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Ecological dynamics of a synthetic bacterial community growing in a gradient of nutrients and toxicity

Thèse de doctorat ès sciences de la vie (PhD)

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Faculté de biologie et de médecine
de l'Université de Lausanne

par

Rita DI MARTINO

M.Sc. en Biologie, orientation biomoléculaire
Université de Naples "Federico II"

Jury

Prof. Tanja Schwander, Présidente
Prof. Sara Mitri, Directrice de thése
Prof. Jan-Willem Veening, Co-directeur de thése
Prof. Melanie Blokesch, Experte
Prof. Orkun Soyer, Expert

Lausanne
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A mio nonno Domenico,
che mi ha sempre spronato a fare ciò che mi piaceva.
L'ho fatto.

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Abstract

Bacterial communities are composed of many species that can interact in intricate ways. However, how the environment affects bacterial interactions and coexistence over time is still poorly understood. This PhD thesis aims to provide answers to this question by investigating the impact of a single compound that creates a nutrient-toxic gradient on community dynamics. We used a small synthetic community composed of *Agrobacterium tumefaciens* (At), *Comamonas testosteroni* (Ct), *Microbacterium saperdae* (Ms) and *Ochrobactrum anthropi* (Oa). These four species can degrade Metal Working Fluids (MWF), oil-based fluids used in manufacturing that is designed to be toxic for bacteria. A former study found that inter-species interactions within this community were consistent with the predictions of the Stress Gradient Hypothesis (SGH), which posits that positive interactions are more likely to arise in harsh environments.

To reduce the complexity of the MWF chemical composition, we designed a chemically-defined system. We tested the effect of several MWF compounds on the four species and chose to focus on Linoleic acid (LA), which was a nutrient for At and Ct at low concentration, and toxic for At but not for Ct at high concentration. We combined mathematical models and lab experiments to test the predictions of the SGH. Our model predictions and experimental results were consistent: in co-culture, competition for the single nutrient source occurred at low LA concentration. Instead, facilitation arose at high LA concentration, because as Ct consumed LA to grow, its concentration decreased, allowing At to survive and grow.

Next, we investigated the mechanism behind LA toxicity and found that reactive oxygen species (ROS) were accumulating over time as a consequence of LA oxidation. By quantifying ROS, we found that at high LA concentration, it was high in At's monoculture, but low in the presence of Ct (both mono- and co-culture). This result proved that facilitation towards At occurred because Ct reduced ROS concentration. Furthermore, removing ROS from the high LA medium using an antioxidant rescued At in monoculture and reverted the interaction sign back to competition in the co-culture with Ct. This meant that, in absence of toxicity, negative interactions prevailed once again.

Finally, we tested whether the two species could coexist on the single LA resource. The mathematical model predicted that only short-term coexistence was possible and that only Ct would survive in the long-term, as expected from the competitive exclusion principle, whereby coexistence between two species is not possible on a single nutrient source. The experimental results of a transfer experiment at both low and high LA concentrations confirmed model predictions.

Overall, we have shown that one can manipulate interactions just by changing the concentration of a single compound that has a toxic effect. We have advanced our understanding of the role of toxicity in determining inter-specific interactions. We also highlight that further exploration of the impact of ROS on community dynamics is needed to better understand the circumstances under which the SGH operates.

Résumé

Les communautés bactériennes sont composées de nombreuses espèces qui peuvent interagir de manière complexe. Cependant, la façon dont l'environnement affecte les interactions entre bactéries et leur coexistence au fil du temps est encore mal comprise. Cette thèse de doctorat vise à apporter des réponses à cette question en étudiant l'impact d'un seul composé à l'origine d'un gradient à la fois toxique et nutritif, sur la dynamique des communautés. Nous avons utilisé une petite communauté synthétique composée d'*Agrobacterium tumefaciens* (At), *Comamonas testosteroni* (Ct), *Microbacterium saperdae* (Ms) et *Ochrobactrum anthropi* (Oa). Ces quatre espèces peuvent dégrader le metal working fluid (MWF), un fluide à base d'hydrocarbure utilisé dans l'industrie et conçu pour être toxique pour les bactéries. Une étude antérieure a montré que les interactions interspécifiques au sein de cette communauté correspondaient aux prédictions de l'hypothèse de gradient de stress, qui prédit que les interactions positives comme la facilitation sont plus susceptibles de se produire dans des environnements stressants ou toxiques.

Afin de réduire la complexité de la composition chimique du MWF, nous avons conçu un système défini chimiquement. Nous avons testé l'effet de plusieurs composés du MWF sur les quatre espèces et avons choisi de nous concentrer sur l'acide linoléique (AL), qui est un nutriment pour At et Ct à faible concentration, et est toxique pour At, mais pas pour Ct, à forte concentration. Nous avons combiné des modèles mathématiques et des expériences de laboratoire pour tester les prédictions de l'hypothèse de gradient de stress. Les prédictions de notre modèle et les résultats expérimentaux concordent : à faible concentration en AL, on observe une compétition entre At et Ct pour la source unique de nutriment. Inversement, à haute concentration en AL, on observe de la facilitation, car à mesure que Ct consomme l'AL pour sa croissance, sa concentration diminue, permettant ainsi la survie et la croissance de At. Nous avons ensuite étudié le mécanisme à l'origine de la toxicité de l'acide linoléique et montré que son oxydation conduit à une accumulation en espèces réactives de l'oxygène (ROS). En quantifiant les ROS, nous avons constaté qu'à une concentration élevée en acide linoléique, leur quantité était élevée dans la monoculture d'At, mais faible en présence de Ct (mono- et co-culture). Ce résultat montre que le mécanisme de la facilitation de At par Ct est la réduction de la concentration de ROS par Ct. De plus, en éliminant les ROS à forte concentration en AL à l'aide d'un antioxydant, At survi en monoculture, et est en compétition avec Ct en co-culture. Cela signifie qu'en absence de toxicité, les interactions négatives dominent.

Enfin, nous avons testé si les deux espèces peuvent coexister avec l'acide linoléique comme seule ressource. Le modèle mathématique prédit que seule la coexistence à court terme est possible et que seul Ct devrait survivre à long terme, comme prédit par le principe d'exclusion compétitive, selon lequel la coexistence entre deux espèces n'est pas possible en présence d'une seule source de nutriments. Les résultats expérimentaux d'une expérience de transfert par dilutions sérielles à des concentrations en AL faibles et

élevées ont confirmé les prédictions du modèle.

Dans l'ensemble, nous avons montré que l'on peut manipuler les interactions entre deux espèces en modifiant la concentration d'un seul composé qui a un effet toxique. Nous avons fait progresser notre compréhension du rôle de la toxicité dans la détermination des interactions interspécifiques. Nous soulignons également qu'une exploration plus approfondie de l'impact des ROS sur la dynamique des communautés microbiennes serait nécessaire pour mieux comprendre les circonstances dans lesquelles l'hypothèse du gradient de stress opère.

