LT adaptors with cutadapt and quality filtered reads using illumina-utils (Eren et al. 2013). We used MEGAHIT to co-assemble reads from the same mesocosm across the four time points (Li et al. 2015). We merged all contigs and removed those less than 2,500 bp. We then independently mapped reads from each sample to contigs using Bowtie2 and SAMtools (Langmead and Salzberg 2012, Danecek et al. 2021). We identified prokaryotic genes using Prodigal (Hyatt et al. 2010). We used hidden Markov models for collections of 71 bacteria, 76 archaea, and 83 protist single-copy core genes (SCGs) as well as for transfer RNA and ribosomal RNA 5S, 12S, 16S, 18S, 23S, and 28S to identify and recover them from contigs (Parks et al. 2018, Lee 2019, Manni et al. 2021). We functionally annotated genes using the Clusters of Orthologous Genes (COG), Pfam, Kyoto Encyclopedia of Genes and Genomes (KEGG) and InterPro databases (Jones et al. 2014, Galperin et al. 2021, Mistry et al. 2021, Kanehisa et al. 2023, Paysan-Lafosse et al. 2023). We assigned gene-level taxonomies using Centrifuge (Kim et al. 2016).

We clustered contigs into bins using CONCOCT and MetaBAT2, which we then dereplicated and aggregated into metagenome-assembled genomes (MAGs) using DAS Tool (Alneberg et al. 2014, Sieber et al. 2018, Kang et al. 2019). We estimated completeness and redundancy of MAGs based on SCG collections. We determined prokaryotic taxonomic identities of MAGs via the most frequent of 22 bacterial SCGs from the Genome Taxonomy Database (GTDB) using DIAMOND (Buchfink et al. 2015, Parks et al. 2022). We grouped MAGs with a completeness >90% and redundancy <10% along with the most dominant MAG at the end of the experiment based on mean coverage within samples from each phase I treatment

and time point. We used these MAG groups to identify enriched gene clusters via a pangenomics approach incorporating a generalized linear model with the logit linkage function to calculate enrichment scores and false discovery rate adjusted q-values (Storey and Tibshirani 2003, Delmont and Eren 2018, Shaiber et al. 2020). For the dominant MAG at the end, we used a reference genome approach to identify single nucleotide polymorphisms (SNPs) occurring in at least two samples (Kiefl et al. 2023). We also calculated pairwise fixation index (F_{ST}) and the ratio of non-synonymous to synonymous rates of polymorphisms (pN/pS) between samples (Schloissnig et al. 2013, Kiefl et al. 2023). We determined significant differences in pN/pS ratios of genes between phase I treatments at each time point via two-sided T-tests and Holm-Bonferroni adjusted p-values (Student 1908, Holm 1979).

Three major genera (*Acidiphilium*, *Acidocella*, and *Granulicella*) comprised phase II communities based on 16S rRNA amplicon and MAG results. We further assessed population microdiversity of species within these genera using InStrain (Olm et al. 2021). We downloaded reference genomes from the three genera from NCBI RefSeq and merged them with MAGs to create a custom genome database that we then dereplicated using dRep, checkM, MASH, and fastANI with a MASH sketch size of 10,000 and a minimum overlap between genomes of 0.3 (Parks et al. 2015, O'Leary et al. 2016, Ondov et al. 2016, Olm et al. 2017, Jain et al. 2018). We used Prodigal to profile genes for each genome, and we competitively mapped reads against reference genomes and MAGs using Bowtie2 and SAMtools (Hyatt et al. 2010, Langmead and Salzberg 2012, Danecek et al. 2021). We called SNPs using a minimum coverage threshold of 5 and a minimum SNP frequency of 0.05 using InStrain (Olm et al. 2021). We calculated scaffold-level metrics including average nucleotide identity (ANI) and nucleotide diversity (π) (Nei and Li 1979, Konstantinidis and Tiedje 2005, Olm et al. 2021). We also calculated allele frequency

change of polarized major alleles through subtracting the frequency of the major allele at each SNP by the frequency of that same allele in a subsequent time point, resulting in a negative frequency change. We longitudinally compared pairwise ANI and π as well as allele frequency changes between pre-exposed and naïve communities using Dunn's test and assessed for statistical significance via Holm-Bonferroni adjusted p-values (Dunn 1964, Holm 1979). For the most dominant species identified in phase II, we used permutation tests randomizing the mesocosm of each SNP to assess the significance of the number of shared SNPs present in both phase II and the end of the experiment between populations in pre-exposed and naïve communities.

2.4 Results

2.4.1 16S rRNA

An average of 28,387 16S rRNA reads were produced per sample. Rarefaction retained 134 (95.71%) samples after removing six samples that produced less than 5 reads. Alpha diversity differed significantly between pre-exposed and naïve communities indicating the effect of pre-exposure on ASV richness and evenness (Fig. 2A). The experiment began with all mesocosms at approximately a Shannon's index of 5.6, but pre-exposure caused a significant decrease by the end of phase I (Kruskal-Wallis, q=4.3E-05) (Fig. S1). Although both pre-exposed and naïve communities decreased in alpha diversity due to severe acidification, naïve communities experienced a significantly greater decline resulting in significantly lower alpha diversity than pre-exposed communities in phase II (Kruskal-Wallis, q=1.1E-05). Control communities remained unchanged. From phase II to the end of the experiment, pre-exposed communities continued to decrease in alpha diversity while naïve communities recovered slightly such that all

communities besides controls ultimately converged regardless of pre-exposure (Kruskal-Wallace, q=0.28). The observed recovery in alpha diversity as measured by Shannon's index of naïve communities coincided with a small but significant increase in the number of observed ASVs (Wilcoxon signed-rank, p=6.2E-05) (Fig. S2). The change in alpha diversity of pre-exposed communities was significantly negative between all time points (Wilcoxon signed-rank, start-phase I p=9.8E-04, phase I-II p=0.001, phase II-end p=0.032) (Fig. S3). This was also true of naïve communities except for during phase I (Wilcoxon signed-rank, start-phase I p=0.17, phase I-II p=9.2E-05, phase II-end p=9.2E-05). Change in alpha diversity was significantly different between pre-exposed and naïve communities across all time points (Mann-Whitney U, start-phase I p=2E-05, phase I-II p=7E-05, phase II-end p=1E-05). Dispersal did not have an obvious effect on alpha diversity throughout the experiment.

We used a total of 6,206 V4 16S rRNA sequences (6,003 "fresh water" and 203 "freshwater) to weight taxonomic assignment towards those found previously in freshwater environments (Kaehler et al. 2019). Community composition of mesocosms shifted drastically due to acidification in phases I and II (Fig. 2B). The top ten genera-level classifications in order of decreasing relative frequency included "Chloroplast", "Acidiphilium", "Acetobacteraceae_uncultured", "Acidocella", "Mucilaginibabcter", "Granulicella", "Mitochondria", "Polynucleoacter", "Acidisoma", and "Sporichthyaceae_hgcl_clade". Most sequences were classified as bacteria, but a significant portion of reads assigned to "Chloroplast" and "Mitochondria", likely reflecting the presence of photosynthetic eukaryotic algae, especially at the start and end of the experiment. All communities began with a large diversity of bacteria, mainly from the Bacteroidota and Proteobacteria phyla. They are further classified into the classes Bacteroidia, Alphaproteobacteria, and Gammaproteobacteria, and the orders

Burkholderiales, Caulobacterales, Cytophagales, Flavobacteriales, Rhodobacterales, Rickettsiales, Sphingomonadales among many others. Pre-exposed communities became dominated by the family Acetobacteraceae and genera Mucilaginibacter and Granulicella by the end of phase I. In contrast, naïve communities remained highly diverse with an increase in the genus Polynucleobacter and the family Sporichthyaceae, which were also present in relatively high frequency in control communities. Dispersal did not affect species composition in either pre-exposed or naïve communities. In phase II, communities within pre-exposed mesocosms continued to shift, with an increase in Acidocella and Acidosoma and the disappearance of most genera not among the top ten. A single genus, Acidocella, overwhelmingly dominated naïve communities in phase II. By the end of the experiment, community composition of both pre-exposed and naïve communities regardless of dispersal converged and became dominated by Acidiphilium followed by Acetobacteraceae, Granulicella, and Acidocella.

2.4.2 Metagenomics

An average of 242,096,037 raw read pairs were sequenced per sample with a minimum of 9,883,771 and maximum of 550,897,325 read pairs. As intended, samples from before phase II yielded approximately two- to three-fold the number of read pairs compared to samples from after phase II (start: 204,301,033; phase I: 369,637,547; phase II: 142,871,337; end: 99,883,771). On average, 93.77% of read pairs passed quality filtration (227,096,142 read pairs per sample). Each mesocosm co-assembly contained an average of 711,468 contigs with an average total base count of approximately 1.95 Gb (2,875 bp per contig). An average of 89.58% of read pairs mapped successfully to contigs resulting in 407,520,384 forward and reverse read pairs mapped per sample. A significantly lower proportion of reads mapped in samples from the start of the

experiment than at any other time point ($\chi 2$ test, $p<0.001$) consistent with a high proportion of reads originating from photosynthetic algae and the relatively low abundance of eukaryotic genomes detected. The final contig database consisted of 3,104,372 contigs totaling approximately 20.8 Gb, and as called by Prodigal it contained 2,0934,491 genes from an estimated 2,328 bacterial and 64 eukaryotic genomes based on SCGs. A total of 180,619 transfer RNAs, 912 16S rRNA, 277 18S rRNA, 1,635 23S rRNA, and 510 28S rRNAs were identified, and 7,902,772 COG categories and functions, 2,128,414 COG pathways, and 17,805,329 Pfam protein families were annotated.

We binned contigs into 81 MAGs accounting for approximately 4 Mb, which represented 1.90% of all nucleotides. Bacterial MAGs ranged from 524 Kb to 15.6 Mb and varied in quality (52.11-100% completeness, 0-85.92% redundancy). Two eukaryotic MAGs were also binned (23.95 Mb and 19.14 Mb). Twenty-two MAGs with a minimum of five bacterial SCGs were taxonomically classified to the phyla Actinobacteriota, Bacteroidota, Proteobacteria, and Verrucomicrobiota based on at least 75% supporting SCGs. These corresponded to 14 unique genera and 12 species, which included *Acidiphilium rubrum* and Acidocella also present in the 16S rRNA data. The MAG assigned to *A. rubrum* constituted 97-98% of all mapped reads at the end of the experiment across both pre-exposed and naïve communities. Pangenomic functional analysis utilized 18 MAGs that had a completeness of >90% and redundancy <10% along with the *A. rubrum* MAG. Significantly enriched gene clusters included 234 COG functions, 32 COG categories, 9 COG pathways, and 483 Pfam protein families (adjusted $q<0.05$) (Supplementary tables 1-4, not shown due to length).

Because the MAG assigned to *A. rubrum* was virtually the only one present at the end of the experiment, we reran the anvi'o pipeline by mapping all reads to the *A. rubrum* reference

genome (NCBI RefSeq assembly GCF_900156265.1, strain ATCC 35905). Pairwise F_{ST} of *A. rubrum* among communities across time exhibited the maximum range of values between 0 and 1 (Fig. 3). At the start of the experiment, *A. rubrum* across communities were genetically similar. By the end of phase I, F_{ST} had increased significantly between pre-exposed and naïve communities. We also observed differentiation among certain naïve communities during phase I. In phase II, the F_{ST} difference between pre-exposed and naïve communities as well as among naïve communities decreased. By the end of the experiment, F_{ST} of *A. rubrum* between all communities regardless of pre-exposure decreased similarly to that observed at the beginning. The notable exceptions were between naïve mesocosm F4 and pre-exposed mesocosms K3 and K7 as well as among naïve mesocosms F3 and F4. We were unable to calculate F_{ST} of *A. rubrum* populations in control communities because no SNPs were shared with populations in pre-exposed and naïve communities. We identified four *A. rubrum* genes that exhibited significantly different pN/pS ratios between pre-exposed and naïve communities in phases I and II whereas *A. rubrum* in control communities differed significantly from pre-exposed and naïve communities in eight genes (Table 1).

We obtained 44 reference genomes from 17 species within Acidiphilium, Acidocella, and Granulicella from NCBI RefSeq and merged them with MAGs to create a custom genome database, which we dereplicated to 29 reference genomes and 29 MAGs. Reads from pre-exposed and naïve communities successfully mapped competitively to five Acidiphilium species (*A. iwatense*, *A. multivorum*, *A. rubrum*, *A.* sp. *C61*, *A.* sp. *PA*), three Acidocella species (*A. aminolytica*, *A. facillis*, *A.* sp. *KAb 2-4*), *Granulicella* sp. *5B5*, and a single MAG assigned to an unnamed species within the order Rickettsiales (family *SXRF01*, genus *RFOF01*). Reads from control communities mapped only to other MAGs. Mean coverage of genomes differed

significantly across species and between pre-exposed and naïve communities across time (Fig. S4). Despite focusing sequencing on phase I samples, the total number of mapped reads in phase I was dwarfed by phase II samples (Fig. S5A). Samples from the beginning of the experiment and from naïve mesocosms in phase I yielded relatively few mapped reads, mapping only to *A.* sp. *KAb 2-4* (average of 17,875 and 44,997 mapped read pairs per sample resulting in approximately 1X and 2.3X coverage respectively). Reads from pre-exposed communities in phase I mapped across all 10 species (Fig. S5B). Reads from phase II samples mapped mostly to *A. facilis* with much more mapped from naïve than pre-exposed communities. At the end of the experiment, most reads mapped to *A. rubrum* followed by *A.* sp. *PA*. Despite this observed heterogeneity in read mapping, changes in genome-wide SNP allele frequencies showed similar patterns across all species within Acidiphilium and Acidocella (Fig. 4). For pre-exposed communities, all five Acidiphilium and all three Acidocella species exhibited significantly less change in allele frequencies from phase II to the end of the experiment than from phase I to II. Additionally, except for *A. iwatense* and *A.* sp. *KAb 2-4*, species in pre-exposed communities also exhibited less change in allele frequencies than naïve communities from phase II to the end. By far the most dominant species at the end of the experiment was *A. rubrum* (Fig. S4-5). Mean pairwise ANI of *A. rubrum* scaffolds was significantly lower at the end of the experiment than during phase II despite lower variance ($p<0.05$) (Fig. 5A). In addition, ANI of *A. rubrum* was significantly lower in pre-exposed than naïve communities with greater variance in both phase II and the end of the experiment ($p<0.05$) (Fig. 5A). The same patterns were true for π of *A. rubrum* scaffolds where π was significantly higher at the end of the experiment than during phase II as well as in pre-exposed than naïve communities in both phase II and at the end of the experiment ($p<0.05$) (Fig. 5B). The population of *A. rubrum* in pre-exposed communities shared

36 SNPs and in naïve communities shared 31 SNPs with 12 SNPs shared across populations in communities (Fig. 6A). Permutation tests indicated that the number of shared SNPs within pre-exposed and naïve communities were significantly higher than neutral expectations (N=10,000, $p<0.05$) (Fig. 6B).

2.5 Discussion

2.5.1 Community response to pre-exposure

Environmental stress can negatively impact biodiversity structure and functioning, but pre-exposure to sublethal levels of stress has the potential to protect biological communities from more severe stress in the future. Understanding the contributions of species sorting and evolutionary adaptation towards this process will shed light on the underlying mechanisms of community recovery after disturbance. Such insights may inform the contexts under which pre-exposure confers benefits, the extent of its effects as well as its limitations. Here, we provide evidence that pre-exposure to pulse treatments of acidification was able to bolster aquatic microbial communities against severe acidification, ecologically through changes in community composition and evolutionarily via genome-wide shifts of genetic variation across multiple species.

2.5.2 Species sorting

Pre-exposure to acidification had profound effects on bacterial communities through species sorting by the end of phase I, as indicated by significantly lower alpha diversity. Although pre-exposure was initially detrimental to taxonomic richness and evenness, pre-exposed communities

exhibited greater resistance to severe acidification in phase II; alpha diversity not only decreased significantly less than naïve communities but also exhibited significantly higher absolute values.

This increased community resistance may be due in part to species sorting of acidophiles that were previously at very low frequency in pre-exposed communities at the start of the experiment such as the family Acetobacteraceae, which contains several acidophilic genera including Acidiphillium, Acidisoma, and Acidocella (Komagata et al. 2014). Pre-exposure also selected for Granulicella, a genus of acidophiles within the family Acidobacteriaceae (Dedysh 2017), and Mucilaginibacter, a diverse genus within the family Sphingobacteriaceae that contains species previously isolated from acidic forest soils (Nguyen et al. 2018) and documented to grow in acidic conditions as low as pH 2 (Madhaiyan et al. 2010). In contrast, these taxonomic groups were of low abundance or absent in naïve communities at the end of phase I. Instead, there was an increase in the genus Polynucleobacter, a ubiquitous and diverse freshwater bacterioplankton that can tolerate a wide range of environmental conditions (Newton et al. 2011, Jezbera et al. 2011, 2012, Hahn et al. 2016), and the family Sporichthyaceae, which contains four named species of motile facultative anaerobes with aerial hyphae isolated from soil, lake sediment, and human skin (Rainey et al. 1993, Tamura et al. 1999, Tamura 2014, Lee et al. 2018, Qu et al. 2018). Both Polynucleobacter and Sporichthyaceae were also observed in control communities at the end of phase I, so their presence likely indicates seasonal effects or selection by the mesocosm environment.