Summary

In our daily lives, we encounter bacteria in almost every environment on the planet: they are present in soil, in marine environments and in multiple areas of the human body, such as the lungs, skin and gut. In all these contexts, bacteria are not only surrounded by cells of the same species, but they rather live in multispecies communities. Multispecies microbial communities play an important role in human wellness and also in economical activities: a healthy gut microbiome is crucial for human well-being and some soil communities are used in agriculture to burst crop growth. Within these communities, bacteria interact with each other and with the surrounding environment. We can broadly categorize interaction types of one species towards another as positive if it brings an advantage to the recipient, negative if it damages the recipient, and neutral otherwise. It is important to understand the forces that drive bacterial interactions and the role of the environment in determining the sign of interaction (positive, negative or neutral).

In my PhD project, I focused on studying how different the environment affects interactions among bacteria that belong to the same small community. More specifically, I tested how nutrient abundance and toxicity for bacteria can affect their interactions and whether they can continue to coexist in the same environment. In line with former studies, I found that in an environment with a small quantity of a single nutrient source, bacteria competed for the sole available food. In a second environment, the same nutrient source was present in higher quantities, but there was also the accumulation of a compound that was toxic for one of the bacterial species. In this mixed toxic-nutrient environment, toxin-sensitive species grew better in the presence of the toxic-resistant one. We discovered that this facilitation was occurring because the resistant species could reduce the toxic compound, creating a more permissive environment for the sensitive species. However, we also discovered that if we artificially removed the compound ourselves, the sign of interaction changed from positive back to negative, bringing the species to compete once again.

We achieved these results combining both experiments performed in the lab and analysing the predictions of mathematical models. We also used this combined experimental-mathematical approach to test whether these diverse interactions would allow these two species to coexist in the long term or if competition would lead to only one or the other species to survive. We found that the two species can coexist in the short-term, but that eventually, the toxic-resistant species will prevail.

This PhD work has shed light on the impact of environmental toxicity on interactions and coexistence within a bacterial community. We showed how one species' ability to reduce toxicity does not only benefit itself, but also other species coexisting in the same environment. The findings of this thesis can have important implications on our understanding of microbial communities and on which factors to take into account when designing new communities to apply in diverse environmental conditions, such as designing soil communities to increase plant growth or human gut communities to recover from diseases.

Résumé pour le grand publique

Dans notre vie quotidienne, nous rencontrons des bactéries dans presque tous les environnements de la planète : elles sont présentes dans le sol, dans les environnements marins et dans de multiples zones du corps humain, telles que les poumons, la peau et les intestins. Dans tous ces contextes, les bactéries ne sont pas seulement entourées de cellules d'une même espèce, elles vivent dans des communautés multispécifiques. Les communautés microbiennes multi-espèces jouent un rôle important dans le bien-être humain ainsi que dans les activités économiques : un microbiome intestinal sain est crucial pour le bien-être humain et certaines communautés du sol sont utilisées en agriculture pour améliorer la croissance des cultures. Au sein de ces communautés, les bactéries interagissent entre elles et avec le milieu environnant. Nous pouvons globalement catégoriser les types d'interaction d'une espèce envers une autre comme positive si elle apporte un avantage au receveur, négative si elle nuit au receveur, et neutre sinon. Il est important de comprendre les forces qui régissent les interactions bactériennes et le rôle de l'environnement dans la détermination du signe de l'interaction (positif, négatif ou neutre).

Dans mon projet de doctorat, je me suis concentrée sur l'étude de la façon dont les différents environnements affectent les interactions entre les bactéries appartenant à la même communauté d'un petit nombre d'espèces. Plus précisément, j'ai testé comment l'abondance des nutriments et le degré de toxicité pour les bactéries peuvent affecter leurs interactions et si elles peuvent continuer à coexister dans le même environnement. Conformément à des études antérieures, j'ai montré que dans un environnement avec une petite quantité d'une seule source de nutriments, les bactéries se disputent la seule nourriture disponible. Dans un deuxième environnement, où la même source de nutriments était présente en plus grande quantité, on a également observé l'accumulation d'un composé toxique pour l'une des espèces bactériennes. Dans cet environnement mixte toxique et nutritif, les espèces sensibles aux toxines ont eu une meilleure croissance lorsqu'elles étaient cultivées en présence de celles résistantes aux toxines. Nous avons montré que cette facilitation se produisait parce que les espèces résistantes pouvaient réduire la concentration du composé toxique, créant un environnement plus permissif pour les espèces sensibles. Cependant, nous avons également montré que si nous supprimions artificiellement le composé nous-mêmes, le signe d'interaction revenait de positif à négatif, amenant les espèces à être à nouveau en compétition.

Nous avons obtenu ces résultats en combinant à la fois des expériences réalisées en laboratoire et en analysant les prédictions de modèles mathématiques. Nous avons également utilisé cette approche combinée expérimentale et théorique pour tester si ces diverses interactions permettraient à ces deux espèces de coexister à long terme ou si la compétition conduirait à la survie d'une seule ou de l'autre espèce. Nous avons constaté que les deux espèces peuvent coexister à court terme, mais qu'à terme, les espèces résistantes à la toxicité excluent les sensibles.

Ce travail de thèse a mis en lumière l'impact de la toxicité environnementale sur les interactions et la coexistence au sein d'une communauté bactérienne. Nous avons montré comment la capacité d'une espèce à réduire la toxicité ne bénéficie pas seulement à elle-même, mais également à d'autres espèces coexistant dans le même environnement. Les résultats de cette thèse peuvent avoir des implications importantes sur notre compréhension des communautés microbiennes et sur les facteurs à prendre en compte lors de la synthèse de nouvelles communautés à appliquer dans diverses conditions environnementales, telles que la biosynthèse de communautés du sol pour augmenter la croissance des plantes ou les communautés intestinales humaines pour guéri de maladies.

Contents

Chapter 1

Introduction

1.1 Prelude

> *No man is an island, entire of itself; every man is a piece of the continent, a part of the main; if a clod be washed away by the sea, Europe is the less, as well as if a promontory were, as well as if a manor of thy friend's or of thine own were; any man's death diminishes me, because I am involved in mankind, and therefore never send to know for whom the bell tolls; it tolls for thee.*
>
> "No Man is an Island", John Donne, *Devotions Upon Emergent Occasions*, 1623

"No Man is an Island" is an extract from John Donne's book *"Devotions Upon Emergent Occasions"*. Poet and priest, in his work John Donne emphasized the importance of community and interactions among human beings, as people thrive better together rather than when in isolation. "No man is an island" means that no one is an isolated, separated entity from their metaphorical continent, which is overall human society.

Similarly to human beings, bacteria are no islands either. Bacteria colonize almost every environment on the planet and they tend to coexist in multispecies communities rather than living in isolation. Many bacterial communities are actively and positively involved in several human-related processes. For example, the impact of gut microbiota is crucial in preserving human health [54] and plant growth-promoting bacteria are widely exploited in agriculture to improve nutrient availability and to accelerate their assimilation by plants [6]. Within these communities, bacteria are bound to interact. Multiple levels of interaction occur in a multi-species microbial community, potentially reaching an equilibrium among community members. For example, one bacterial species can be affected by the presence of a different species positively, negatively or neutrally. Similarly, the environment in which bacteria are living has a huge impact on their surviving and thriving capacities.

I have always found fascinating how similar the dynamics are that rule bacterial and human communities. If we compare these two community types at their core structure, we can see that they both strongly

depend on the balance of interactions occurring between community members and that the interactions themselves are affected by the surrounding environmental conditions. I experienced myself how different environments and conditions can shape the way I establish interactions with other people in one or another direction, and as a passionate microbiologist I tried to convey this parallelism in my PhD work.

Unfortunately, as opposed to people, we cannot simply ask bacteria what the motives are that lead them to interact and coexist within a community. Former studies showed that environmental conditions such as pH, temperature and presence of different types of metabolites can affect interactions and coexistence dynamics. However, still little is known about the underlying principles are in how the environment can influence the sign of interactions in a network. Additionally, natural bacterial communities can be made of even thousands of different species, making them a very complex system to investigate.

During my PhD, I focused on understanding how an environment with various concentrations of nutritious and toxic substances can affect the interaction networks among bacteria and how it impacts their chances of coexisting. To make the study of microbial community dynamics less challenging, I set up a system composed of a synthetic microbial community with defined species and environmental compositions. I used this simple setting to address some of the current open questions in microbial ecology, such as which are the major mechanisms ruling interactions, how do they affect coexistence and how can we use this knowledge to predict further ecological circumstances that go beyond the observed conditions.

1.2 Classifying bacterial interactions

As briefly mentioned above, bacteria can interact with each other when sharing an environment. Largely, we can differentiate bacteria in two groups depending on their genetic features: cells of identical genotype for the trait of interest and cells of different genotype for the same trait of interest.

Overall, the net effect of a a clonal group of cells of identical genotype on cells of different genotype can be of three different signs: positive (+), negative (-) or neutral (0). A way to assess the sign of an interaction occurring between two bacteria is to measure the population size of the species of interest when cultured alone and to compare it to its population size when in co-culture with a second species. If the population size of the focal genotype is higher in the co-culture compared to the monoculture, it is a positive interaction. If the population size of the focal species is impaired in the co-culture compared to monoculture, it is a negative interaction. Finally, if there's no variation of population size of our species of interest when in co-culture compared to its monoculture, it means that the interaction is neutral. All the possible combinations of the sign of interactions between two species result in six different interaction types, as summarised in Fig. 1.1. If one species in unaffected by the presence of another species and this second one experiences a positive (0/+) or negative (0/-) effect, we call it commensalism or amensalism, respectively. If the positive effect is bidirectional (+/+), it is cooperation. Both combinations (-/+) and (-/-) fall in the competition spectrum: if one species provides positive effect to its partner and this second species, in return, impacts the first one negatively, it is exploitative competition; if the two species both negatively affect each other, it is mutual inhibition.

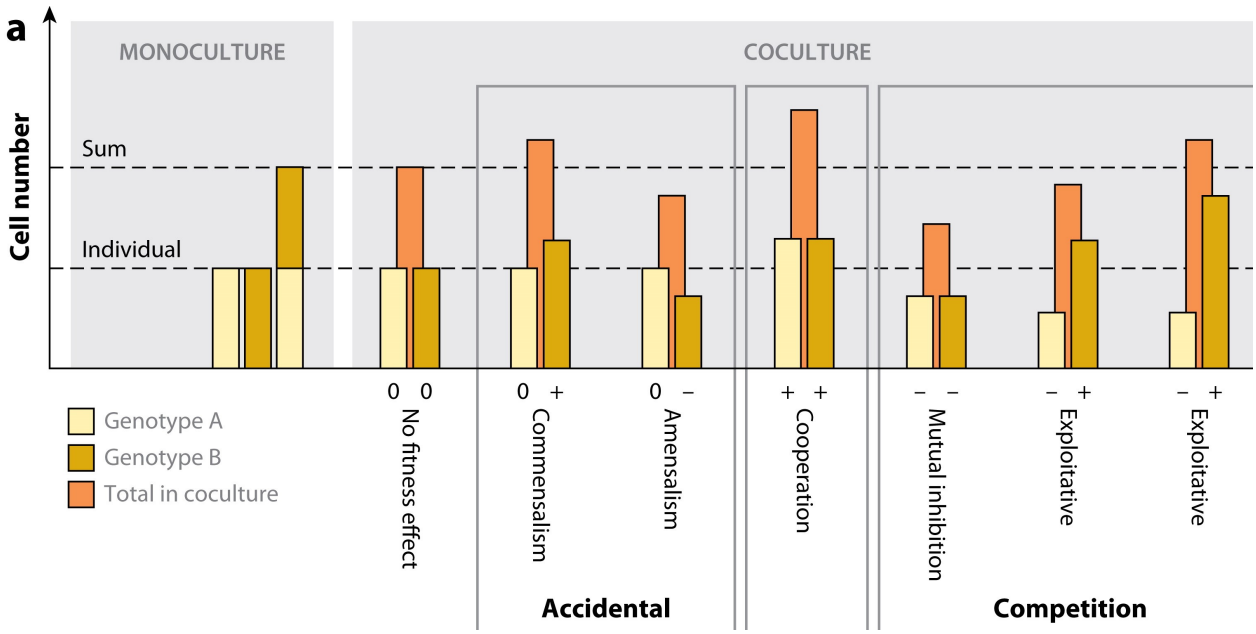


Figure 1.1 – To classify social interactions between two strains or species, two genotypes are growth both in their respective monocultures and in co-colture at the same conditions. The plot shows all possible outcomes of such an experiment and the types of interactions that might be inferred from them, as their are described above in the main text. Adapted from [79].

1.2.1 Evolved versus accidental interactions

So far we have described the different types of interactions based on a measured effect of one species towards a partner species. But if we want to predict how these interactions may change over time, we need to understand why two genotypes interact in a given way, or, in other words, their evolutionary history. We can thereby classify interactions into "accidental" ones that are mediated by molecules that are secreted regardless of the presence of other species, in contrast to "evolved" interactions that occur because of the presence of bacteria sharing the same environment [79].

Some interactions have evolved, at least partially, because of their effect on the surrounding cells in the environment [79].

Cooperative interactions often evolve when a cell is most likely to be surrounded by others that are genetically identical to it, such that these traits bring an advantage to the whole group. Examples include biofilm production, where the participant cells cooperate by the secretion of polysaccarides to form the extracellular matrix [69], or quorum sensing , whereby signaling molecules can trigger the entire bacterial population to synchronize their collective behavior [78]. Another classical example of evolved cooperation is the production of siderophores, which are iron-scavenging molecules released by bacteria to trap iron more efficiently in environments in which iron is a limiting source. Despite the high cost of siderophore production, siderophore-producing cells are favoured over the non-producing ones in iron-limiting conditions, but non-producers still persist because they can exploit the siderophores produced by the other cell type [15]. The evolutionary stability of cooperation in all of these examples is expected as long as genetic identity between neighboring cells remains high, and the environment favors cooperators.