In phase II, diversity within naïve communities crashed and became overwhelmingly dominated by Acidocella with minor and inconsistent contributions from Acetobacteraceae, Acidisoma, and Granulicella. In contrast, pre-exposed communities retained significantly greater evenness among these taxa as well as the presence of chloroplast DNA possibly indicating the

survival of photosynthetic algae in certain mesocosms. Acetobacteraceae and Granulicella were among the selected taxa in pre-exposed communities during phase I, indicating that pre-exposure had strengthened these taxa against severe acidification. Thus, we provide evidence for our hypothesis that pre-exposure improved initial community resistance to severe acidification through species sorting for acidophiles.

However, contrary to our hypothesis, pre-exposure did not improve long-term community resistance, with both alpha diversity and community composition ultimately converging across pre-exposed and naïve communities by the end of the experiment. Despite this convergence, pre-exposure did cause a significantly different species sorting trajectory in phase II. Additionally, pre-exposure did not improve community resilience as alpha diversity failed to recover and decreased throughout the experiment, albeit at lower rates between each successive time point.

Interestingly, Shannon's index and the observed number of ASVs in naïve communities did recover significantly between phase II and the end of the experiment. Although the 16S rRNA analysis included mesocosms under dispersal during phase I, we did not observe any significant effects on taxonomic richness, evenness, or composition at any time point. Furthermore, there was no additional input from the source lake to mesocosms after the start of the experiment. Thus, seemingly novel ASVs at the end of the experiment such as those assigned to the genus Acidiphilium were most likely previously present, but at undetectably low absolute or relative abundances. Aerial dispersal is another possibility. Regardless, sufficient positive selection was necessary to overcome loss through ecological drift since Acidiphilium would have started at very low abundance in either scenario. Our findings are consistent with a previous study on the same source lake that successfully recovered non-obligate acidophilic bacteria capable of surviving at pH 2, which suggests that such acidophiles are ever present in ordinary

freshwater (Low-Décarie et al. 2016). Recovery of Shannon's index in naïve communities was therefore driven by a combination of population growth of initially undetected acidophiles and increases in taxonomic evenness.

All communities besides controls converged to a single profile composed of mostly Acidiphilium at the end of the experiment. Acidiphilium (meaning "acid lover") is a genus of gram negative, motile, flagellated, photosynthetic, straight rod Proteobacteria containing eight named species (Bhattacharyya et al. 1991, Okamura et al. 2015, Hiraishi and Imhoff 2015). Acidiphilium are known to be mesophilic and obligately acidophilic, growing between pH 2.0-5.9 but not above 6.1 (Hiraishi and Imhoff 2015). Here, we show that while Acidiphilium may not grow well under neutral pH conditions, it does persist at low levels in natural lake freshwater of approximately pH 8.5 and can rapidly increase if environmental conditions become sufficiently acidified. Putative acidophiles selected through species sorting in pre-exposed communities (Acetobacteraceae and Granulicella) also persisted at the end of the experiment and at slightly greater frequencies than in naïve communities, potentially suggesting long-term effects of pre-exposure.

2.5.3 Functional enrichment

Pangenomic analysis indicated that communities were enriched for different gene clusters across time. Pre-exposure to acidification enriched for glycosyl hydrolases according to COG functions/Pfam protein families and for defense mechanisms through cell wall/membrane/envelope biogenesis based on COG categories. These gene clusters were uniquely enriched in pre-exposed communities at the end of phase I, demonstrating that pre-exposure caused significant changes in community functional profiles. In contrast, naïve

communities did not exhibit any functional enrichment. Only a single MAG was associated to each time point after severe acidification leading to a wide diversity of significantly enriched gene clusters in phase II and even more at the end of the experiment. Because no other MAGs were grouped at these time points, any unique gene within these two MAGs would appear enriched so this may be an artifact.

2.5.4 Evolutionary adaptation

We identified and tracked SNPs across time points in nine reference genomes (five Acidiphilium species, three Acidocella species, and *Granulicella sp. 5B5*) and a single MAG (an unknown Rickettsiales bacterium). Significant changes in genome-wide allele frequencies, including SNPs where the major allele was completely replaced, were observed in all genomes between at least two time points, demonstrating the role of evolutionary processes in community responses to acidification. Because these genomes were not detected in control communities, we were unable to characterize and directly compare SNPs from populations that did not experience severe acidification. However, of the six MAGs that did map reads from control communities, genome-wide allele frequency changes were considerably lower as expected (Fig. S6).

The only genome that mapped reads from both pre-exposed and naïve communities across all four time points was *Acidocella* sp. *KAb 2-4*. Although average coverage of *A.* sp. *KAb 2-4* was only between 1-3X at the start of the experiment due to its low abundance, we were still able to track hundreds of SNPs through phase I. Our analyses did not reveal significant differences in allele frequency change between pre-exposed and naïve communities. However, between phases I and II, *A.* sp. *KAb 2-4* in pre-exposed communities did exhibit significantly greater allele frequency change than in naïve communities, suggesting stronger selection

pressures. Like Acidiphilium, Acidocella (meaning “acid cell”) is also a genus of gram negative Proteobacteria (Hiraishi 2015). It can be differentiated from Acidiphilium by its slightly curved rods and coccobacilli, lack of photosynthetic pigments and bisphosphatidylglycerol, and presence of 2-hydroxy fatty acid (Hiraishi 2015). Acidocella contains five named species, and like Acidiphilium are mesophilic and obligately acidophilic growing between pH 2.5-6.0 and not above 6.1 (Kimoto et al. 2010, Jones et al. 2013, Hiraishi 2015, Okamoto et al. 2017). We could only compare between pre-exposed and naïve communities during phase I and between phases I and II with *A.* sp. *KAb 2-4*, so it is unclear how generalizable these observations are.

We tracked SNPs in pre-exposed communities between phases I and II as well as in both pre-exposed and naïve communities between phase II and the end of the experiment across all species of Acidiphilium and Acidocella. These eight acidophilic species independently exhibited strikingly similar patterns of genome-wide allele frequency changes (Fig. 4). In pre-exposed communities, severe acidification caused significantly greater change between phases I and II than between phase II and the end of the experiment. This suggests that the severe press acidification treatment initially caused relatively strong selection. Interestingly, between phase II and the end of the experiment, six of the eight acidophilic species (excluding *A. iwatense* and *A.* sp. *KAb 2-4*) exhibited significantly lower changes in genome-wide allele frequency in pre-exposed communities than naïve communities. These patterns were consistent across species with varying demographic trajectories as reflected by changes in genome coverage (Fig. S4). This result provides strong parallel evidence from six independent species that pre-exposure to acidification in phase I resulted in weaker selection during phase II, presumably because species had already been pre-adapted to severe acidification through pre-exposure.

Besides Acidiphilium and Acidocella, the two other genomes present were *G.* sp. *5B5* and an unnamed Rickettsiales bacterium in the family SXRF01. *G.* sp. *5B5* was only observed in pre-exposed communities and exhibited similar allele frequency changes as other acidophiles, which is unsurprising given that this species has been previously shown to grow well under acidic conditions of pH 4-5 (Fig. 4) (Campanharo et al. 2016). However, between phase II and the end of the experiment, *G.* sp. *5B5* exhibited significantly less SNPs than between phases I-II. This was due to a drop in sequencing coverage that likely reflected population decline, perhaps due to intolerance of severe acid conditions or competition from other acidophiles (Fig. S4). Allele frequencies of observable genome-wide SNPs in *G.* sp. *5B5* were significantly lower, which, in combination with population decline, signals the lack of adaptive alleles to severe acidification. While most Rickettsiales are known to have obligate intracellular lifestyles, with the most notorious being pathogens that cause typhus, the SXRF01 family represents a basal lineage that is flagellated, exhibits chemotaxis and aerotaxis, and is likely free-living in aquatic environments (Yu and Walker 2006, Moncadas et al. 2023). Rickettsiales are not known to be acidophiles, which is consistent with it having the only genome to exhibit a distinct pattern of allele frequency change. Unlike the other acidophiles including *G.* sp. *5B5*, allele frequency change in Rickettsiales from pre-exposed communities was significantly lower between phases I and II than between phase II and the end of the experiment.

2.5.5 The dominance of *Acidiphilium rubrum*

16S rRNA and MAG analysis indicated that all communities regardless of pre-exposure contained a single genus, Acidiphilium, by the end of the experiment, and competitive metagenomic read mapping of Acidiphilium reference genomes revealed that communities were

dominated specifically by *Acidiphilium rubrum* with *Acidiphilium* sp. *PA* as a minor member (Fig. S5). *A. rubrum* is a highly acidophilic purple bacterium that can be isolated from acid mine drainage sites of pH 2-3 (Wichlacz et al. 1986, Johnson et al. 2001, Aytar et al. 2015). Its dominance at the end of the experiment suggests the possibility that *A. rubrum* has a selective advantage over other acidophiles at such low pH. *A. rubrum* across pre-exposed and naïve communities at the end of the experiment were relatively homogenous based on F_{ST} calculations, but populations between pre-exposed and naïve communities were significantly differentiated by the end of phase I, indicating an independent evolutionary trajectory caused by the pre-exposure treatment (Fig. 3). This genetic differentiation decreased slightly in phase II, implying that severe acidification imposed similar selection pressures as pre-exposure did in phase I. However, *A. rubrum* in pre-exposed communities were even more differentiated from pre-exposed and naïve populations at both the beginning and the end of the experiment, suggesting that the pre-exposure treatment was not just quantitatively but qualitatively different, and that pre-exposed *A. rubrum* at the end of phase I continued to adapt under severe acidification in phase II.

The pN/pS ratio characterizes selection constraints by comparing observed with expected ratios of non-synonymous and synonymous substitutions (dN/dS) from pools of sequences without haplotype information (Schloissnig et al. 2013). Almost all pN/pS ratios greater than 1 in *A. rubrum* genes were observed in pre-exposed and naïve communities at the end of the experiment, suggesting strong selection pressures on these 10 genes due to severe acidification despite significantly less genome-wide change in allele frequency during phase II (Supplementary table 4, not shown due to length). Pre-exposure caused greater selection in several *A. rubrum* genes including speE, sdhA, and prpB compared to naïve communities after phase I (Table 1). A single gene, dnaE, was found to be under greater selection in *A. rubrum* of

pre-exposed than control communities. This same gene was also under greater selection in *A. rubrum* from naïve compared to control communities after severe acidification in phase II, showing that pre-exposure and severe acidification caused similar selection pressures on this gene. However, a different gene was under greater selection in naïve than pre-exposed communities in phase II, indicating the selection pressures of pre-exposure and severe acidification were not identical. Interestingly, the same gene, ctaD, was found to exhibit significantly higher pN/pS ratios in control communities compared to both pre-exposed and naïve communities in phase II, which may suggest a relaxing of selection on this gene in the communities exposed to acidification in phase II. However, ctaD pN/pS ratios across all communities was less than 1 so selection was weak overall. Lastly, the same six genes in *A. rubrum* were under greater selection in both pre-exposed and naïve communities compared to control communities, demonstrating similar selection caused by severe acidification regardless of pre-exposure.

The shielding effects of pre-exposure on *A. rubrum* evolution are also revealed by pairwise ANI and π of *A. rubrum* scaffolds that show genomes in pre-exposed communities were able to retain significantly greater genetic diversity after severe acidification in phase II (Fig. 5). By the end of the experiment, *A. rubrum* in both pre-exposed and naïve communities increased significantly in genetic diversity, but the beneficial effect of pre-exposure was still evident as *A. rubrum* in pre-exposed communities were still significantly more diverse than in naïve communities. Further evidence for the impact of pre-exposure on *A. rubrum* evolution is provided by the number of shared SNPs within pre-exposed communities. Significantly more SNPs were shared between *A. rubrum* populations among pre-exposed and among naïve

communities than neutrally expected, which suggests that pre-exposure independently selected for alleles not under selection in naïve communities (Fig. 6).

In this study, we show that pre-exposure to environmental stress can protect biological communities against future severe stress. Despite the convergence of community composition between pre-exposed and naïve communities, we demonstrate that species sorting due to pre-exposure generated greater community resistance and mitigated taxonomic loss. Additionally, we show that pre-exposure caused independent evolutionary processes that resulted in lower changes in genome-wide allele frequencies and greater levels of genetic diversity. Thus, we provide evidence for the dual roles of species sorting and evolutionary adaptation in community response to severe stress, as well as the utility of pre-exposure for pre-adaptation (Tagkopoulos et al. 2008, Mitchell et al. 2009, Costantini et al. 2010, Bell 2013a, Bernhardt et al. 2020, Zhou and Wang 2023). These results suggest that pre-exposure to stress could potentially be useful as a biodiversity management strategy to improve ecological and evolutionary responses of natural complex communities.

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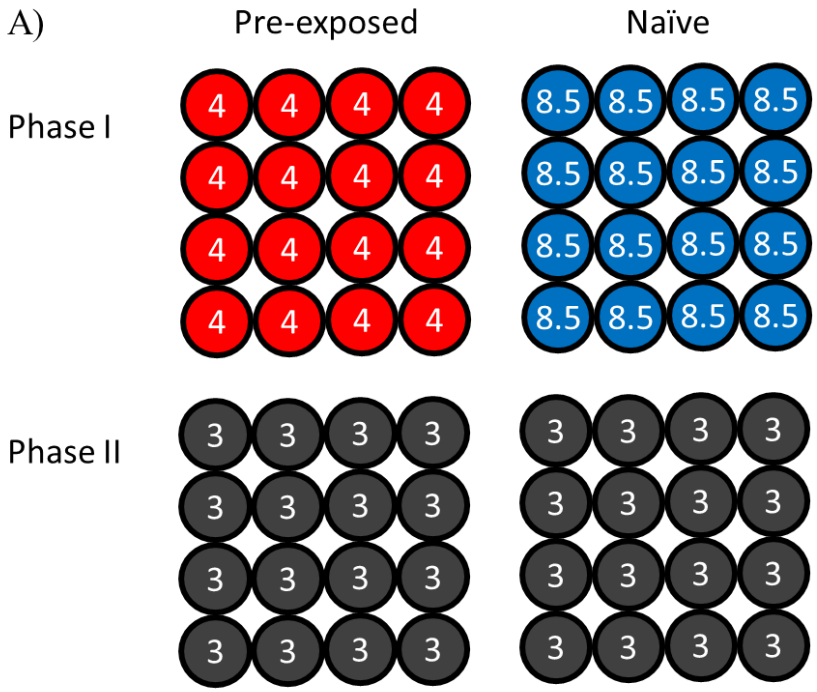
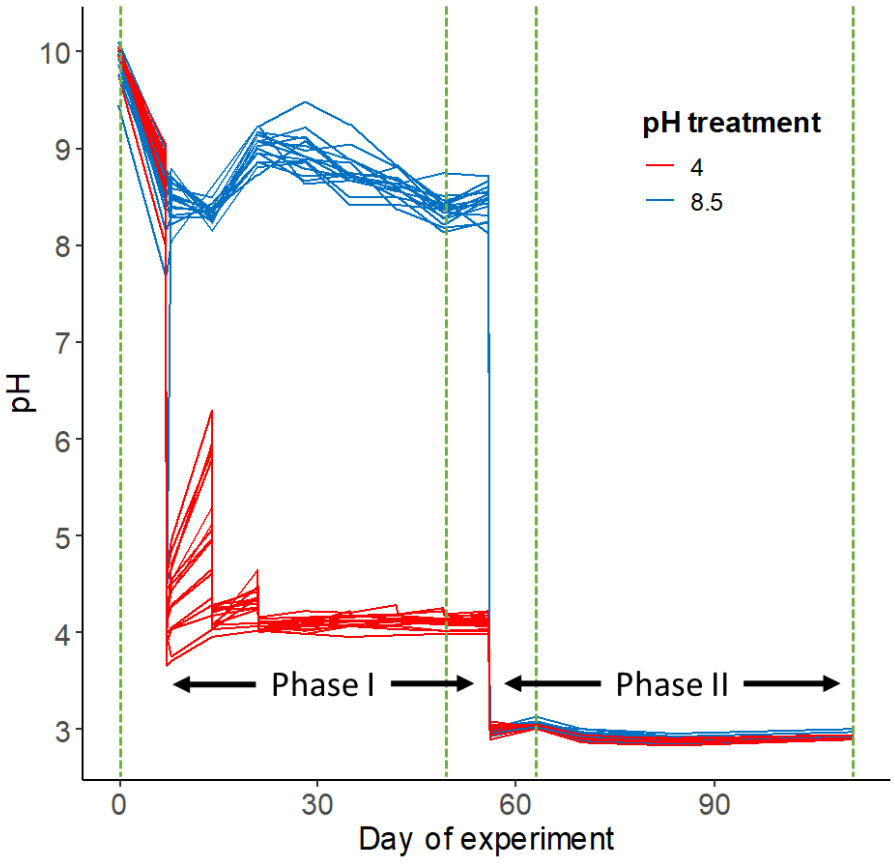
2.8 Figures and tables

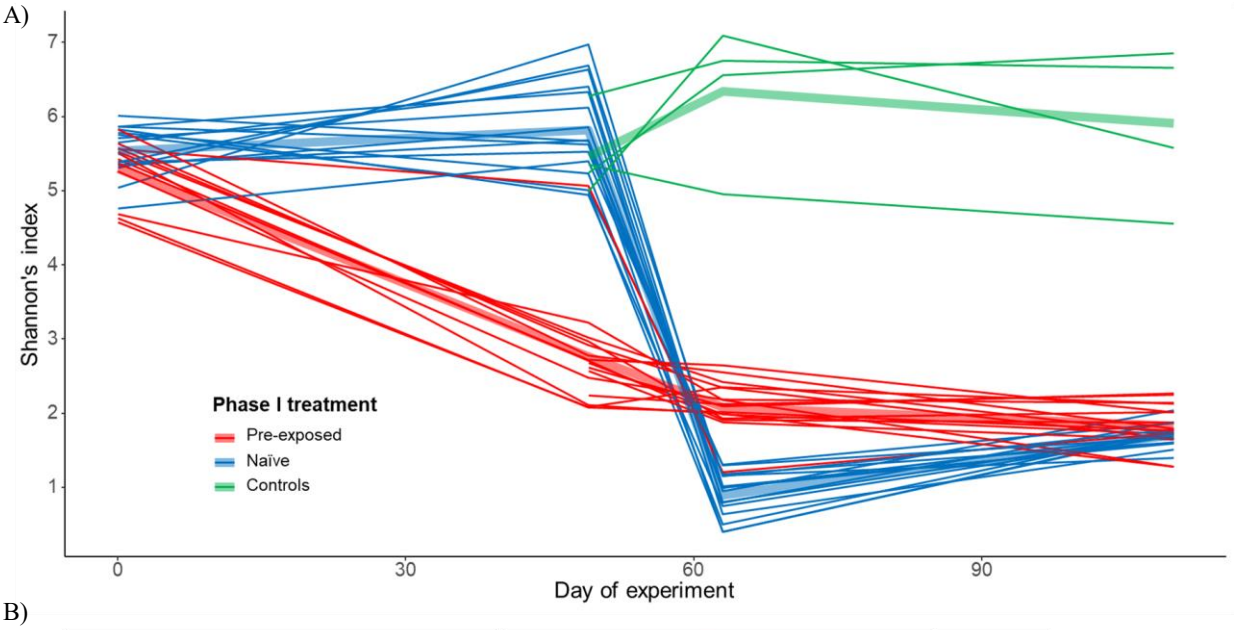


B)

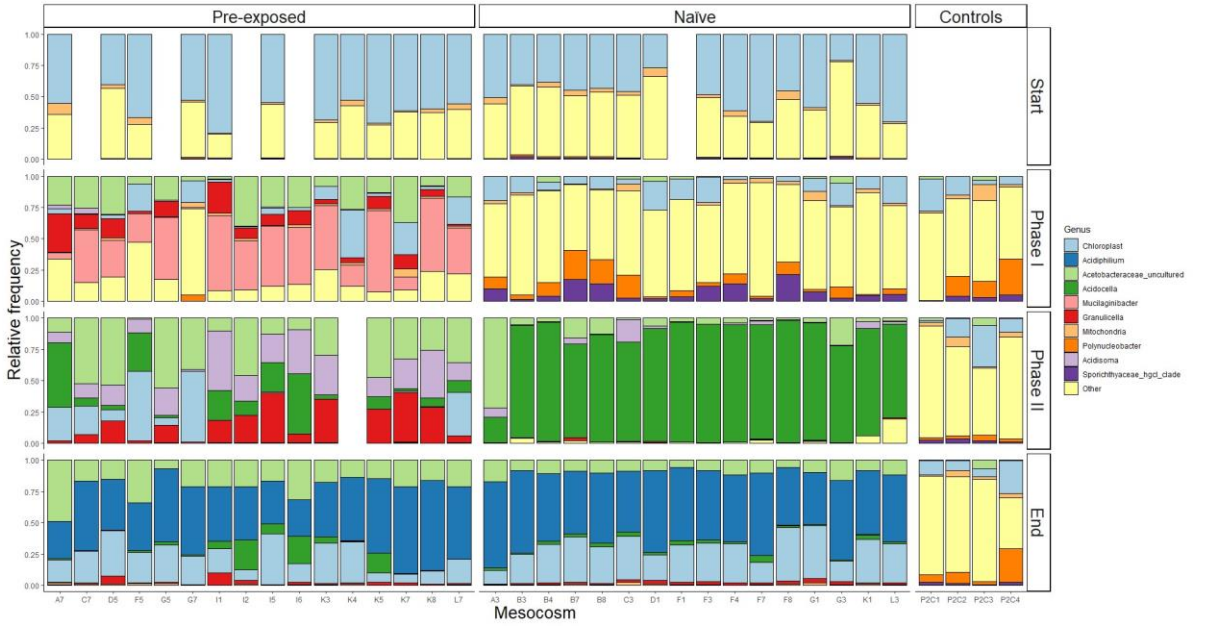


2.8.1 Fig. 1. Experimental design and pre-exposure treatments. A) Schematic representation of the subset of mesocosms from the two-phase experiment included in this study. Circles represent mesocosms. Colors and numbers indicate pH of pre-exposure treatments. Half of pre-exposed and naïve mesocosms were under a global dispersal regime while the other half were isolated (dispersal regimes not shown). Phase II also included four control mesocosms which were not acidified (not shown). B) Measured pH of each mesocosm throughout the experiment. Each line represents an individual mesocosm and colors indicate pH of pre-exposure treatments. In phase II, all mesocosms were acidified to pH 3 on August 2/day 56 until the end of the experiment. Green dashed lines mark the four time points during the experiment when samples were taken (day 0: June 7, day 49: July 26, day 63: August 9, and day 110: September 25) referred to as “Start”, “Phase I”, “Phase II”, and “End”.

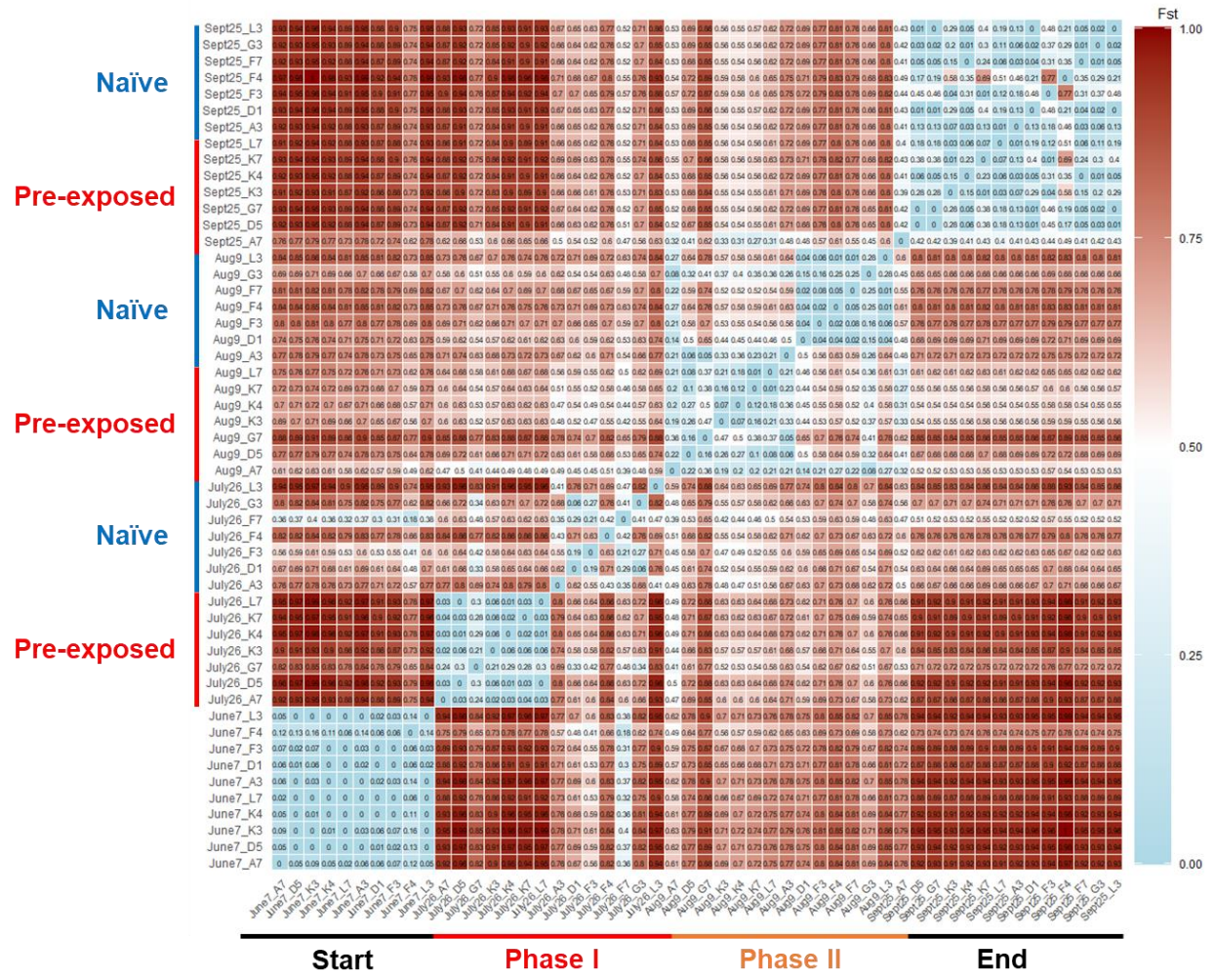
A)


Shannon's index
Day of experiment
Phase I treatment
Pre-exposed
Naïve
Controls

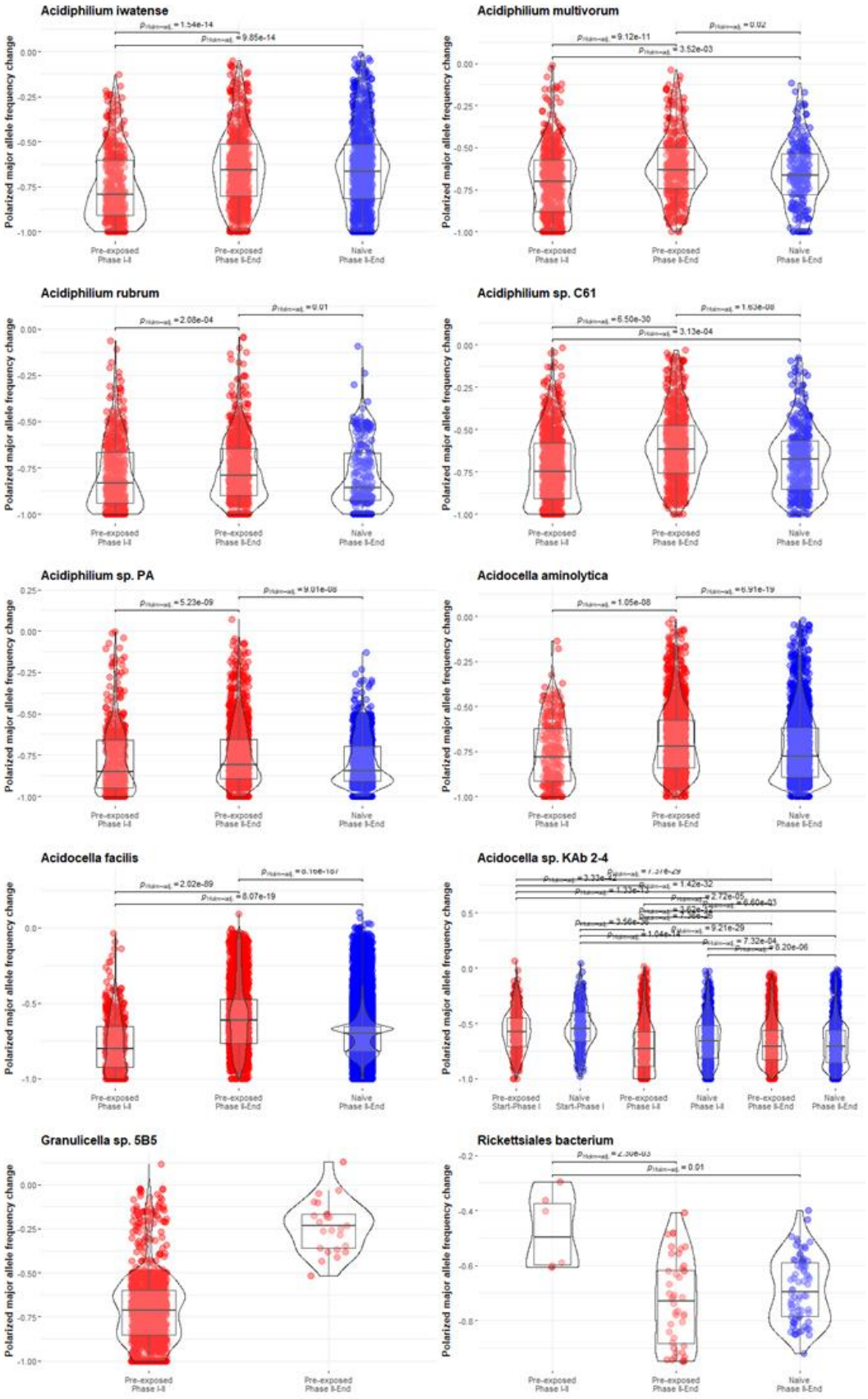
B)


Pre-exposed
Naïve
Controls
Start
Phase I
Phase II
End
Relative frequency
Mesocosm
Genus
Chloroplast
Acidiphilium
Acetobacteraceae_uncultured
Acidocella
Mucilaginibacter
Granulicella
Mitochondria
Polynucleobacter
Acidisoma
Sporichthyaceae_hgcI_clade
Other

2.8.2 Fig. 2. Pre-exposure caused significant changes in alpha diversity and community composition. A) Shannon's index of pre-exposed (red), naïve (blue), and control (green) communities over time. B) Genus-level taxonomic composition of pre-exposed, naïve, and control communities. The top ten genera are colored individually, and all others are grouped together in yellow.

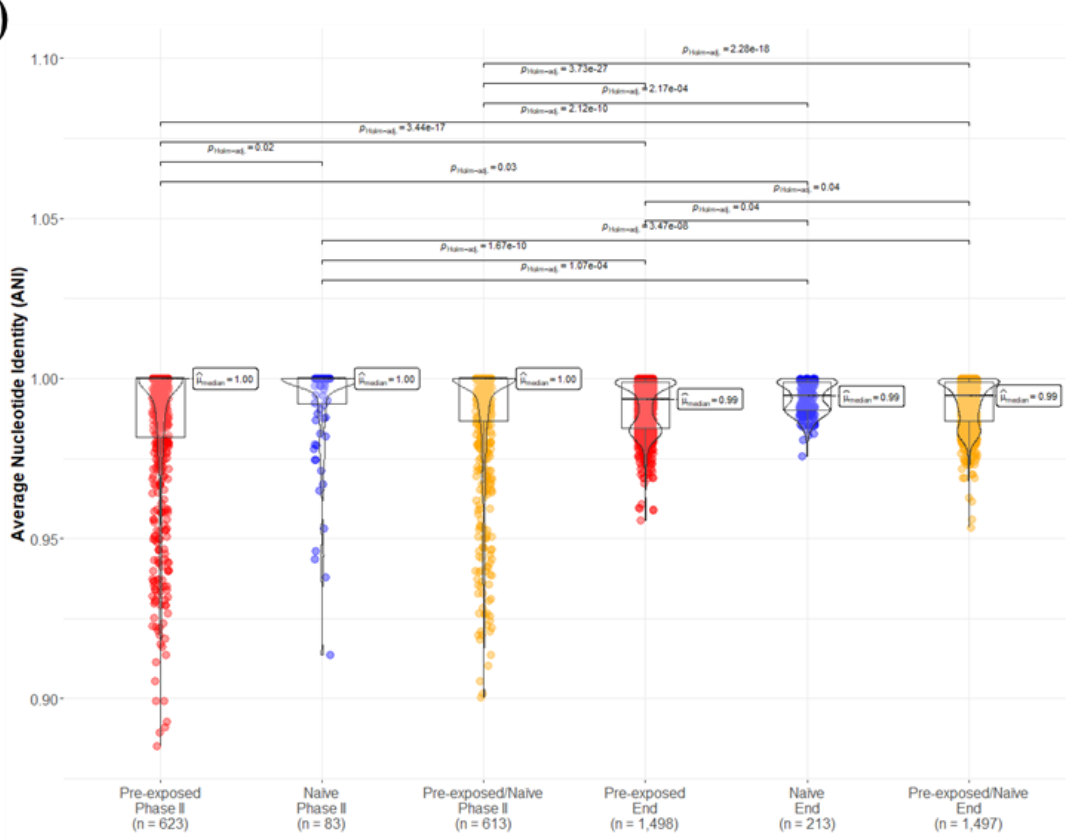

Naïve
Pre-exposed
Naïve
Pre-exposed
Naïve
Pre-exposed
Fst
1.00
0.75
0.50
0.25
0.00
Start
Phase I
Phase II
End

2.8.3 Fig. 3. Pairwise fixation index (F_{ST}) of *Acidiphilium rubrum* between each sample. Samples are chronological from left to right and from bottom to top. Time points are marked horizontally as start, phase I (red), phase II (orange), and end. Phase I treatments are marked vertically as pre-exposed (red) and naïve (blue). F_{ST} values ranged from 0 (light blue) to 1 (dark red).