Competitive interactions can also evolve to inhibit other cells, even if they are costly. Examples of such behaviors are the production of antimicrobial compounds and/or the use of weaponry, such as phage-like

particles or Type VI secretion systems [97, 110, 45].

Many interactions, however, occur not necessarily as a consequence of natural selection, but rather via molecules that are secreted regardless of the presence of other species. When measured experimentally, the two types of interactions will look identical (Fig. 1.1), but the long-term dynamics that result from these interactions is expected to be very different [37].

Accidental positive interactions are often due to crossfeeding, when the byproduct of one species becomes the nutrient substrate of another [50]. Cross-feeding can be unidirectional (one species benefits from another) or bidirectional (both species benefit from each other's products). An example of unidirectional crossfeeeding is found in [93], where a sub-population of *E. coli* cells started to feed on acetate which was released as a waste of glucose metabolism by the majority of the *E. coli* population. Bidirectional cross-feeding is often found in soil, where different species involved in distinct steps of the nytrogen cycle release byproducts that are mutually exchanged for nitrification [70, 58].

Detoxification is also a strong driver of accidental positive interactions, which involves the removal of toxic harmful substances through extracellular degradation [28]. A recent study showed that positive interactions arose in toxic copper stress conditions between copper-sensitive and copper-resistant species. This happened because the copper-resistant species were detoxifying the environment through the release of metal-detoxifying siderophores for their own good. By doing so, co-inhabiting species that produced fewer metal-detoxifying siderophores could benefit from the environment being less toxic [49]. This work highlights both the importance of public goods in interactions dynamics and how the presence of toxicity in the environment can shift the sign of interactions towards more positive ones. This pattern invokes the Stress Gradient Hypothesis (SGH), a concept that will be further discussed in the upcoming paragraphs.

Although it may be challenging to distinguish which interactions arise through natural selection and which are accidents, it is nevertheless important to keep the concepts in mind, particularly as we think about long-term dynamics between species, as we will below.

1.3 Coexistence of different species in a community

In the former section, we discussed the different interactions between bacteria, but how do these dynamics affect the coexistence of species within a community? We know that bacterial communities can include hundreds of different species, so there must be some rules that determine which species coexist and which exclude each other.

1.3.1 Coexistence theory

If we focus again on a metabolic-centered point of view, we can identify the chemical composition of the environment and the pool of available metabolites as crucial factors. A limited nutrients and co-factors availability will force different species to compete for these scarce resources, letting negative interactions prevail. An important hypothesis on how resource complexity can establish coexistence is given by the competitive exclusion principle, which states that two species that occupy the exact same ecological and geographical niche cannot coexist [46]. In these conditions, the species that has even the smallest fitness advantage (meant as faster growth rate) will dominate and the weaker competitor will go to local extinction.

A different way to phrase this principle is that complete competitors cannot coexist: eventually, one species will prevail over the other.

A corollary to the competitive exclusion principle is the concept of limiting similarity, which suggests that two species will coexist only if their niches don't overlap entirely [76]. Overall, a lot of the theoretical work on coexistence supports that the number of species coexisting is defined by the number of limiting factors that they share. This statement has been tested in the honey bee gut microbiota by Brochet et al.: their *in vitro* and *in vivo* experiments showed that coexistence between four *Lactobacillus* species was possible only when they could feed on the nutritionally complex pollen compared to simple sugars [13]. This result is consistent with number of available niches in the two conditions: in simple sugars, the species compete for the same limiting resources, but in complex pollen they consume distinct pollen components and this niche partitioning allows coexistence.

However, even when starting with restricting conditions, bacteria have the capacity to modify the chemical composition of their environment, which may make increase the ability of others to survive. Environmental modifications can, for example, change the pH to better tolerated values, but it can also include the secretion of waste products that potentially can become new substrates for a co-inhabiting species that originally was competing for the same initial resource [38, 91]. This means that, even if the starting conditions of a community might not suggest the possibility of coexistence, specific bacterial activities can increase the number of available niches and change the chances of coexistence success [14, 17].

1.3.2 Introduction on oxidative and chemical stress

So far, we have explored how limited resources can result in competition that negatively affects the chances of coexistence unless new niches are created. However, nutrients and beneficial co-factors are environmental players that can impact bacteria survival in a positive way, but there are multiple other factors that can provide stress to bacteria. Overall, we can describe as "stress" any condition that negatively impact the fitness [1] of an organism. The adverse conditions (or "stressors") can be physical/abiotic (temperature, water availability, pH, salinity, limiting resources and more) or biotic (shared resources with co-habitating competitors, presence of predators/pathogens).

Reactive oxygen species (ROS) as a source of stress

One example of abiotic stress that bacteria can experience is oxidative stress induced by the presence of high quantities of reactive oxygen species (ROS). ROS are molecules that contain one or more unpaired electrons. ROS can react with other molecules to try to increase their stability: this process happens by either donating their unpaired electron to another molecule or by taking an electron away from another molecule, generating a new ROS molecule in a chain-reaction mechanism [55]. The sequential oxygen reduction through the addition of electrons leads to the formation different types of ROS, as shown in Fig. 1.2

ROS damage involves nucleotide oxidation, protein carbonylation (i.e. the introduction of carbon monoxide in the molecule) and lipid peroxidation. In this last reaction, ROS oxidises an unsaturated lipid

1. Following the Darwinian definition of fitness, with this term we indicate the ability of an individual to survive and to successfully reproduce to pass its genes to the next generation.

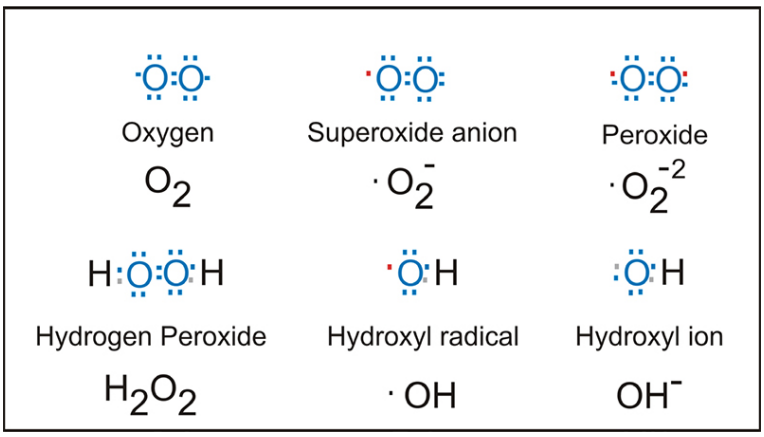


Figure 1.2 – Electron structures of common reactive oxygen species. Each structure is provided with its name and chemical formula. The red • designates an unpaired electron. Taken from [11].

chain, leading to the formation of a hydroperoxidised lipid and another radical that can continue the peroxidation chain reaction [55].

In a biological context, ROS form in various ways: as natural byproducts of aerobic metabolism or of enzymes such as NADPH oxidases (NOXs), UV irradiation and also exogenous sources, such as pollution [55]. In addition, ROS are formed as necessary intermediates of metal catalyzed oxidation reactions, with this last mechanism being particularly relevant for this PhD work [11].