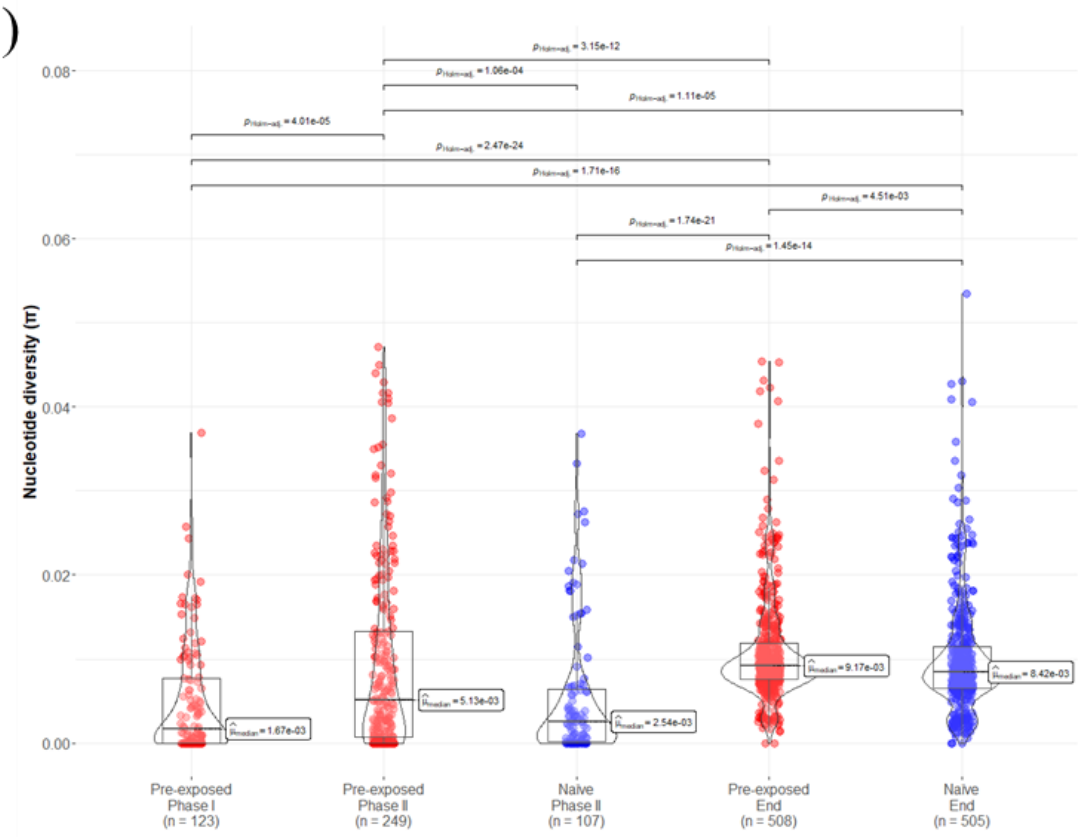

Acidiphilium iwatense
Acidiphilium multivorum
Acidiphilium rubrum
Acidiphilium sp. C61
Acidiphilium sp. PA
Acidocella aminolytica
Acidocella facilis
Acidocella sp. KAb 2-4
Granulicella sp. 5B5
Rickettsiales bacterium
Polarized major allele frequency change
Pre-exposed Phase I-II
Pre-exposed Phase II-End
Naïve Phase II-End
Pre-exposed Start-Phase I
Naïve Start-Phase I
Naïve Phase I-II

2.8.4 Fig. 4. Allele frequency change of single nucleotide polymorphisms (SNPs). SNPs were polarized by taking the frequency of the major allele, and allele frequency change was calculated through subtracting that frequency by the frequency of that same allele in a subsequent time point, resulting in a negative frequency change. Colors indicate pre-exposed (red) and naïve (blue) communities. Bars indicate statistical significance based on Holm-Bonferroni adjusted p-values of Dunn's test ($p<0.05$).

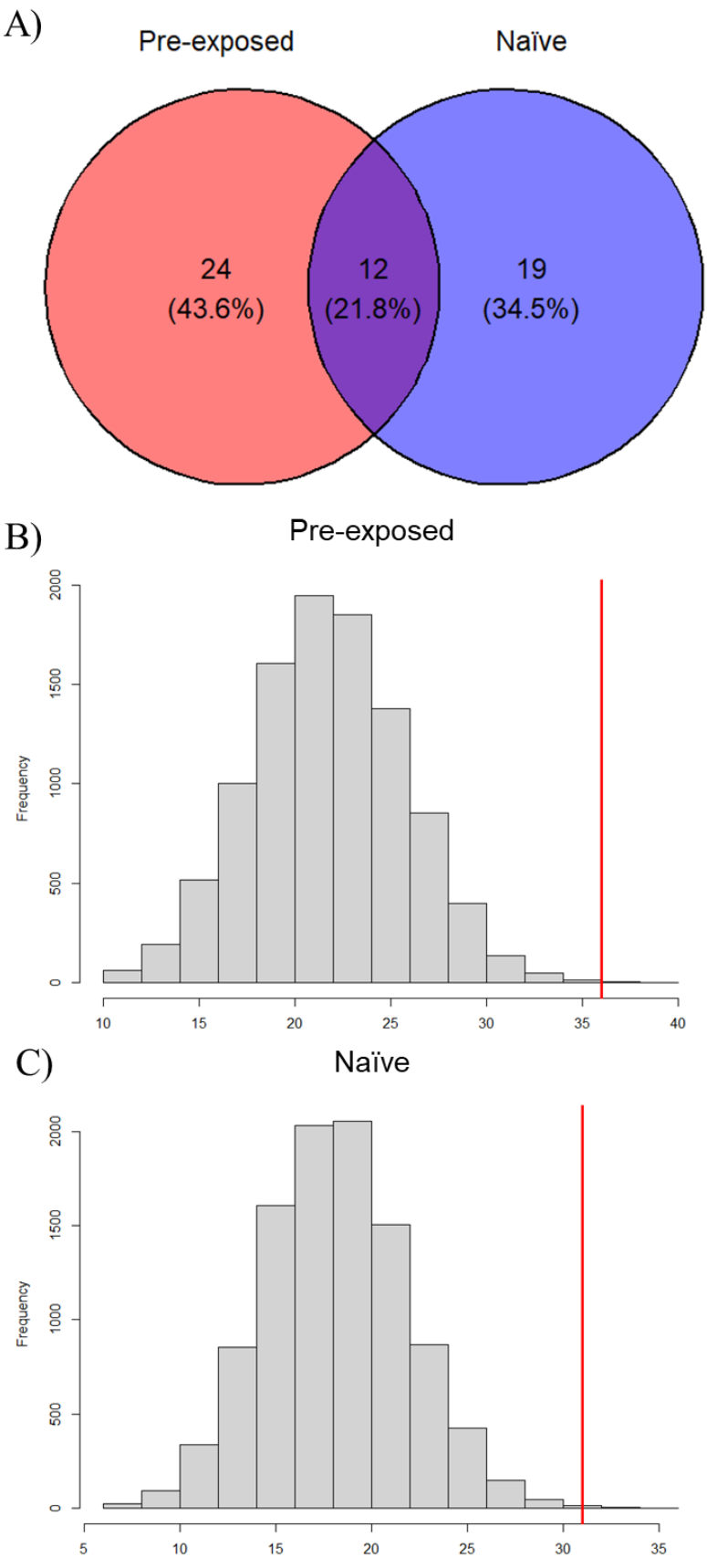
A)


Average Nucleotide Identity (ANI)
1.10
1.05
1.00
0.95
0.90
$p_{Holm-adj.}$ = 2.28e-18
$p_{Holm-adj.}$ = 3.73e-27
$p_{Holm-adj.}$ = 2.17e-04
$p_{Holm-adj.}$ = 2.12e-10
$p_{Holm-adj.}$ = 3.44e-17
$p_{Holm-adj.}$ = 0.02
$p_{Holm-adj.}$ = 0.03
$p_{Holm-adj.}$ = 0.04
$p_{Holm-adj.}$ = 0.04
$p_{Holm-adj.}$ = 3.47e-08
$p_{Holm-adj.}$ = 1.67e-10
$p_{Holm-adj.}$ = 1.07e-04
$\hat{p}_{median}$ = 1.00
$\hat{p}_{median}$ = 1.00
$\hat{p}_{median}$ = 1.00
$\hat{p}_{median}$ = 0.99
$\hat{p}_{median}$ = 0.99
$\hat{p}_{median}$ = 0.99
Pre-exposed Phase II (n = 623)
Naïve Phase II (n = 83)
Pre-exposed/Naïve Phase II (n = 613)
Pre-exposed End (n = 1,498)
Naïve End (n = 213)
Pre-exposed/Naïve End (n = 1,497)

B)


Nucleotide diversity (π)
0.08
0.06
0.04
0.02
0.00
$p_{Holm-adj.}$ = 3.15e-12
$p_{Holm-adj.}$ = 1.06e-04
$p_{Holm-adj.}$ = 1.11e-05
$p_{Holm-adj.}$ = 4.01e-05
$p_{Holm-adj.}$ = 2.47e-24
$p_{Holm-adj.}$ = 1.71e-16
$p_{Holm-adj.}$ = 4.51e-03
$p_{Holm-adj.}$ = 1.74e-21
$p_{Holm-adj.}$ = 1.45e-14
$\hat{p}_{median}$ = 1.67e-03
$\hat{p}_{median}$ = 5.13e-03
$\hat{p}_{median}$ = 2.54e-03
$\hat{p}_{median}$ = 9.17e-03
$\hat{p}_{median}$ = 8.42e-03
Pre-exposed Phase I (n = 123)
Pre-exposed Phase II (n = 249)
Naïve Phase II (n = 107)
Pre-exposed End (n = 508)
Naïve End (n = 505)

2.8.5 Fig. 5. Effects of pre-exposure on evolution of *Acidiphilium rubrum*. A) Pairwise average nucleotide identity (ANI) of scaffolds from *A. rubrum* genomes within pre-exposed (red) and naïve (blue) communities as well as between pre-exposed and naïve (orange) communities in phase II and at the end of the experiment. B) Nucleotide diversity (π) of scaffolds from *A. rubrum* genomes within pre-exposed (red) and naïve (blue) communities across time. Bars indicate statistical significance based on Holm-Bonferroni adjusted p-values of Dunn's test ($p<0.05$). Number of scaffolds within each group are in parenthesis.

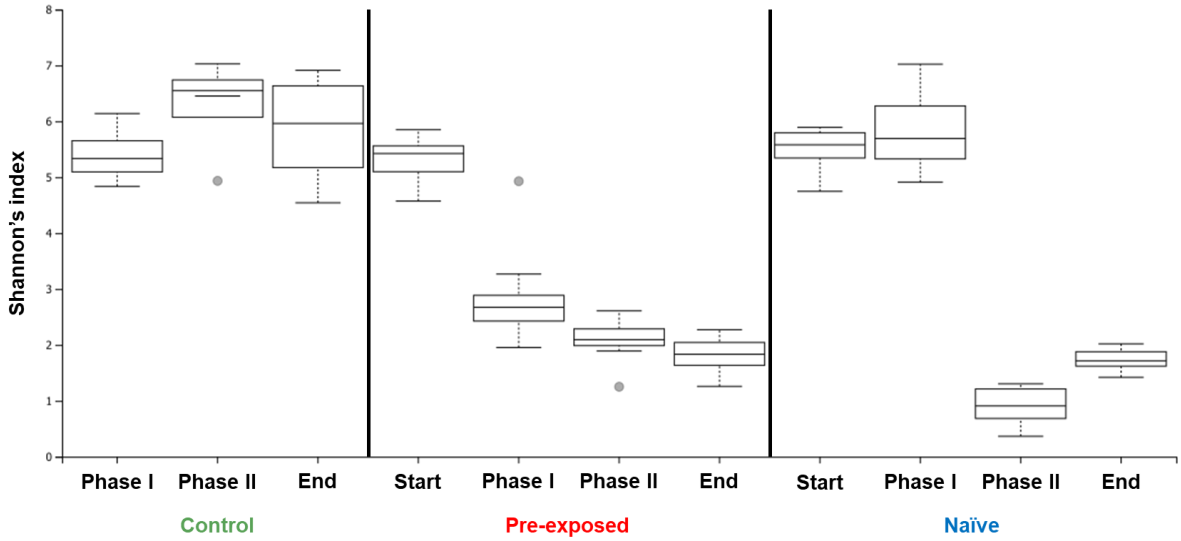

A)
Pre-exposed
Naïve
24
(43.6%)
12
(21.8%)
19
(34.5%)
B)
Pre-exposed
Frequency
0
500
1000
1500
2000
10
15
20
25
30
35
40
C)
Naïve
Frequency
0
500
1000
1500
2000
5
10
15
20
25
30
35

2.8.6 Fig. 6. Shared single nucleotide polymorphisms (SNPs) of *Acidiphilium rubrum* populations. A) Number of shared SNPs between and among pre-exposed and naïve communities present in both phase II and at the end of the experiment. Permutations of mesocosms over SNPs indicate that the number of shared SNPs within B) pre-exposed and C) naïve communities are significantly greater than neutral expectations (N=10,000, $p<0.05$). Red lines indicate the observed number of shared SNPs.

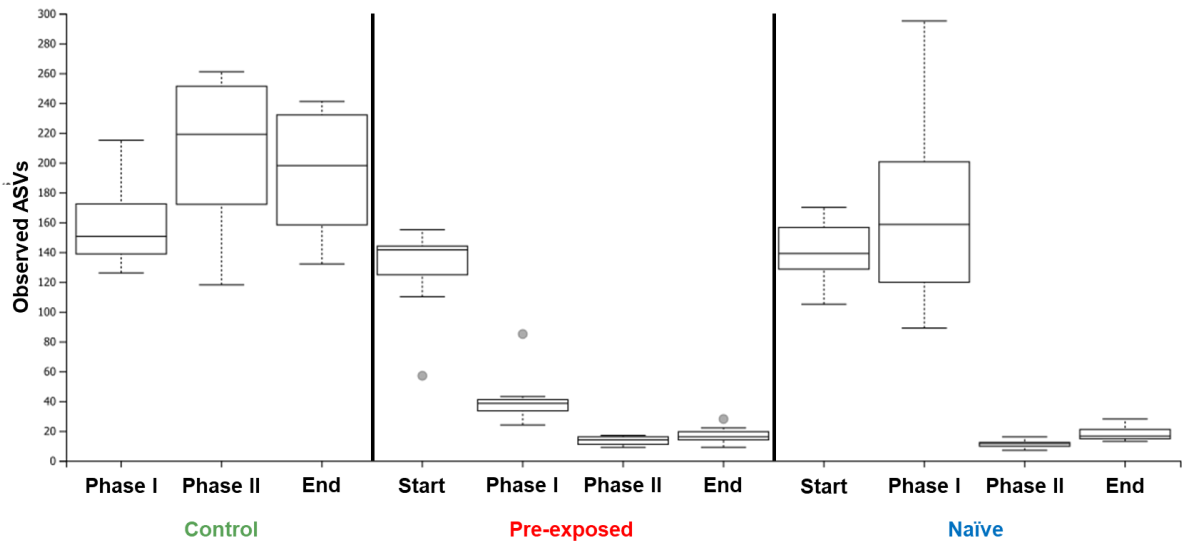
2.8.7 Table 1. Significant non-synonymous to synonymous rates of polymorphism (pN/pS) ratio comparisons of *Acidiphilium rubrum* genes. "pN/pS comparison" indicates directionality of comparisons. Statistical testing was conducted using two-sided T-tests and p-values were adjusted via Holm-Bonferroni.

Time	pN/pS comparison	Gene	p-value
Phase I	Pre-exposed > Naïve	polyamine aminopropyltransferase (speE)	0.024
		succinate dehydrogenase flavoprotein subunit (sdhA)	0.001
		methylisocitrate lyase (prpB)	9.52E-08
	Pre-exposed > Controls	DNA polymerase III subunit alpha (dnaE)	0.012
Phase II	Naïve > Pre-exposed	acetolactate synthase 3 large subunit	0.002
	Controls > Pre-exposed	cytochrome c oxidase subunit I (ctaD)	0.006
	Controls > Naïve	cytochrome c oxidase subunit I (ctaD)	0.023
	Naïve > Controls	DNA polymerase III subunit alpha (dnaE)	0.020
		DNA translocase FtsK	0.042
		pyruvate dehydrogenase complex E1 component subunit beta	0.020
End	Pre-exposed > Controls	adenosylhomocysteinase (ahcY)	0.015
		transcription termination factor Rho (rho)	0.001
		DNA polymerase III subunit alpha (dnaE)	2.06E-06
		chlorophyllide a reductase iron protein subunit X	0.015
		pyruvate dehydrogenase complex E1 component subunit beta	0.001
		cyanase (cynS)	6.15E-06
	Naïve > Controls	adenosylhomocysteinase (ahcY)	2.38E-05
		transcription termination factor Rho (rho)	2.24E-04
		DNA polymerase III subunit alpha (dnaE)	3.42E-06
		chlorophyllide a reductase iron protein subunit X	0.031
		pyruvate dehydrogenase complex E1 component subunit beta	0.004
		cyanase (cynS)	7.99E-08

2.9 Supplementary materials

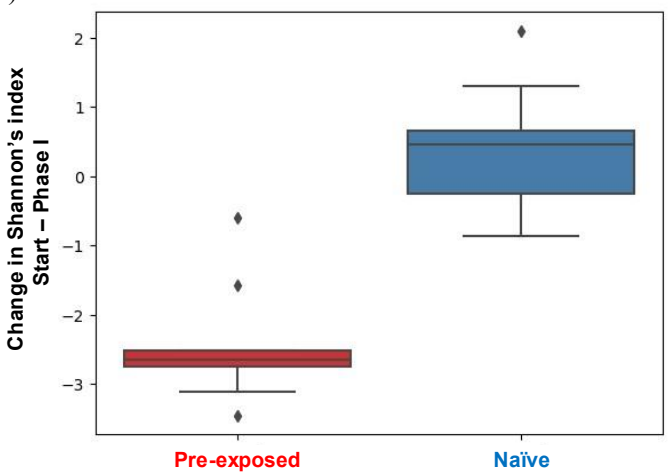


2.9.1 Fig. S1. Shannon's index. Significant differences were observed in pre-exposed communities between the start of the experiment and phase I (q=4.3E-05) and between pre-exposed and naïve communities in phase II (q=1.1E-05). Pre-exposed and naïve communities converged by the end of the experiment (q=0.28). Statistical testing was conducted using Kruskal-Wallace tests and q-values were corrected via Benjamin & Hochberg.

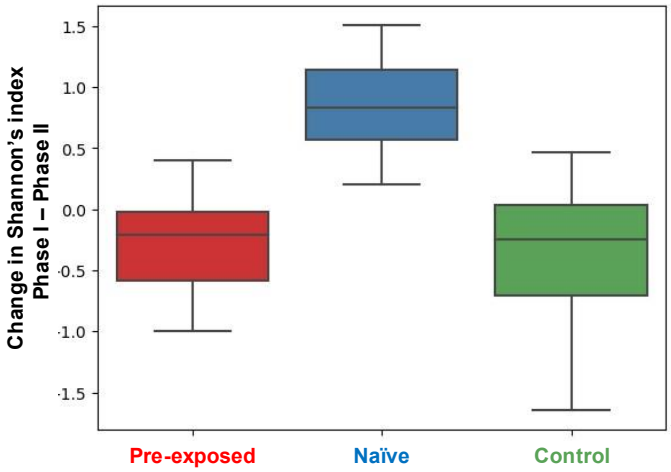

Observed ASVs
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
Phase I
Phase II
End
Start
Phase I
Phase II
End
Start
Phase I
Phase II
End
Control
Pre-exposed
Naïve

2.9.2 Fig. S2. Observed number of amplicon sequence variants (ASVs). Observed ASVs were significantly greater in naïve communities at the end of the experiment than in phase II (q=6.2E-05). Statistical testing was conducted using Wilcoxon signed-rank test and q-value was adjusted via Benjamini & Hochberg.

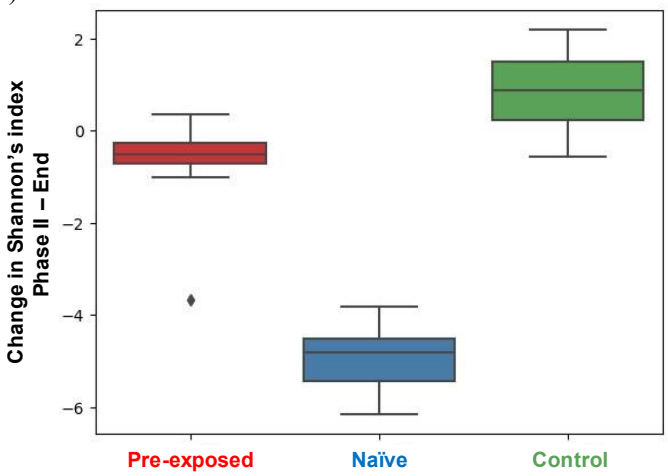
A)


Change in Shannon's index
Start – Phase I
2
1
0
−1
−2
−3
Pre-exposed
Naïve

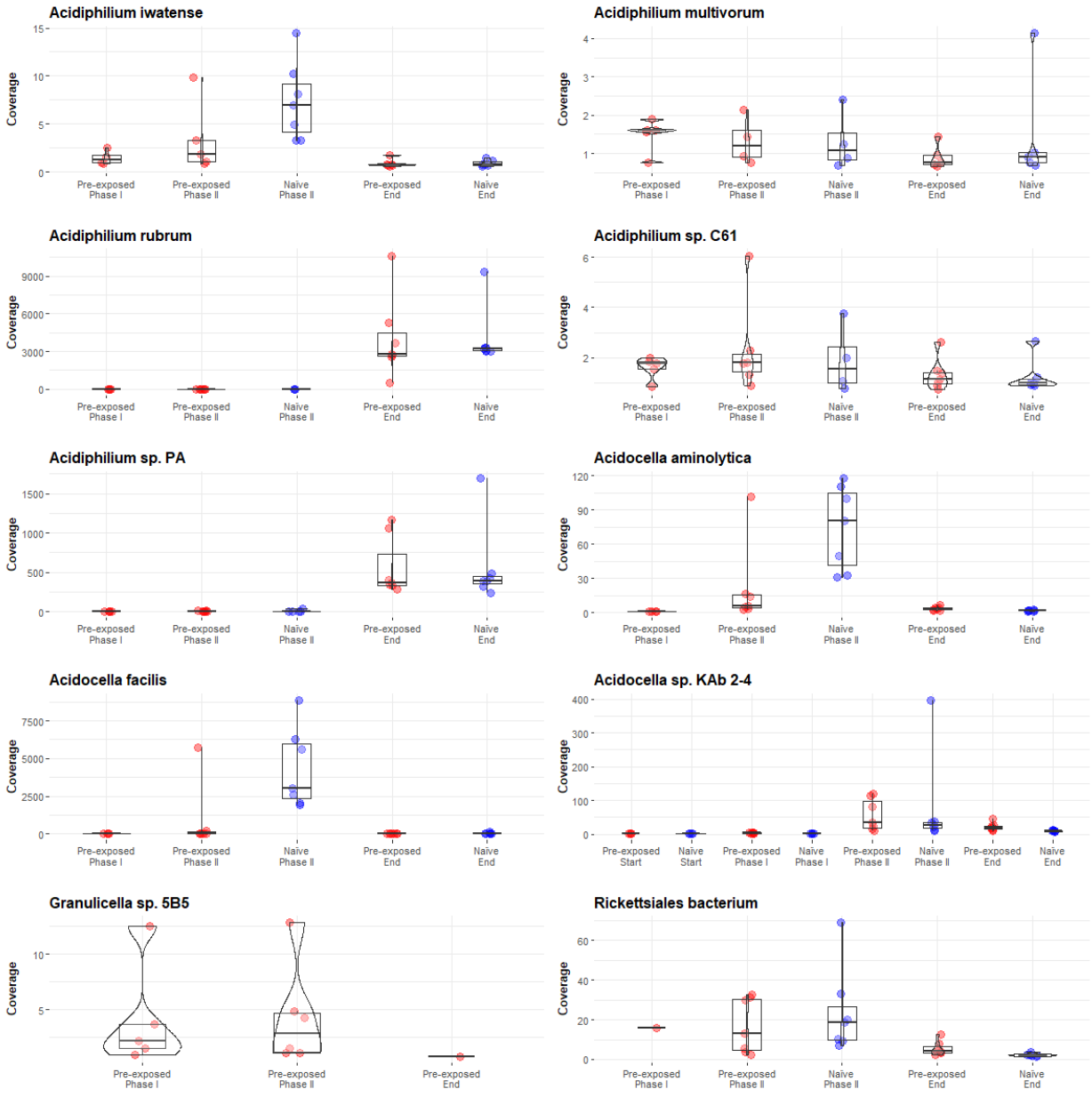
B)


Change in Shannon's index
Phase I – Phase II
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
Pre-exposed
Naïve
Control

C)

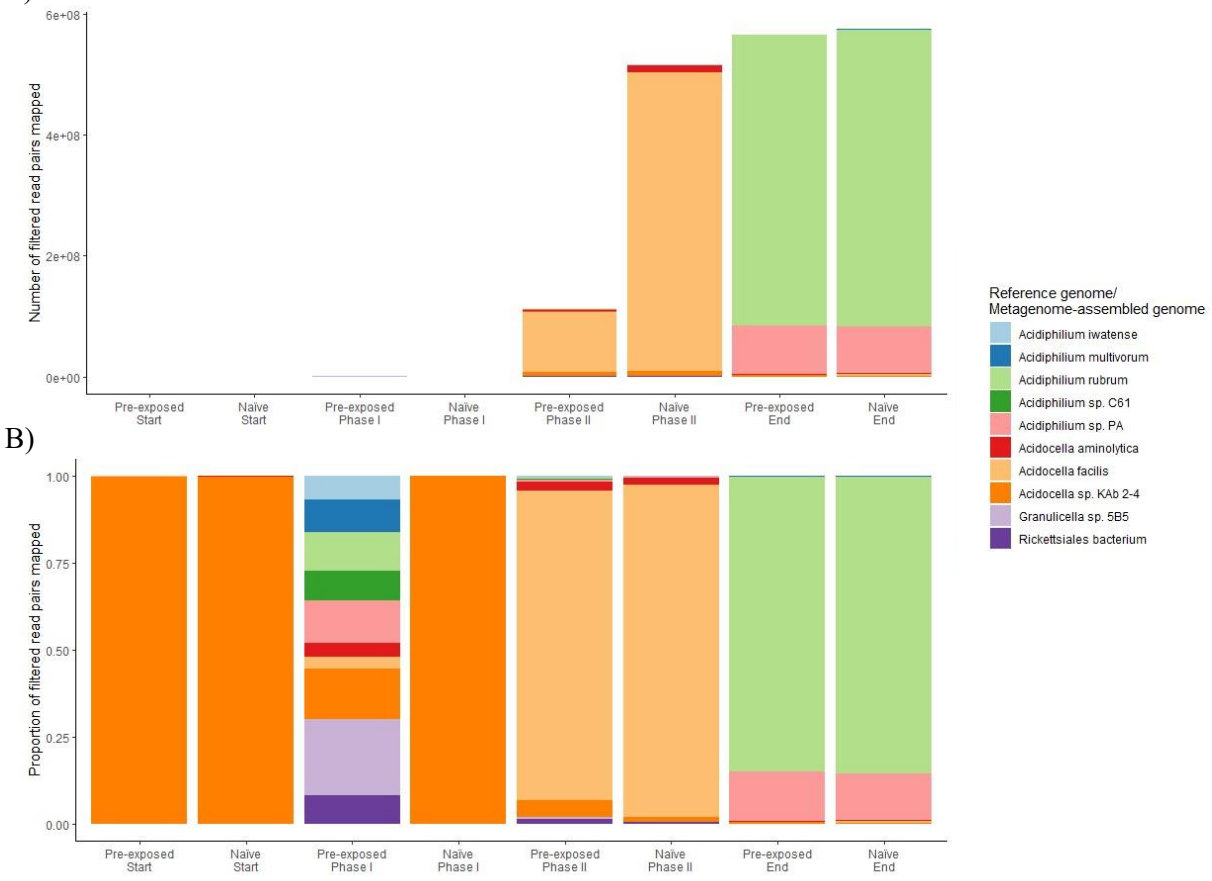

Change in Shannon's index
Phase II – End
2
0
−2
−4
−6
Pre-exposed
Naïve
Control

2.9.3 Fig. S3. Change in Shannon's index. Significant differences were observed between pre-exposed and naïve communities at A) the start of the experiment and phase I (p=2E-05), B) phase I and phase II (p=7E-05, and C) phase II and the end of the experiment (p=1E-05). Statistical testing was conducted using Mann-Whitney U tests and q-values were adjusted via Benjamini & Hochberg.


Acidiphilium iwatense
Acidiphilium multivorum
Acidiphilium rubrum
Acidiphilium sp. C61
Acidiphilium sp. PA
Acidocella aminolytica
Acidocella facilis
Acidocella sp. KAb 2-4
Granulicella sp. 5B5
Rickettsiales bacterium
Coverage
Pre-exposed Phase I
Pre-exposed Phase II
Naïve Phase II
Pre-exposed End
Naïve End
Pre-exposed Start
Naïve Start
Naïve Phase I

2.9.4 Fig. S4. Mean coverage of genomes. Significant variation is observed between pre-exposed (red) and naïve (blue) communities across time.

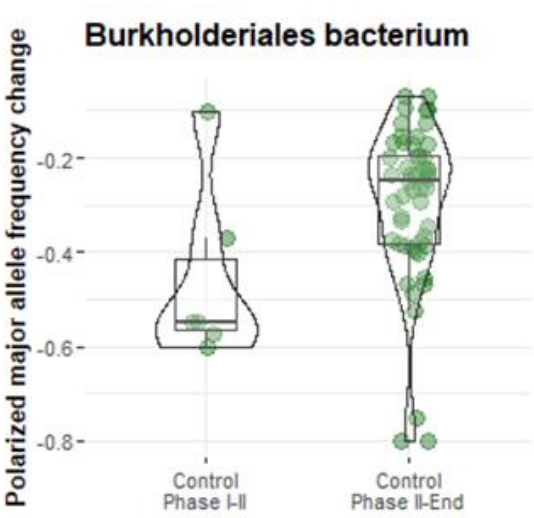
A)

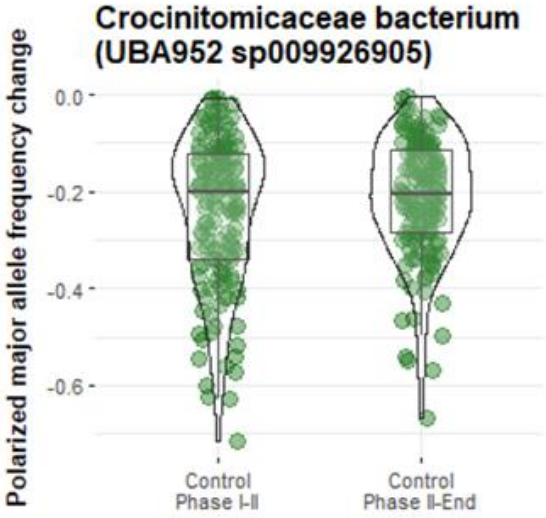

Number of filtered read pairs mapped
6e+08
4e+08
2e+08
0e+00
Pre-exposed Start
Naïve Start
Pre-exposed Phase I
Naïve Phase I
Pre-exposed Phase II
Naïve Phase II
Pre-exposed End
Naïve End
Reference genome/ Metagenome-assembled genome
Acidiphilium iwatense
Acidiphilium multivorum
Acidiphilium rubrum
Acidiphilium sp. C61
Acidiphilium sp. PA
Acidocella aminolytica
Acidocella facilis
Acidocella sp. KAb 2-4
Granulicella sp. 5B5
Rickettsiales bacterium

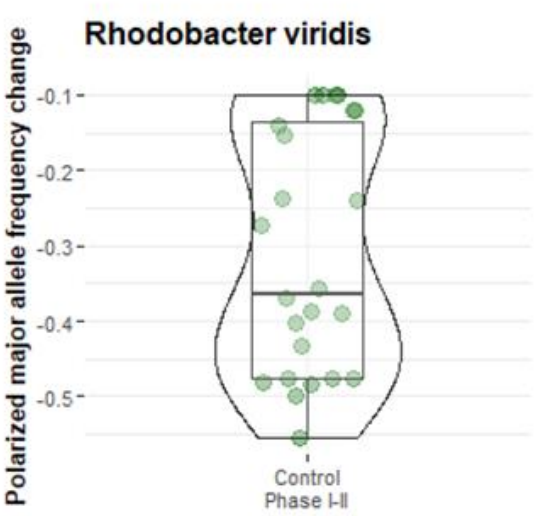
B)

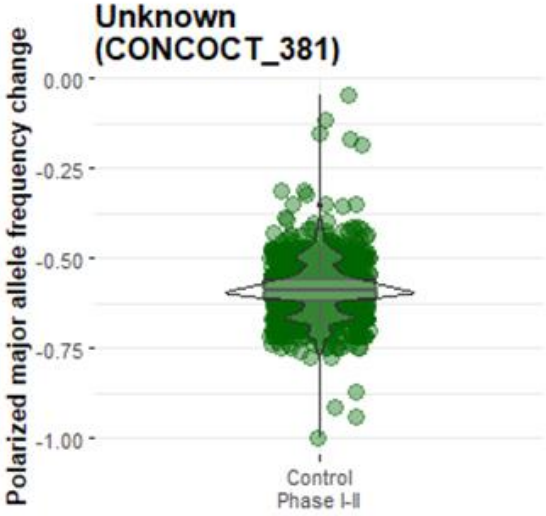
Proportion of filtered read pairs mapped
1.00
0.75
0.50
0.25
0.00
Pre-exposed Start
Naïve Start
Pre-exposed Phase I
Naïve Phase I
Pre-exposed Phase II
Naïve Phase II
Pre-exposed End
Naïve End

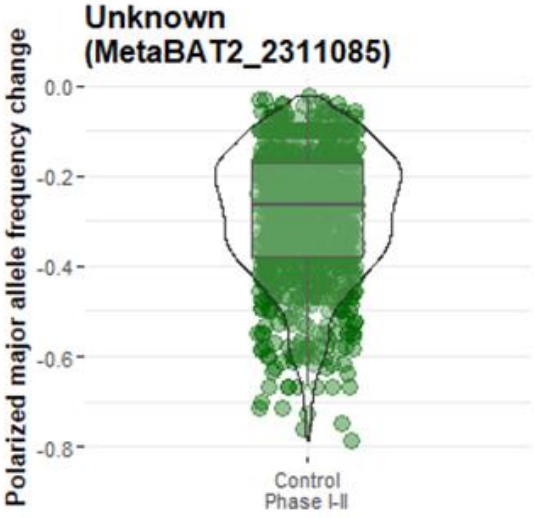
2.9.5 Fig. S5. Read pairs mapped to genomes. A) Total read pairs mapped. B) Proportion of reads pairs mapped.