Microbes find themselves involved with ROS in a variety of ways. Endogenous ROS production originates during the transfer of electrons from redox enzymes to oxygen, generating a mixture of O_2- and H_2O_2 [53]. H_2O_2 has been detected in both fresh and oceanic water, and the detected levels are high enough to plausibly trigger oxidative-stress response in water bacteria [66, 75]. The impact of ROS toxicity on bacteria has strong human relevance. Studies have shown that the presence of ROS contributes to both antibiotic-mediated and host-mediated pathogen killing [103]. The first is obtained often in form of a secondary damage effect, meaning that the antibiotic is responsible for the first damage, but this triggers ROS production and it causes additional damage to bacteria [51]. On a more direct host mechanism of action, mammalian phagocytes and plants respond to bacteria by engulfing them and then saturating them with superoxide [9]. Particularly, in plants, ROS production is not only a weapon against pathogen invasion, but it is also involved in signalling pathways to activate further defense mechanisms against bacteria [31, 102, 64].

But how can bacteria defend themselves from ROS toxicity? Several enzymes have been identified as capable of deactivating ROS with different target-preferences depending on the peculiar ROS species [99, 53].

It is true that Fe2+ ions contribute to ROS production [23, 62], but great ROS accumulation interfere with iron stability and bacteria wellness: ROS attack releases free iron which binds to DNA and induces DNA damage [52]. Additionally, it has been shown in *E.coli* that ROS can attack iron-cofactors of a dehydrogenase causing the deactivation of the enzyme and subsequent growth inhibition **??**. Given these fatal possibilities, it is not surprising that some species defend themselves from oxidative stress by upregulating their siderophore production. By doing so, they both absorb precious iron and they recruit the free Fe, preventing to continuation of oxidation chain reaction and the aforementioned damages [20]. A similar

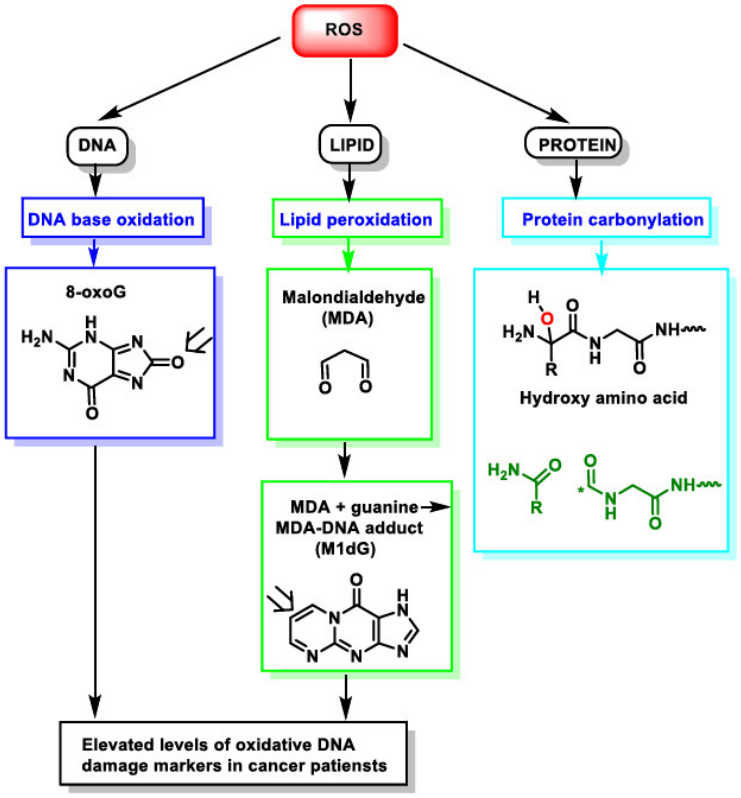


Figure 1.3 – ROS action on DNA, lipids and proteins lead to DNA base oxidation, lipid peroxidation and protein carbonylation, respectively. * Unpaired electron. Taken from [55]

situation has been observed in *E.coli*, in which an enterobactin was involved in both iron scavenging and reduction of oxidative stress [3].

The last two example show ways to cope with oxidative stress by the activation of a system that acts extracellularly. This means that not only the secreting species benefit from the activation of these countermeasures, but also other species that coexist in the same environment. This process could generate accidental facilitation and it should be taken into account when addressing interactions in a microbial community in the presence of oxidative stress.

Diverse toxic compounds as a source of stress

Toxic compounds are an important source of stress that can impact the survival of a species and its persistence within a community. Like nutrients, these can either be present in the environment independently of microbial presence, or they can be produced by the microbes themselves.

In the latter category, bacteriocins are microbial weapons produced by bacteria to kill closely related species: the bacteriocin-sensitive species are eliminated and the producer gains more access to the formerly shared resources [101, 92]. Similarly, bacteria also produce various antibiotics to kill or inhibit their competitors. Studies performed on Streptomyces strains have shown that different species tend to increase the production of their own antibiotic and to induce the suppression of competitor antibiotic production in a co-culture [2]. Antibiotics are of course also often added to an environment, for example during treatments.

Independently of their origin, the presence of toxic concentrations of antibiotics doesn't automatically

mean that a sensitive species is bound to go extinct. Both theoretical and experimental works performed on multispecies biofilms showed that a sensitive species can survive when co-cultured with a resistant one [82, 27]. The detoxification performed by the resistant species modifies the environment and creates favorable conditions for the survival of the sensitive species too. The level of intermixing between the two species and the consequent spatial structure are important to determine the success of facilitation through detoxification in promoting coexistence [27]. This example shows how a toxic environment can lead to facilitation in a co-culture.

1.3.3 The Stress Gradient Hypothesis

The role of environmental toxicity in the establishment of facilitation and positive interactions is described by the Stress Gradient Hypothesis (SGH). The SGH proposes that facilitation should be more common in stressful environments, compared with favourable environments where competition should prevail [10]. SGH has been tested and discussed in depth in plant communities, for example in plants growing in presence of different gradient of water availability or alpine altitudes as abiotic stress [10, 73, 40]. However, it hasn't been tested much outside plant ecology. An example of evidence of SGH in microbial communities has been highlighted by a study performed by Li et al. on biological soil crusts (BSC) [68]. BSC are soil aggregates containing an abundance of microbial species and phototrophic microorganisms; soil samples were collected in different location characterized by diverse stress intensity (as lower or higher nutrient abundance) and they found that there was an increase of negative interactions and competition among members of communities that were sampled in the less stressful sites. In a different study, the biomass increase was one of the parameter evaluated to rate the fitness of several communities and it was observed that the number of communities that could give biomass growth decreased when abiotic stress was present [29]. Some pertinent results on the occurrence of SGH in microbial world come from a recent study focusing the interactions between plants and microbes in which it was found that soil microbes facilitate germination with increasing enviromental stress [22]

Despite these remarkable examples specifically addressing SGH, I would like to underline that a recent work performed by screening an extensive list of search terms related to stress in the published ecological literature showed that, in microbial ecology, the SGH is rarely mentioned or invoked to explain findings, even when data would support it [85]. This could mean that SGH might apply to many more contexts than the ones validated so far.