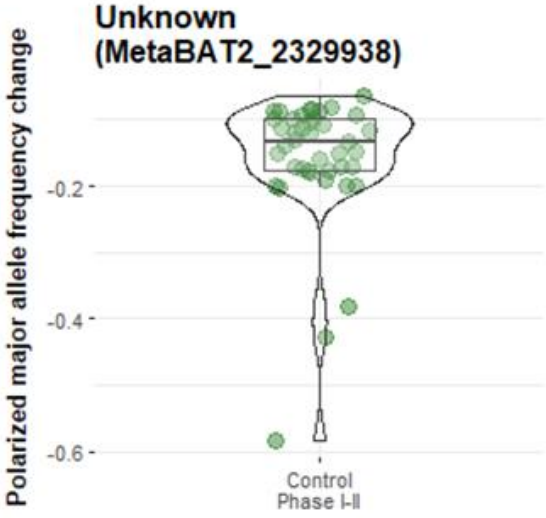

Burkholderiales bacterium
Polarized major allele frequency change
-0.2
-0.4
-0.6
-0.8
Control
Phase I-II
Control
Phase II-End


Crocinitomicaceae bacterium
(UBA952 sp009926905)
Polarized major allele frequency change
0.0
-0.2
-0.4
-0.6
Control
Phase I-II
Control
Phase II-End


Rhodobacter viridis
Polarized major allele frequency change
-0.1
-0.2
-0.3
-0.4
-0.5
Control
Phase I-II


Unknown
(CONCOCT_381)
Polarized major allele frequency change
0.00
-0.25
-0.50
-0.75
-1.00
Control
Phase I-II


Unknown
(MetaBAT2_2311085)
Polarized major allele frequency change
0.0
-0.2
-0.4
-0.6
-0.8
Control
Phase I-II


Unknown
(MetaBAT2_2329938)
Polarized major allele frequency change
-0.2
-0.4
-0.6
Control
Phase I-II

2.9.6 Fig. S6. Allele frequency changes in single nucleotide polymorphisms (SNPs) of metagenome-assembled genomes (MAGs) in control communities. SNPs were polarized by taking the frequency of the major allele, and allele frequency change was calculated through subtracting that frequency by the frequency of that same allele in a subsequent time point, resulting in a negative frequency change. MAG names are listed in parentheses if no taxonomic identity was able to be assigned. Unnamed genus and species are also listed in parentheses.

2.10 Bridging Chapter 2 – Chapter 3: From academic to applied

Chapters 1 and 2 investigated the impact of environmental change on bacterial communities in two sharply different systems, addressing fundamental knowledge gaps of community response to rapid change. Chapter 2 in particular grew from a long line of scientific inquiries regarding evolutionary and community rescue that has generated novel hypotheses and an impressive body of theoretical and empirical literature (Bell and Gonzalez 2009, 2011, Barrett and Hendry 2012, Lachapelle and Bell 2012, Bell 2013a, 2013b, 2017, Gonzalez and Bell 2013, Gonzalez et al. 2013, Kovach-Orr and Fussmann 2013, Fussmann and Gonzalez 2013, Low-Décarie et al. 2015, Samani and Bell 2016, Bell et al. 2019, Fugère et al. 2020, Peniston et al. 2020, O'Connor et al. 2020, van Moorsel et al. 2021, Barbosa Da Costa et al. 2021, 2022, Hébert et al. 2021, Jewell and Bell 2022). This research avenue has clearly received much attention in recent years due in part to its relevance across broad agricultural, environmental, and medical issues such as agrochemical and antibiotic resistance, industrial pollution, and biological invasions (Bell 2017). However, real-world applications of evolutionary and community rescue findings typically remain incidental and abstract as selection has demographic costs and the outcome of rescue is challenging to predict (Bell 2013a). Extension to the evolution of major fitness components in natural populations of nonmicrobial organisms like vertebrates are even more scarce and often impractical within conservation contexts (Kinnison et al. 2007, Vander Wal et al. 2013).

Results from Chapter 2 suggest that pre-exposure to sublethal levels of environmental stress can protect communities from severe future stress, but to claim that such pre-exposure should be used as part of an active management or conservation strategy tackling issues like those listed previously within any specific context is premature at best and irresponsible at worst (Raffensperger and Tickner 1999, Dorman 2005). Indeed evolution has been demonstrated to

operate on time-scales relevant to conservation biology, and examples exist of negative conservation outcomes when evolution is ignored (Stockwell et al. 2003, Ashley et al. 2003). However, adaptation is not a panacea. Understanding the limitations of evolution in preventing biodiversity decline will be crucial for conservation applications (Bridle and Vines 2007, Bell 2013a). Further research is needed to make informed predictions regarding when and how the benefits of stress pre-exposure arise in different systems and environments. Though, it would be prudent to note that history is rife with examples of biological management decisions with negative unintentional evolutionary, ecological, and social consequences such as rhinoceros dehorning (Berger and Cunningham 1996), overuse leading to increased resistance (*e.g.*, of antibiotics (Davies and Davies 2010), herbicides (Délye et al. 2013), pesticides (Bouwman et al. 2011, Gould et al. 2018), and fungicides (Lucas et al. 2015)), intentional biological introductions of cane toads and European rabbits to Australia (Coman 2010, Shanmuganathan et al. 2010), kudzu to the U.S. (Forseth and Innis 2004), mongooses to Hawaii (Baldwin et al. 1952), Nile perch to Lake Victoria (Barel et al. 1985, Pringle 2005), various fish species within and between countries (Kohler and Courtenay Jr 1986), and wolves to Yellowstone and Isle Royale National Parks (Smith and Peterson 2021), in addition to certain historical vaccination programs for dengue (Sridhar et al. 2018), measles (Fulginiti 1967), respiratory syncytial virus (RSV) (Kim et al. 1969), rotavirus (Murphy et al. 2001, Kapikian 2008), smallpox (Belongia and Naleway 2003), and the 1976 Swine Flu (Sencer and Millar 2006). These examples are not to argue that biological management and public health action should never be taken because of negative risks, particularly with regards to vaccinations, but the plethora of such cases does demonstrate that outcomes in practice ultimately involve significant uncertainty (McCarthy 2014). Conservation decision analysis suggests one approach is to choose actions that achieve a minimum level of

performance regardless of uncertainty (Williams and Johnson 2013). Unfortunately, this criterion applies poorly to stress pre-exposure as mechanisms and interactions with other factors are not well understood (Badyaev 2005). Thus, such research will most likely remain primarily as an academic albeit intellectually stimulating pursuit, at least for the foreseeable future.

Given the unprecedented biodiversity threats in the Anthropocene, action is needed despite risks (McGill et al. 2015). Synthetic biology and advances like CRISPR, which have revolutionized capacities to fundamentally manipulate life in potentially irreversible ways, is of particular concern as the significance of their impact towards biodiversity and across society is widely recognized (Shinwari et al. 2018, Schleidgen et al. 2020, Wang and Doudna 2023). In an effort to directly contribute towards the protection of global biodiversity by translating knowledge into action, Chapter 3 proposes and demonstrates proof-of-concept that environmental DNA (eDNA) methods can be used for monitoring of genetically modified (GM) animals and their transgenes (Xu et al. 2021). While eDNA is currently being successfully used for active biodiversity assessment programs of natural organisms (Petruniak et al. 2021), the application of eDNA to detect GM animals had not been demonstrated prior to the publication of Chapter 3. There are concerns that GM animals could disturb natural biodiversity if released, especially since GM animals are typically designed to maximize certain competitive life history traits like growth or resistance (Muir and Howard 1999, Moreau 2014, Lara-Flores and Rivera-Arriaga 2019). They could directly drive biodiversity dynamics (*e.g.*, as predators of natural organisms) or indirectly through inducing environmental change like those explored in the previous chapters (*e.g.*, population displacement or ecosystem engineering). Thus, released GM animals represent an emerging but significant threat to natural biodiversity, and eDNA could become a powerful tool to mitigate potential harm.

CHAPTER 3. TRANSGENES OF GENETICALLY MODIFIED ANIMALS DETECTED NON-INVASIVELY VIA ENVIRONMENTAL DNA

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[2]Department of Biology, McGill University, Montreal, Quebec, Canada

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3.1 Abstract

We demonstrate that simple, non-invasive environmental DNA (eDNA) methods can detect transgenes of genetically modified (GM) animals from terrestrial and aquatic sources in invertebrate and vertebrate systems. We detected transgenic fragments between 82-234 bp through targeted PCR amplification of environmental DNA extracted from food media of GM fruit flies (*Drosophila melanogaster*), feces, urine, and saliva of GM laboratory mice (*Mus musculus*), and aquarium water of GM tetra fish (*Gymnocorymbus ternetzi*). With rapidly growing accessibility of genome-editing technologies such as CRISPR, the prevalence and diversity of GM animals will increase dramatically. GM animals have already been released into the wild with more releases planned in the future. eDNA methods have the potential to address the critical need for sensitive, accurate, and cost-effective detection and monitoring of GM animals and their transgenes in nature.

3.2 Introduction

Environmental DNA (eDNA) is DNA extracted from environmental samples such as soil, sediment, water, air, feces, dust, as well as bulk DNA from artificial and natural collectors like Malaise insect traps, ocean sponges, and spider webs (Turner et al. 2015, Xu et al. 2015, Taberlet et al. 2018, Mariani et al. 2019). eDNA techniques commonly employ PCR, qPCR, and recently ddPCR to amplify taxonomically informatic DNA markers including 16S and 18S rRNA, cytochrome c oxidase subunit I (COI), and the internal transcribed spacer (ITS) from traces of DNA found in the environment for detection of specific species (Giovannoni et al. 1990, Doi et al. 2015). Compared to traditional methods, eDNA has proven to be more sensitive and accurate while requiring less time and lower costs (Rees et al. 2014b, Turner et al. 2014b). High-throughput next-generation sequencing of DNA markers and shotgun sequencing have also been utilized to generate large genetic data sets that span across taxonomic groups for community-level studies (Lodge et al. 2012, Evans et al. 2016, Olds et al. 2016, Evans et al. 2017, Stat et al. 2017). These eDNA methods have revolutionized biodiversity research and are increasingly used by academic biologists, environmental regulatory agencies, and private industry for biomonitoring purposes (Bohmann et al. 2014).

In parallel to the development of eDNA methods for biomonitoring, the advent of CRISPR-based genome-editing technologies have revolutionized molecular biology by vastly simplifying the process of creating genetically modified (GM) organisms, which has allowed transgenic research and production to advance dramatically (Jinek et al. 2012). This sudden democratization of genome-editing is leading to an explosion in the diversity of genetic modifications, the kinds of species targeted, and the contexts in which these methods are applied (Kaufman and Egender 2019). For example, do-it-yourself CRISPR kits are currently available

for purchase online with little to no restriction (Smalley 2018). Additionally, CRIPSR-based gene drives have been developed that enable a transgene to quickly spread across a population by favoring the inheritance of the transgene over natural genes (Grunwald et al. 2019). The use of GM animals outside laboratory environments has begun with AquaAdvantage® Atlantic salmon in the aquaculture industry (Ledford 2015). GM mosquitos have also been released in several locations around the world, and there are plans to release gene-driven GM white-footed mice onto human-populated islands (Reardon 2016, Buchthal et al. 2019, Waltz 2021). Although the application of GM methods to animal populations in natural settings is expected to increase rapidly in the coming years, there are currently no methods to detect and track GM animals that are efficient, accurate, and sensitive (Reardon 2016, Komor et al. 2017).

GM plants have been heavily utilized in agriculture and their transgenes have already been detected from environmental samples (Widmer et al. 1996). The environment has been found to serve as a reservoir for transgenes from GM plants with short-term persistence (hours to days) in aquatic environments and long-term persistence (days to years) in terrestrial soils (Barnes and Turner 2016). However, to our knowledge, detection of transgenes via eDNA from GM animals in nature has yet to be reported in the literature despite their recent proliferation including insect vectors, livestock, and pets (Reardon 2016). Because GM animals are indistinguishable from natural individuals based on appearance alone, eDNA methods could be especially useful for early detection and monitoring purposes. Just like wild species, GM animals are expected to shed eDNA through feces, skin cells, decomposition, and other natural processes that can be difficult if not impossible to control. Detectability, persistence, and environmental consequences of animal transgenes left in the environment are still unexplored issues.

In this study, we hypothesized that transgenes of GM animals are deposited in their environment and that this extra-organismal DNA could be used to detect the presence of GM animals. We report that fragments of transgenes from GM animals are indeed detectable non-invasively via environmental DNA across three different animal systems: invertebrates (fruit flies; *Drosophila melanogaster*), mammals (laboratory mice; *Mus musculus*), and fish (black tetras; *Gymnocorymbus ternetzi*) (Fig. 1).

3.3 Methods

3.3.1 Sample collection

For the invertebrate system, we extracted eDNA from approximately 3 g of food media from a laboratory fruit fly strain carrying a transgene encoding the green fluorescent protein fused to the *vasa* gene (*eGFP-vas*). The food media contained no observable flies or fly parts. The *eGFP*-tagged full length *vasa* gene was inserted using the *attB/attP* system. We used the FavorPrep Stool DNA Isolation Mini Kit (FAVORGEN Biotech) following the standard protocol except for a 90-minute (instead of 20-minute) incubation at 60°C during the lysis step. We also included an extraction blank using the same extraction method. A positive control from fly tissue was extracted using the following protocol: 1) A single frozen fly was homogenized into buffer containing 10 mM Tris-HCl, 1 mM EDTA, 25 mM NaCl, and 200 μg/mL proteinase K, 2) Fly-buffer mixture was incubated at 37°C for 20 minutes, 3) Supernatant was extracted and incubated at 95°C for 1 minute, 4) DNA was stored at 4°C.

For the mammalian system, we used a laboratory mouse strain carrying the tdTomato transgene (JAX stock number: 007905, Strain Name: B6;129S6-*Gt(ROSA)26Sor*$^{tm9(CAG\text{-}tdTomato)Hze}$/J) obtained from the McGill Integrated Core for Animal Modeling. We extracted non-

invasive extra-organismal DNA from feces inside the housing cage, from ~0.2 mL of urine, and from a cotton oral swab (~ 30 seconds) collected from a single individual. While these samples are technically not true eDNA samples, feces, urine, and saliva are animal eDNA sources in nature and thus provide a useful proof-of-concept since no transgenic mammals have been released to date. DNA extractions were conducted using the DNeasy PowerSoil Kit (QIAGEN) following the standard protocol. We also included an extraction blank and a positive control from an ear punch sample using the same extraction method.

For the fish system, we obtained water from a single 40-gallon aquarium containing approximately 40 GloFish® Cosmic Blue®, Electric Green®, Galactic Purple®, Moonrise Pink®, Starfire Red®, and Sunburst Orange® tetras (GloFish LLC, hereafter called GloFish tetras) from a local pet store (Montreal, Quebec, Canada). We filtered approximately one liter of aquarium water through 0.22 μM and 0.7 μM polyethersulfone filter papers (Millipore) separately using a handpump (Mityvac). Both filter pore sizes were used to maximize detection probability since the particle size of transgenic eDNA is unknown. We extracted eDNA from filter papers using the DNeasy PowerWater Kit (QIAGEN) following the standard protocol. We also included an extraction blank using the same extraction method.

All sample collection was non-invasive and did not involve any entire living materials. This "A" level of invasiveness did not require animal use approval at McGill University.

3.3.2 Primer design

We designed three different sets of primers to amplify 82-187 bp of the *eGFP* gene for detection of GM fruit flies (Table 1). A single pair of primers were used to amplify a 196 bp fragment of the *tdTomato* gene from GM laboratory mice (Table 1). For detection of GM GloFish tetras, we

designed three sets of primers targeting: 1) 213 bp of *dsRed2*, 2) 210 bp of *ZsGreen1*, and 3) 234 bp of *ZsYellow1* fluorescent genes (Blake et al. 2010) (Table 1). All primers were designed based on publicly available sequences obtained from the National Center for Biotechnology Information (NCBI) GenBank database using the Primer3 software (Untergasser et al. 2012, Sayers et al. 2020).

3.3.3 PCR amplification and analysis of products

DNA concentrations of samples were quantified using the Quant-iT™ High-Sensitivity dsDNA Assay (Invitrogen). DNA samples were amplified in polymerase chain reactions (PCR) of 10 µL containing 6.36 µL of ultrapure water (Milli-Q), 1 µL of 10X PCR Buffer (Invitrogen), 0.3 µL of 50 mM $MgCl_2$ (Invitrogen), 0.3 µL of 10 mM dNTP Mix (Invitrogen), 0.5 µL 10 mM forward primer, 0.5 µL 10 mM reverse primer, 0.04 µL of Platinum© Taq DNA Polymerase (Invitrogen), and 1 µL (<0.2-102.16 ng/µL) of genomic DNA. Negative control reactions with ultrapure water instead of DNA were included in every PCR to test for contamination. Gel electrophoresis was conducted using 5 µL of PCR product mixed with 1 µL TriTrack DNA Loading Dye (6X) (Thermo Scientific) and amplicon length was estimated using GeneRuler 100 bp DNA ladder (Thermo Scientific). Bi-directional Sanger sequencing was conducted on an ABI 3730xl 96-capillary sequencer by the Centre d'expertise et de services Génome Québec. DNA sequences were aligned using BioEdit v.7.2.5 and ClustalW (Hall 1999, Thompson et al. 2003).

3.4 Results

Genomic DNA concentrations of eDNA extractions ranged from <0.2 ng/µL (threshold of quantification assay) to 102.16 ng/µL. All target transgenes were successfully detected based on

estimated amplicon sizes except for *dsRed2* from the 0.7 μM filter while extraction blanks and PCR negative controls yielded no amplification (Fig. 2). DNA sequences were obtained via Sanger sequencing. Forward and reverse reads of each sample were aligned, and primer sequences were then removed. Transgene identities of aligned amplicons were confirmed by NCBI BLAST using default settings and alignment with reference genes downloaded from the GenBank Nucleotide database (Sayers et al. 2021). All raw DNA sequences and reference alignments are accessible on DRYAD at https://doi.org/10.5061/dryad.866t1g1pp.

3.5 Discussion

Our results demonstrate that transgenes from a diversity of GM animals can be detected from non-invasive environmental DNA samples thus providing proof-of-concept that eDNA has the potential to be a powerful tool in biomonitoring of GM animals. The single failed amplification of *dsRed2* from the 0.7 μM filter is likely due to low total DNA concentration (<0.2 ng/μL), which is consistent with weak amplification of *ZsGreen1* and *ZsYellow1* from the same sample. Despite DNA concentrations of less than the threshold of the quantification assay, the 0.7 μM filter along with the mouse urine and mouth swab samples still successfully amplified and produced clear chromatograms from Sanger sequencing suggesting that eDNA methods are highly sensitive in detecting transgenes. Our results also suggest that transgenes are more likely to be detected using 0.22 μM rather than 0.7 μM filters in aquatic environments. While both mouse urine and mouth swab samples yielded less than quantifiable amounts of total DNA, only the urine sample showed weak amplification, which indicates that concentration of transgenic DNA may not always correspond with total DNA concentration. This relationship is predicted to

change depending on the type of eDNA sample collected and the amount of nontarget DNA present (Barnes and Turner 2016).

The samples used in this study were collected under laboratory conditions and commercial settings, which likely biased detection success. Application of eDNA methods for detection of transgenes from GM animals in nature is expected to be more complicated due to environmental exposure and fluctuating conditions (Barnes et al. 2014, 2020). Typical eDNA assays target short gene fragments because eDNA is readily susceptible to degradation, influenced by factors such as temperature, turbidity, acidity, salinity, and bacterial abundance (Harrison et al. 2019). Determining the particle size, degradation, persistence, and ecological fate of animal transgenes in the environment will be important in developing eDNA methods for tracking GM animals (Turner et al. 2014a, Barnes and Turner 2016, Goldberg et al. 2016). Nonetheless, this proof-of-concept demonstration is the first step towards future validation studies conducted in field settings using more sensitive methods such as qPCR and ddPCR. Metabarcoding and metagenomic methods also hold promise for simultaneous detection of multiple transgenes across multiple GM species (Garlapati et al. 2019).

One important factor affecting the sensitivity of eDNA methods is the copy number of the target DNA sequence. Most eDNA studies use mitochondrial DNA like 16S rRNA or the COI gene to maximize detection probability because of their high copy numbers per cell (Thomsen and Willerslev 2015). Additionally, eDNA studies using multicopy nuclear genes like 18S rRNA and ITS have also been successful (Drummond et al. 2015). While some transgenes are present in tandem multiple copy arrays across the nuclear genome, many are single genes that have either been edited or inserted (Henikoff 1998). Single transgenes may thus be relatively harder to detect than conventional eDNA markers due to copy number differences. Additionally,

if the eDNA detection method targets a specific transgenic allele, genotype may also influence sensitivity (homozygous allele copy number is twice that of heterozygous and hemizygous alleles in diploid species) (Lodish et al. 2021). Transgenes are also often inserted inside transposons, which can lead to multiple independent insertion events and positively bias eDNA detection. Another unexplored research frontier is the consequence of newly available epigenome-editing tools on the efficiency of eDNA amplification and sequencing of epigenetically modified genes due to potential structural changes (Pulecio et al. 2017).

Concerns have been raised about the potential for transmission of transgenes from GM organisms and the subsequent ecological effects. Methods of transmission into unintended populations and species include cross-pollination, hybridization, and horizontal gene transfer (HGT) (Traavik 1999). For example, despite the presence of a dominant lethal transgene, reportedly sterile GM mosquitoes in Brazil have been able to create viable hybrids with wild individuals (Evans et al. 2019). Additionally, there are demonstrated ecological impacts of viable hybrids created from GM Atlantic salmon and wild brown trout, which are able to grow faster and competitively suppress both GM and wild salmon (Oke et al. 2013). HGT through a natural ability to uptake naked plasmids and fragments of chromosomal DNA directly from the environment has been observed in many bacterial species across a variety of habitats (Johnsborg et al. 2007). While there has been no documented case of HGT from GM animals in nature, there is evidence for HGT of transgenes from GM plants to bacteria and fungi despite transmission and establishment barriers (although these events are rare and mostly limited to transgenes of bacterial origin that are often already abundant in the environment) (Keese 2008). Despite these valid concerns, GM organisms have many significant benefits for the environment, human

health, agriculture, and industry that have improved global human well-being and have led to valuable scientific discoveries (Reardon 2016).

The advantages of using eDNA to detect GM organisms could synergize well with artificial DNA barcodes. Used as identification tags for transgenes, artificial DNA barcodes can be synthesized to contain a unique DNA sequence not found in nature (Gressel and Ehrlich 2002a, Marillonnet et al. 2003a). These silent barcodes are neither transcribed nor translated and their sole purpose is to track neighboring transgenes. Artificial DNA barcodes can be linked to metadata associated with the barcoded GM individual (e.g., identities and number of transgenes present, geographic location and date of creation, intended usage, etc.), and multiple barcodes within a single individual can also be used to independently track multiple transgenes using a metagenomics approach. The design of artificial DNA barcodes would incorporate primer binding sites to facilitate efficient eDNA detection, enabling sensitive, non-invasive, and ubiquitous biomonitoring of GM organisms. By providing a method for quick and easy differentiation of GM organisms, artificial DNA barcodes may help to alleviate public and governmental concerns and inform policies regarding their potential release. In addition, artificial DNA barcodes may be incorporated into gene-drives to track their spread across populations, which has been a major concern for application of gene-drives in nature (Oye et al. 2014). Although the idea of artificial DNA barcodes is not new, and they have been used to 'watermark' artificially synthesized genomes, we are unaware of wide adoption by regulatory agencies or industry (Arita and Ohashi 2004, Gibson et al. 2008, 2010). Further development of biotechnologies like artificial DNA barcodes and their use with emerging biomonitoring methods like eDNA could become an important tool for transgenic producers and regulators to mitigate potential environmental and human health risks of creating and releasing GM animals.

3.6 Conclusion

Potential escape of GM animals from their intended locations and potential introgression of transgenes into unintended populations and species could have significant ecological, evolutionary, and bioethical implications. eDNA methods will improve our ability to locate and manage released GM animals and their transgenes across diverse species and environments in these scenarios.

3.7 Acknowledgements

In memory of Dr. Cameron R. Turner, a pioneer in eDNA research, mentor, and friend. Wherever you are now, we hope you are 'living the dream'. We also thank Michelle Gros for filtration equipment and Nobuko Yamanaka for primer information.

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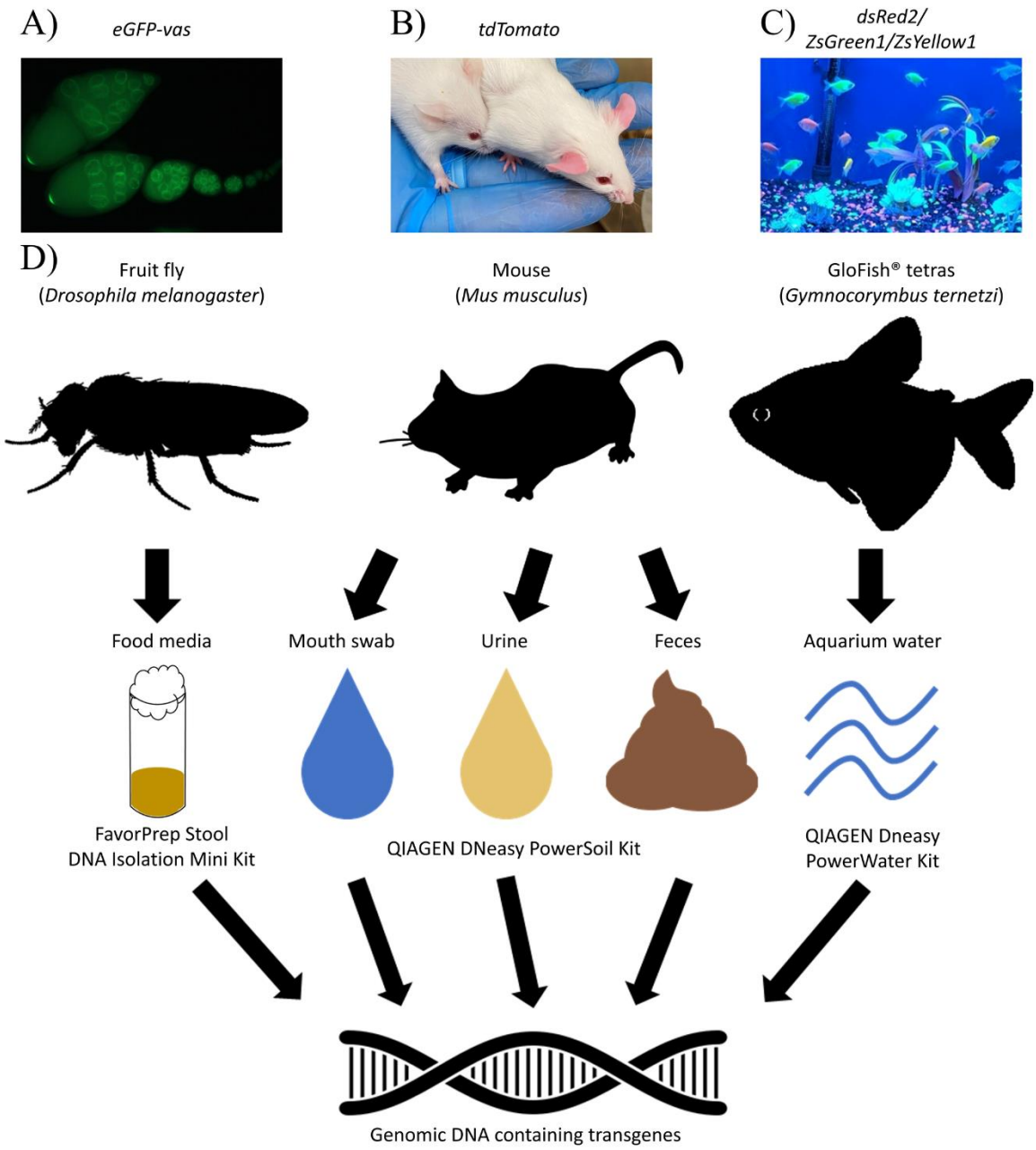
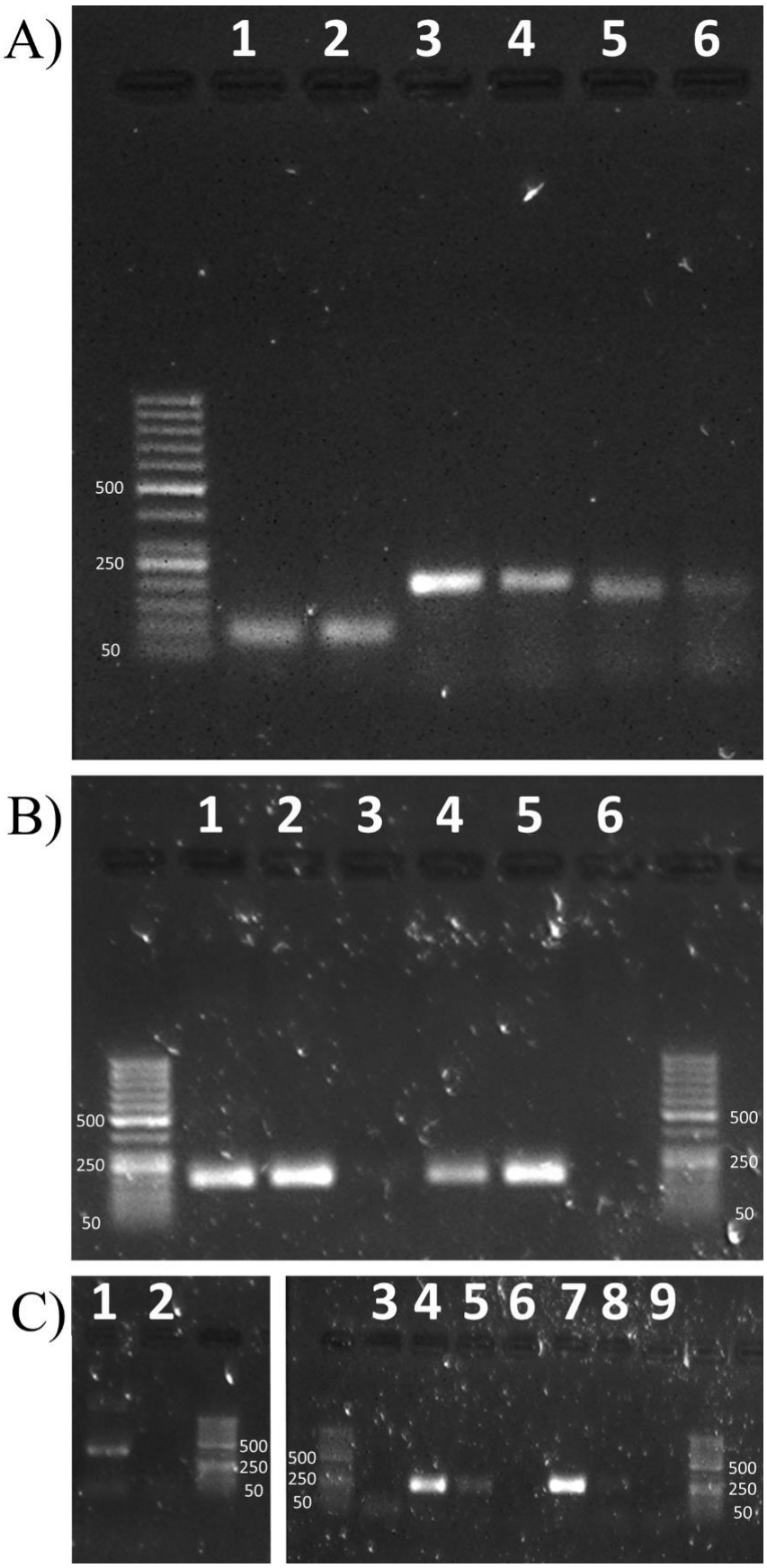
3.9 Figures and tables



3.9.1 Fig. 1. Study design. A) Green fluorescent ovary tissue of genetically modified fruit fly (*Drosophila melanogaster*) expressing green fluorescent protein tagged vasa gene (eGFP-vas). B) Laboratory mice (*Mus musculus*) genetically modified to express tdTomato transgene exhibiting reddish skin (right) compared to without tdTomato (left). C) Multicolored fluorescent GloFish® tetras (*Gymnocorymbus ternetzi*) expressing combinations of transgenic fluorescence genes in a commercial pet store aquarium. D) Non-invasive environmental DNA samples from a diversity of sources (food media, saliva, urine, feces, and aquarium water) were collected and processed using standard commercial DNA extraction kits.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3.9.2 Fig. 2. Agarose gel electrophoresis showing A) amplification of *egfp* from genetically modified fruit fly (*Drosophila melanogaster*) – 1: fly tissue with egfp_F1/R1, 2: fly food media with egfp_F1/R1, 3: fly tissue with egfp_F2/R2, 4: fly food media with egfp_F2/R2, 5: fly tissue with egfp_F3/R3, 6: fly food media with egfp_F3/R3, B) amplification of *tdTomato* from feces, urine, and saliva of genetically modified mouse (*Mus musculus*) – 1,2: mouse feces from different cages, 3: mouse urine (weak amplification), 4: mouse mouth swab, 5: mouse ear punch, 6: DNA extraction negative control (no amplification), and C) amplification of *dsRed2*, *ZsGreen1*, and *ZsYellow1* from filtered aquarium water of GloFish® tetras (*Gymnocorymbus ternetzi*) – 1: 0.22 μM filtered water with dsRed2 F1/R1, 2: 0.7 μM filtered water with dsRed2 F1/R1 (no amplification), 3: DNA extraction negative control (no amplification), 4: 0.22 μM filtered water with ZsGreen1 F1/R1, 5: 0.7 μM filtered water with ZsGreen1 F1/R1 (weak amplification, 6: DNA extraction negative control (no amplification), 7: 0.22 μM filtered water with ZsYellow1 F1/R1, 8: 0.7 μM filtered water with ZsYellow1 F1/R1 (weak amplification), 9: DNA extraction negative controls (no amplification). Gel images were captured using Quantum Vilber Lourmat (MBI) and were cropped to only relevant lanes.