One critique often directed to the SGH is that it lacks of consistent understanding of the specific forms of stress that drive a shift of from negative to positive interactions. In a plant-microbe system, it was found that the way through which bacteria facilitate plants in stressful environments is by promoting germination [22].

A study performed in our research lab by Piccardi et al. addressed this issue by investigating the SGH in a four species synthetic community growing in different gradients of nutrients and toxicity. The use of a small synthetic community with defined species composition allowed them to easily decipher the interaction network in all pairwise combinations. They compared the cumulative growth of every monoculture versus the cumulative growth achieved by each species when co-cultured in pairwise combinations with every community partner: higher growth in the co-culture meant there was facilitation, while lower growth

Figure 1.4 – The classic SGH as formulated by [10]. Negative interactions (competition) are expected to be found in benign environments, while positive interactions (facilitation) should be found in stressful environments. Adapted from [43]

meant there was competition. They found that competition was prevalent in a more permissive and nutrient rich environment, while facilitation arose following the increase of environmental toxicity [87]. These results support the SGH, but the complex and unknown chemical composition of the chosen environment made it difficult to understand which are the molecular mechanism underlying facilitation in the more toxic condition. In sum, no clear molecular mechanism has been found to explain the SGH in this MWF system.

1.4 Approaches to testing the SGH

1.4.1 Synthetic communities

It is difficult to find evidence in support of the SGH in natural microbial communities because they are very complex systems. This is even more challenging in plant communities. The complexity is due to the number of species in the community and the undefined environmental composition. A promising way to overcome these obstacles is to build synthetic communities [41]. A synthetic community is an artificial community created by culturing selected species in a defined medium. These communities are designed to reproduce the main features of natural ones in a simpler and more controlled environment. This approach has already proven successful in studying different patterns of interactions. For example, the use of a two species phototrophic community helped to elucidate how the fluctuations in organic and inorganic nutrient availability can affect community stability by shifting interactions from cooperation to competition [115]. In a study by Balagadde et al., two genetically engineered *E. coli* populations constitute a solid synthetic community to investigate how predator-prey dynamics may vary depending on operating conditions [8].

It is possible to modulate the level of complexity of synthetic communities by varying the number of species and culture conditions. The screening of multiple candidate communities to obtain the ones with the optimal desired featured doesn't necessarily have to be experimentally tedious and time-consuming. In recent work, Pacheco et al. developed a method based on machine learning and metabolic modelling to identify which environments can select for a synthetic community with a specific taxonomic composition and set of metabolic exchange reactions [85]. The application of this method could span from facilitating the experimental design of complex microbiomes to providing insights on how to create environments with diverse stress gradients to test the SGH.

1.4.2 SGH environments: Metal-Working Fluids

To recap, the SGH proposes that a shift of interactions from competition towards facilitation will occur following an increase in stress. To investigate the SGH then, one does not only need to carefully select the community, but the chosen environmental settings must provide some sort of gradient of stress. In plant ecology, gradients of altitude and temperature are important abiotic sources of stress [19]. Wind exposure and desert-correlated stressors determine morphological development of soil crust phototrophyc communities [68], but these parameters are hard to test in laboratory conditions. Fetzer et al. built a system in which a stress gradient was created in lab conditions in form of increasing concentration of benzoate: in this work, benzoate was both the single carbon source available and a toxic, stressful element [29].

This dual nature of nutrient-toxic input is also a characteristic of Metalworking Fluids (MWFs). MWFs are mineral oil-based fluids commonly used as lubricants and coolants of industrial machinery [35]. MWF composition is a mix of compounds that could be potential nutrient sources for bacteria, like mineral oil and fatty acids, but also biocides that are intended to prevent bacterial contamination [18, 89]. MWF waste is a potential environmental pollutant and one of the approaches to properly handle it is the treatment with bacteria that can degrade it. For this purpose, a small bacterial community was assembled and species were chosen depending on both their abundance in diverse MWF samples and their growth yield [34].

This four species community was used by Piccardi et al. as a model system to explore how abiotic and biotic interactions impact community productivity. Additionally, the presence of both nutrients and toxic compounds in MWF made this system a great setting to also test the SGH in microbial communities [87]. Interaction network analysis showed that only positive or neutral interactions were detected in toxic MWF (Fig. 1.5, panel A), while at lower toxicity/higher nutrient availability, negative interactions emerged, in compliance with the SGH (Fig. 1.5, panel B and C).

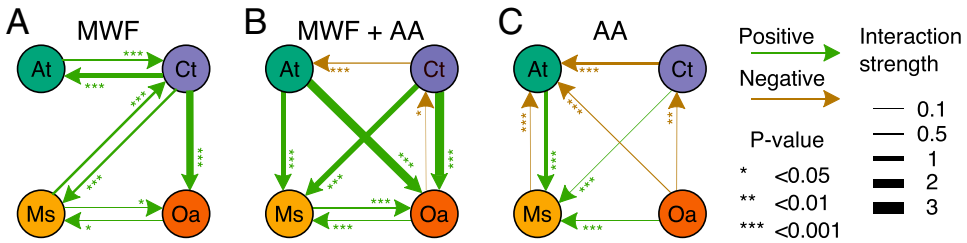


Figure 1.5 – Pairwise interaction networks under different environmental conditions. Positive/negative interactions indicate that the species at the end of an arrow grew significantly better/worse in the presence of the species at the beginning of the arrow in (A) MWF, (B) MWF + AA, and (C) AA medium. Arrow thickness represents interaction strength as the 10-fold change in the coculture AUCs compared with monoculture AUCs, i.e., by how many orders of magnitude a species changed the AUC of another. Statistical significance and interaction strengths were calculated based on data showed in [87]. Figure adapted from [87].

1.5 Goal of the thesis

In this PhD work, I explore more specifically the possible mechanisms through which the SGH operates in the context of a small synthetic bacterial communities and I study how they affect the interaction network

and the probability of coexistence. The species that I worked with are the four species described by Piccardi et al.: *Agrobacterium tumefaciens*, *Comamonas testosteroni*, *Ochrobactrum anthropi* and *Microbacterium saperdae* (from now on referred as At, Ct, Ms and Oa, [87]).

I coupled experimental data with theoretical models developed by a former lab member, Dr. Aurore Picot. The constant interplay between experiment and models allowed us to support the interpretation of our data and to predict outcomes that go further than our tested conditions.

In Chapter 3, my goal was to reduce the complexity of the MWF environment whose chemical composition remains unknown and to design my own setting that could provide a carefully manipulable stress gradient. Inspired by previous work in the lab by Piccardi et al. [87], I screened multiple compounds derived from MWF and I chose linoleic acid (LA) as the key nutrient source. I found that an increasing concentration gradient of LA resulted not only in more nutrient availability, but also in increasing toxicity, making the high LA concentration environment not permissive to the growth of some species in monoculture. At this stage, we believed that LA was the single molecule responsible for carrying both nutrient and toxic potentials.