3.9.3 Table 1. Primers for PCR amplification of transgenic elements.

Name	**Sequence (5'-3')**	**Forward/ Reverse**	**Length (bp)**	**Tm (C°)**	**GC (%)**	**Target**	**Amplicon size (bp)**	**Reference**
egfp_F1	GAGCAAAGACC CCAACGAGA	Forward	20	59.97	55	eGFP	82	This study
egfp_R1	GTCCATGCCGA GAGTGATCC	Reverse	20	60	60	eGFP		This study
egfp_F2	ACGTAAACGGC CACAAGTTC	Forward	20	60	50	eGFP	187	This study
egfp_R2	AAGTCGTGCTG CTTCATGTG	Reverse	20	60.1	50	eGFP		This study
egfp_F3	TATATCATGGC CGACAAGCA	Forward	20	60.1	45	eGFP	163	This study
egfp_R3	ACTGGGTGCTC AGGTAGTGG	Reverse	20	60.2	60	eGFP		This study
oIMR9103	GGCATTAAAGC AGCGTATCC	Forward	20	60.5	50	tdTomato	196	(Madisen et al. 2010)

oIMR9105	CTGTTCCTGTA CGGCATGG	Reverse	19	60.5	57.9	tdTomato		(Madisen et al. 2010)
dsRed2_F1	GAACGTCATCA CCGAGTTCA	Forward	20	59.7	50	dsRed2	213	This study
dsRed2_R1	GGGTGCTTCAC GTACACCTT	Reverse	20	60	55	dsRed2		This study
ZsGreen1_F1	CCCCGTGATGA AGAAGATGA	Forward	20	61	50	ZsGreen1	210	This study
ZsGreen1_R1	GTCAGCTTGTG CTGGATGAA	Reverse	20	60	50	ZsGreen1		This study
ZsYellow1_F1	GACCGGATCTT CACCGAGTA	Forward	20	60.1	55	ZsYellow1	234	This study
ZsYellow1_R1	CTCCCAGTTGG TGGTCATCT	Reverse	20	60	55	ZsYellow1		This study

DISCUSSION

MUIR with a magnifying glass examining spots in petrified wood.

"*The world is big and I want to have a good look at it before it gets dark.*"

– John Muir (Wolfe 1945)

Research themes

This thesis utilizes a multidisciplinary approach to address hypotheses and challenges of biodiversity dynamics and monitoring during rapid environmental change. By taking advantage of cutting-edge genetic and metagenomic methods within longitudinal experimental designs and innovative study systems across a diversity of disciplines from dermatology to limnology, this thesis provides in-depth investigations into the ecological and evolutionary mechanisms driving biodiversity response to stress and novel habitats. Studies were complementary as observational and manipulative experiments were conducted on both model and non-model systems in well-studied and novel habitats using quantitative and qualitative stressors. While this research builds iteratively upon past work in evolutionary and community rescue, an original model system and

biodiversity monitoring method were proposed for future studies as well as practical applications.

Microbial study designs

The study of any sort of change implies the need for observation over time. Although it is possible to infer temporal processes like species sorting and adaptation from cross-sectional data, longitudinal experimental designs and analyses employing repeated measures of the same individuals or populations across time-series like those described in Chapters 1 and 2 are especially powerful (Barrett and Hoekstra 2011, Faust et al. 2018, Barrett et al. 2019, Pringle et al. 2019). This is particularly true for establishing cause and effect between environmental perturbations and ecological stability (*i.e.*, resilience and resistance) of microbial communities (Stein et al. 2013, Coyte et al. 2015, Ridenhour et al. 2017). Microbial communities like skin microbiomes and lake bacteria serve important roles as study systems due to their relative ease of care and manipulation, complex interspecies relationships, unrivaled diversity, and fast generation times allowing for more rapid ecological and evolutionary responses (Allen and Banfield 2005, Mitchell-Olds et al. 2008). However, the ecological complexity and high dimensionality of anthropogenic environmental change is expected to create no-analog selection regimes in the future (Fitzpatrick and Hargrove 2009, Bay et al. 2017). This is problematic given that experimental evidence of microbial response to environmental change have historically been limited to single or few model species in controlled laboratory environments. Hence, innovative approaches such as using human piercings as a model for microbiome response to novel ecological niches can provide fresh perspectives and uncover previously undescribed dynamics at play in nature (McDonald et al. 2020).

Natural microbial communities are ubiquitous and foundational to community and ecosystem functioning as well as host health in the context of microbiomes. While laboratory experiments of isolate cultures or mock communities offer unparalleled control and high replication, the goal of ecology and evolution is to ultimately understand life as it happens in nature (Gould et al. 2007). Exposure to natural variation of biotic and abiotic environmental factors is the rule and often have important implications for mechanisms underlying biodiversity responses to stress (Drake and Lodge 2004, Sgrò and Hoffmann 2004, Lawson et al. 2015). However, such studies of populations and communities in natural environments are often constrained to observational or retrospective investigations, and for good reason (Farnsworth and Rosovsky 1993, Marsh and Kenchington 2004, Soulsbury et al. 2020, Zemanova 2020). This gap between manipulative laboratory experiments and observational studies in natural environments highlights the opportunity of mesocosm-based semi-natural experiments that can be replicated on an ecological scale (Odum 1984). Such studies like Chapter 2 and other experiments conducted at LEAP and other mesocosm facilities worldwide (www.mesocosm.org) can offer a compromise between experimental power and ecological realism in the study of natural populations and communities (Ledger et al. 2006, 2009). Mesocosm (and on a smaller scale, microcosm) study systems have been useful for ecological risk assessment of pesticides, merging academic and applied interests of understanding the ecological and evolutionary impacts of such chemical stress on biological communities (Graney 2020). However, concerns do exist regarding the extent of ecological realism as species assemblages tend to be diversity poor and the vertical walls of mesocosms exert their own selection pressures, excluding establishment of certain species over others, especially when mesocosm experiments are extended to studies of

invertebrate, plant, and animal communities (Williams et al. 2002, Beketov et al. 2008, Reiber et al. 2022).

Implications

The results of this thesis have implications for both fundamental understanding of biodiversity dynamics and for motivating and enabling measures to proactively avoid potential future ecological catastrophes. Chapters 1 and 2 provide evidence that ecological responses and contemporary adaptation are significant factors contributing to species survival and the maintenance of biodiversity. Specific situations in which rapid environmental change is expected to catalyze rapid ecological and evolutionary recovery after disturbance include sufficient standing genetic variation, taxonomic diversity, population sizes to absorb the cost of initial selection without biological collapse (Orr and Unckless 2008, Messer and Petrov 2013). Local sterilization in Chapter 1 was expected to cause strong and broad negative impacts on taxonomic diversity, but this was not immediately observed despite intensive time sampling beginning after piercing. On the contrary, the novel ecological niche within piercings appeared to have facilitated greater diversification thus demonstrating that rapid environmental change does not always result in stress (Hoffmann and Parsons 1997, Dornelas 2010, Wellborn and Langerhans 2015, Delicado et al. 2018). Like how seasonal rain may cause a bloom of biological activity (Fischer et al. 2022, Checon et al. 2023), normal environmental factors of the human skin including constant exposure and the lack of nutrients may have imposed ecological constraints on carrying capacity that were ameliorated by the novel piercing niche (Byrd et al. 2018, Swaney and Kalan 2021).

Chapter 2 provides a complementary and contrasting perspective whereby despite stress pre-exposure, which was demonstrated to have protective benefits to community resistance and genetic diversities, all communities suffered drastic loss in taxonomic diversity and ultimately converged in the long term. Strong independently convergent responses were observed across isolated mesocosms as expected when gene flow is absent (Smith and Wilson 2002, Vanschoenwinkel et al. 2010, Tenaillon et al. 2012, Kaeuffer et al. 2012, Roesti et al. 2014, Leale et al. 2023). Community resistance was facilitated by temporal heterogeneity of pre-exposure acidification stress, which is predicted to analogously extend to spatial heterogeneity where extreme environmental conditions at the local scale can maintain standing variation and pre-adapt populations that serve as adaptive sources of migration. In other words, gene flow impacts adaptation when there is population structuring due to environmental heterogeneity, which enables the seeding of adaptation and evolutionary rescue of populations across metacommunities (Ralph and Coop 2010, Uecker et al. 2014, Polechová and Barton 2015). Thus, Chapters 1 and 2 demonstrate the complexity of biodiversity responses to rapid environmental change depending on ecological contexts and heterogeneities, which has potential implications for knowledge transfer of evolutionary and community rescue studies towards applications in human health (*e.g.* microbiome dysbiosis after perturbations, evolutionary medicine, and infectious disease prevention), agricultural and natural resource management (*e.g.*, resistance evolution to pesticides and sustainable improvement of production yields), and environmental conservation (*e.g.*, mechanisms underlying maintenance of genetic diversity and population persistence) (Carroll et al. 2014).

Human-induced rapid environmental change such as deforestation followed by land-use change analogously modeled by human piercings in Chapter 1 and acidification of freshwater

habitats as directly tested in Chapter 2 have driven global biodiversity loss at alarming rates risking essential biodiversity and ecosystem functioning (Cardinale et al. 2012). Aside from climate change and environmental degradation, the era of genetic engineering and synthetic biology represents a fundamental change in the relationship between humans and the natural world (Young 2004, Parekh 2004, Dana et al. 2012). GM animals have been heralded as both a potential savior and devastator of biodiversity, especially if they make their way into the wild either intentionally or unintentionally (Redford et al. 2013, 2014). Regardless of the potential future risks of GM animals, which are inherently difficult to accurately identify and quantify (Silver 2012), methods for their accurate and sensitive detection in nature will be useful. In this respect, Chapter 3 has tangible implications towards the management of released GM animals by taking advantage of equally revolutionary advances in biodiversity monitoring through eDNA methods. This will be particularly useful for detecting GM animals that are physically indistinguishable from natural animals and are difficult to observe via traditional means (Xu et al. 2021). Additionally, it can facilitate novel regulations regarding contingency plans as well as the development of novel biosafety technologies (*e.g.*, artificial DNA barcodes as discussed in Chapter 3) in anticipation of the challenges that will arise from the inevitable release of GM animals into the wild before those problems become realized.

Conversely, GM organisms including animals have largely been safe (Beringer 2000). Preventative measures like kill switches, selective sterility, and xenobiology as well as ethical and legal guidelines continue to be developed (Schmidt 2010, EFSA Panel on Genetically Modified Organisms (GMO) 2013, Tan et al. 2013, Aldrich 2015, Chan et al. 2016, Neuhaus 2018, Clark et al. 2018, Rottinghaus et al. 2022, Bohua et al. 2023). Despite this progress, fears about GM organisms currently outweigh the known risks (Lowenthal 2014, Murray and Maga

2016). Thus, Chapter 3 could also increase mainstream acceptance of GM animals by improving the ability to locate and manage them in nature (Lievens et al. 2015).

Strengths

One of the strengths of this thesis is the diversity and complementarity of each chapter in understanding and addressing practical issues regarding how biodiversity responds to rapid environmental change. This thesis presents a number of originalities including the first scientific exploration of the human piercing microbiome in Chapter 1 and the first application of eDNA methods to detect transgenes from GM animals in Chapter 3. In addition, Chapter 2 employs a level of WGS sequencing power that is rarely achieved in such manipulation experiments of natural complex communities, which was able to provide evidence of independent and parallel genome-wide allele frequency shifts across multiple acidophilic species without *a priori* bias regarding specific taxa or targets under selection.

Upon publication, Chapter 3 attracted significant mainstream and popular science media including Canadian Geographic, Yahoo!/Aol. News, popular science magazines Quo (Spanish-language) and Focus (Italian), and the Korea Foundation for the Advancement of Science & Creativity (KOFAC), which indicates broad interest beyond pure scientific or academic spheres thus serving an important role in science communication and public understanding of science (Stocklmayer et al. 2001, Burns et al. 2003, Broks 2006). This becomes especially significant given that addressing contemporary and future biodiversity challenges necessitates collaborating and negotiating with diverse stakeholders and convincing often non-expert audiences the importance of protecting and conserving biodiversity (Bouamrane et al. 2016, Reed et al. 2019, Raymond et al. 2022). Additionally, such public appeal can be a powerful tool in inclusive

science communication (ISC), which can greatly benefit equity, diversity, and inclusion (EDI) as participation of individuals from low-income, minority ethnic backgrounds in science communication are marginalized and primarily limited to science media consumption (Dawson 2018, Judd and McKinnon 2021, Canfield and Menezes 2022). These strengths are also expected to extend to Chapter 1 given the diverse yet wide-spread popularity of skin piercings and human microbiomes across society (DiStefano and Harjani 2021), as well as directly addressing a major barrier to ISC, powerlessness and participatory exclusion related to race/ethnicity and its intersections with class/income, by incorporating a citizen science approach (Bonney et al. 2016, Dawson 2018).

Limitations

An obvious limitation of this thesis is the temporal resolution at which samples were taken and thus at which ecological and evolutionary dynamics could be observed. Sampling intensity in Chapter 1 was limited to reduce burden on study participants who were responsible for self-collecting samples over the course of 2 weeks. Only four out of the 13 time points when samples were collected in Chapter 2 were selected for amplicon and metagenomic sequencing due to prohibitive costs, which was especially true of WGS sequencing where an average of approximately $350 was spent per sample. Similarly, environmental heterogeneity and dispersal are predicted to significantly impact biodiversity responses to stress, yet sequencing was only conducted on samples from isolated mesocosms due to costs despite samples having been collected for other dispersal regimes as well.

Phenotypic data was unavailable in Chapters 1 and 2, which is difficult to collect for natural complex bacterial populations. Thus, the relative contribution of plasticity to biodiversity

dynamics remains unknown. Additionally, the genetic basis of adaptation was not identified in either Chapter 1 or 2, which meant selection coefficients could not be calculated and causality is undetermined despite the importance of such calculations on understanding how selection acts on genetic variation and the outsized impact of certain individual genes on ecological processes (Thurman and Barrett 2016, Skovmand et al. 2018).

While proof-of-concept was demonstrated in Chapter 3, the study did not go beyond captive GM animals as they have yet to be released into the wild. However, a recent study did show that transgenic eDNA of GM salmon could be detected from fish farm effluent but remains unpublished in the peer-reviewed literature (Kajtar 2021). Regardless, this study indicates that eDNA detection of transgenes should translate well to escaped GM animals in nature.

Future directions

Follow-up studies on Chapter 1 could compare community assembly processes across different types of skin piercings on other parts of the human body as well as different sterilization methods to elucidate common patterns. Greater diversity and size of study participants could yield significant correlations with specific participant metadata, which may lead to an integrated ecological understanding of piercing infections to improve safety and health outcomes. Application of metagenomic methods may also connect evolutionary adaptation to ecological processes like in Chapter 2.

Future mesocosm experiments investigating how environmental perturbations affect biodiversity dynamics such as those conducted at LEAP would benefit from direct measures of demography (*i.e.*, population sizes) to address evolutionary and community rescue questions as well as phenotypic measurements of adaptive traits to measure selection coefficients. Such

studies would also benefit from taking a polygenic approach as most adaptive traits are known to be driven by many loci of small effect sizes, which likely includes those that provided resistance to acidification in Chapter 2 as well as increased fitness in the piercing environment in Chapter 1 (Barghi et al. 2020). Accounting for the genetic architecture of adaptation will help address long-standing questions regarding the direction, rate, magnitude, and limits of eco-evolutionary processes and their role in biodiversity recovery (or lack thereof) post-disturbance (Bay et al. 2017). Additionally, it will be important to include metacommunity dynamics at various spatial scales to assess the interactive effects of environmental heterogeneity and dispersal on biodiversity response.

Artificial DNA barcodes, described as "identification tags for transgenes… (that) can be synthesized to contain a unique DNA sequence not found in nature" represent an exciting future direction for eDNA detection of GM organisms (Xu et al. 2021). Although the idea is not new, its adoption does not appear to be widespread (Gressel and Ehrlich 2002b, Marillonnet et al. 2003b). With an explosion in the global diversity and abundance of GM plants and animals on the horizon, the development and validation of such regulatory technologies on genetic engineering and synthetic biology will be needed more than ever.

CONCLUSION

Together, the three chapters of this thesis utilize the power of DNA sequencing to discover, investigate, and help protect biodiversity – from ecosystems to nucleotides – during rapid environmental change. In summary, this thesis shows that rapid environmental change has the potential to drastically reshape natural biological communities across multiple levels of biodiversity from species composition to genomic diversity. Under certain contexts, environmental change can increase biodiversity despite disturbance as was observed in human ear-piercings after local sterilization. Under other contexts like severe acidification of lakes, environmental stress can cause significant biodiversity loss even when communities have been pre-adapted through pre-exposure. Because GM animals have the potential to become major drivers of environmental stress and biodiversity dynamics if released in the future, this thesis also provides proof-of-concept that eDNA methods could become a powerful biomonitoring tool for GM animals in nature. Better understanding of biodiversity responses to environmental stress as well as improved methodologies for such ecological, evolutionary, and biomonitoring studies will be increasingly necessary to keep pace with the unprecedented ways humans are impacting global biodiversity. The chapters of this thesis provide a strong synergistic experimental framework and novel research directions for doing so. Ultimately, as biologists, we study biodiversity because it fascinates and inspires our curiosity. We need to continue bridging our science to action so that the wealth of our accumulated knowledge does not become but historical footnotes of the wonderous biodiversity that used to be.

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