In Chapter 4, I investigated how the different gradients of nutrient availability and toxicity affected the interaction network in pairwise combinations. I found that the toxicity in the system depended on the release of Reactive Oxygen Species (ROS) as a byproduct of LA oxidation, independently of any bacterial presence. As LA concentration increased, ROS abundance increased as well, meaning that nutritious and toxic effects could be caused by two different molecules. At low LA concentration, ROS do not pose a problem for our tested species in monoculture, and we observed that they compete in co-cultures. At high LA concentration, ROS toxicity accumulated creating an environment that did not allow for the survival of some species in monoculture. Experiments showed that the presence of a ROS-resistant species reduced environmental toxicity. The co-culture of ROS-resistant species with ROS-sensitive ones allowed the rescue of the sensitive species, thus shifting the prevalent interaction network toward facilitation. These results are consistent with the SGH and provide a mechanism by which it can occur. We further validated the ROS-dependent molecular mechanism behind toxicity by supplementing an antioxidant compound that could neutralize ROS in our system and return interspecies interactions to competition.

In Chapter 5, I explore how these toxicity-and-nutrient-dependent interaction dynamics affect the possibility of coexistence of ROS-sensitive and ROS-resistant species. I performed transfer experiments at both low and high LA concentration to test whether LA concentration could affect coexistence. We found that coexistence was obtained at both conditions over the tested period of time. However, theoretical work showed that coexistence is not expected to be maintained over longer time-scales in either LA concentration. Additionally, the mechanistic understanding that ROS accumulation was causing toxicity allowed to model nutrients as being distinct from the toxic compounds. This improved model predictions and gave us more insight on how long the ROS-sensitive and the ROS-resistant species are expected to coexist before the ROS-resistant species prevails.

Chapter 2

Materials and Methods

2.1 Cell culture preparation

The species tested in this study are *Agrobacterium tumefaciens* (At), *Comamonas testosteroni* (Ct), *Microbacterium saperdae* (Ms) and *Ochrobactrum anthropi* (Oa). Strains specifics are described in [87].

2.1.1 High cell density

We prepared separate overnight cultures in Tryptic Soy Broth (TSB) starting from a single colony for each species. Cultures were incubated at 28°C, 200 rpm. The day after, OD_{600} was measured. For each species, we measured the OD_{600} after the overnight growth (Ultrospec 10 cell density meter, Amersham Biosciences) and we calculated the volume of overnight culture to add to 20 ml of fresh TSB to have a starting OD_{600} of 0.05. These new cultures were incubated for three hours at the same conditions to reach exponential phase. After three hours, OD_{600} was measured and we calculated the volume of culture to harvest to have a starting OD of 0.1 in 10 ml of minimal medium (MM, Fig. 2.1). We collected the appropriate volume in individual 15 ml tubes for each species. We centrifuged cultures for 20 min at 4'000 rpm at 22°C. We discarded the supernatants and resuspended the pellets in 10 ml of PBS to perform washes to remove any leftover TSB. We centrifuged the samples again as described before, and performed this wash twice. After the two washes, pellets were resuspended in 13 ml of the final medium. We split each 13 ml sample in three glass tubes containing 4 ml of culture each to have triplicates. Glass tubes were incubated at 28°C, 200 rpm.

2.1.2 Low cell density

The initial steps of the protocol are the same as described above. After three hours, OD_{600} was measured and we calculated the volume of culture to harvest to have a starting OD of 0.1 in 10 ml of minimal medium (MM, Fig. 2.1). We collected the appropriate volume in individual 15 ml tubes for each species. We centrifuged cultures for 20 min at 4'000 rpm at 22°C. We discarded the supernatants and resuspended the pellets in 10 ml of PBS to perform washes to remove any leftover TSB. We centrifuged the samples again as described before, and performed this wash twice. After the two washes, pellets were resuspended in 10 ml of MM. Separately, we prepared the growth media for every selected compound as indicated in Fig. 2.2.

We added 4 ml of each compound- supplemented medium in glass growth tubes, having three replicates per condition. After a short spin up, we transferred 40 μl of each bacterial culture in the appropriate growth tube to dilute bacteria at 10^5-10^6 starting CFU/ml. Growth tubes were then incubated at 28°C, 200 rpm for 8 days.

2.2 Minimal Medium (MM) and compound-supplemented MM preparation

Compounds listed in the Fig. 2.1 B, C and D were mixed to prepare the Minimal Medium (MM) (Fig. 2.1 A). The media supplemented with the different compounds (compound-supplemented MM) were prepared adding the proper amount of compound starting from a 50-fold more concentrated stock. We prepared a 50-fold concentrated stock for every tested concentration of the compounds to standardize media preparation. When that was not possible, we took the adequate amount directly from the original reservoir. Fig. 2.2 shows an example of compound-supplemented MM prepared at 4 different concentrations ([C] 1- [C] 4).

A

Minimal Medium (MM)	
Compound	**Quantity**
HMB 50X	10 ml
M9 10X	50 ml
ddH_2O	440 ml
Final volume	500 ml

B

M9 10X	
Compound	**Quantity**
Na_2HPO_4	60 g
KH_2PO_4	30 g
NaCl	5 g
NH_4Cl	10 g
H_2Oup (final)	in 1 l

C

HMB 50X composition	
Compound	**Quantity**
NTA (Nitrilotriacetic acid)	10g
$MgSO_4 * 7H_2O$	14.45 g
$CaCl_2 * 2H_2O$	3.33 g
$(NH_4)_6Mo_7O_{24} * 4H_2O$	0.00974 g
$FeSO_4 * 7H_2O$	0.099 g
Metals 44	50 mL
H_2Oup (final)	1 l

D

Metals 44 preparation	
Compound	**Quantity**
$Na_2EDTA * 2H_2O$	0.387 g
$ZnSO_4 * 7H_2O$	1.095 g
$FeSO_4 * 7H_2O$	0.914 g
$MnSO_4 * H_2O$	0.154 g
$CuSO_4 * 5H_2O$	0.0392 g
$Co(NO_3)_2 * 6H_2O$	0.0248 g
$Na_2B_4O_7 * 10H_2O$	0.0177 g
H_2Oup	in 100 mL

Figure 2.1 – Minimal Medium (MM) preparation. A) Final quantity of each starting solution to mix to have MM. B) M9 10X recipe. C) HMB 50X recipe. D) Metal 44 recipe.

	MM	MM + Compound [C] 1	MM + Compound [C] 2	MM + Compound [C] 3	MM + Compound [C] 4
M9 10X	8 ml	8 ml	8 ml	8 ml	8 ml
HMB 50X	1.6 ml	1.6 ml	1.6 ml	1.6 ml	1.6 ml
Compound 50X [C] 1	Ø	1.6 ml	Ø	Ø	Ø
Compound 50X [C] 2	Ø	Ø	1.6 ml	Ø	Ø
Compound 50X [C] 3	Ø	Ø	Ø	1.6 ml	Ø
Compound 50X [C] 4	Ø	Ø	Ø	Ø	1.6 ml
ddH2O	70.4 ml	68.8 ml	68.8 ml	68.8 ml	68.8 ml
Final volume	80 ml	80 ml	80 ml	80 ml	80 ml

Figure 2.2 – General scheme of compound (C) supplemented media. Five different compound concentrations ([C]) were prepared for each compound, including the no-compound supplemented MM. When possible, we prepared 50X concentrated stocks of the tested compounds to have a more standard procedure.

2.3 Quantification of population size

To quantify bacteria population size over time, we took 20μl aliquots from each growth tube, we performed serial dilutions in 96well plates filled with 180 μl of PBS. Using a multichannel pipette, we took 10μl of each dilution series and we spread them to form a line on tryptic soy agar (TSA) plates or on lysogeny broth (LB) agar. This procedure allows to spread and distribute on the agar the cells that are in each 10μl drop: each cell will divide into clonal cells that will form a colony. The number of colonies detected in a dilution line multiplied per the corresponding dilution factor and per 100 gives the number of colony forming units (CFU) that are present in 1 ml of culture: this data gives information regarding the number of viable cells that are present in the tested conditions. Plates were incubated at 28°C. Ct formed countable colonies after 24 hours of incubation. At, Ms and Oa formed countable colonies after 48 hours of incubation. To distinguish At and Ct when growing in the co-culture, bacteria were also plated on LB agar supplemented with 14.25μg/ml of sulfamethoxazole and 0.75μg/ml of trimethoprim to count only At colonies. Moreover, the GFP marker of At further helped to truly differentiate At and Ct colonies.

2.4 Quantification of compound effect on bacteria

CFU count was used to plot growth curves of CFU/ml over time and we calculated the area under the curve (AUC) to have a better comparison between the different tested conditions and their MM control. We calculated the ratio between the AUC of each replicate per condition and the mean of the AUC of the three

MM control replicates, and used the log2-fold change of these data to build heatmaps showing the effect of each compound on each of the four species (Chapter 3, Figs. 3.6, 3.3, 3.5). T-tests were performed to compare the tested conditions to the MM control.

2.5 ROS detecting assay

We used the Thiobarbituric Acid Reactive Substances (TBARS) assay to indirectly assess the presence of ROS-induced oxidative stress as described in [4]. Malondialdehyde (MDA) is the primarly product of lipid peroxidation, the oxidative degradation induced by ROS. If there is ROS-induced degradation of LA in our media, this process would lead to MDA production. The TBARS assay measures the formation of the new adduct MDA-TBA2 upon reaction between the MDA in the medium and supplemented thiobarbituric acid (TBA). MDA-TBA2 presence is measured by its absorbance at 532 nm and the detected values are transformed in MDA-TBA2 concentrations through interpolation with a calibration curve built using eight MDA-TBA2 strandards at known concentrations. MDA-TBA2 concentration is a valid proxy to assess low or high ROS-induced oxidative stress in the tested environment. For the step-by-step procedure, we followed the detailed protocol described in [4].

2.6 TBHQ solution preparation

Since TBHQ is insoluble in water, we decided to dissolve it in DMSO. We first tested the effect of different concentrations of TBHQ+DMSO and pure DMSO to find the appropriate no-toxic TBHQ concentration to use and to be sure that DMSO was not toxic either. To minimize the risk of having DMSO toxicity, final DMSO concentration in our sample was 0.1%, a concentration below formerly detected toxicity threshold [25]. We tested 8 different TBHQ concentrations spanning from 1.20 mM down to 0.3μM (Fig. 2.3). We prepared 800X concentrated stock of every tested TBHQ concentration to add only a small volume in the final sample tubes to not affect too much the concentration of our growth media. We prepared our At and Ct monocultures and At-Ct co-culture in MM as described in former paragraphs and we supplemented the different TBHQ+DMSO concentrations to both mono- and co-culture in triplicate: we added 5μl of appropriate concentrated TBHQ+DMSO to each designed sample tube and we also added pure DMSO to the same culture conditions as a control. We followed bacterial abundance through CFU plating over 3 days and we found that the optimal final TBHQ concentration that did not affect bacterial abundance neither towards growth or towards death for all culture conditions was 15uM. Additionally, we found that DMSO alone did not affect bacterial abundance in any way.

For the assay in which we added TBHQ+DMSO every day, we prepared fresh TBHQ+DMSO every day to prevent degradation of the antioxidant and putative loss of its function.

[TBHQ] 800X stock	[TBHQ] final
1.2 M	1.50 mM
0.90 M	1.13 mM
0.60 M	0.75 mM
0.30 M	0.38 mM
120 mM	150 µm
12 mM	15 µm
1.20 mM	1.50 µm
0.30 mM	0.38 µm

Figure 2.3 – Tested TBHQ concentrations and relative 800X stock solutions.

2.7 Sample preparation for metabolite extraction

2.7.1 Collecting supernatant aliquots from sample tubes

The steps for sample preparation for metabolite extractions were performed over different days. The first step was collecting the spent media supernatants at the end of the At and Ct monocultures and At-Ct co-culture growth assay in LA 0.1% and LA 0.75% that we described in Chapter 4. We collected 3 ml from each replicate of each liquid culture condition (At monoculture, Ct monoculture, At-Ct co-culture) and media condition (LA 0.1%, LA 0.75% and the MM control) and put it 1 ml aliquots in three separate 2 ml Eppendorf tubes. We centrifuged the tubes at 4°C, 7000 rpm for 7 minutes. Then we quickly transferred the supernatants withouth disrupting the cell pellet in 300μl aliquots into three 500μl Eppendorf tubes and we snap-froze them in liquid nitrogen. We did so to have nine separate aliquots (technical replicates) for every culture tube (biological replicate) to give us enough room to perform several metabolite extractions over time. It is very important to perform these steps as quickly as possible to avoid variation in the metabolic profile. For this reason, supernatants from the sample tubes were collected and processed in batches of 14. We observed that there were non-cell deposits on the Eppendorf tubes walls after centrifugation and supernatant removal. We figured that this happened because our LA media are an emulsion, so we performed an extra step to be sure that we were not losing precious metabolite information in those debrits. We carefully added 50 μl of PBS to our Eppendorf tubes to gently resuspend and remove the bacterial pellet, we removed the PBS and then we added extra 50μl of PBS to do a gentle wash post cell pellet removal. Then, we snap-froze the empty tubes with wall debrits as they were. For us it was very important to avoid as much as possible cell contamination because we really wanted to have the metabolic profiles of the extracellular supernatants and we did not want to risk to have confounding results because of the presence of cells. Eppendorf tubes were transferred in the -80°C and they can be stored in this condition for several months.

2.7.2 MeOH 80% metabolite extraction and ISTD addiction

On a different day, we started performing the first step of the real metabolite extraction. For this first part we can process quite a high number of samples per day (up to 72). We randomly selected the samples choosing from the several 500μl Eppendorf tubes that we had: random tubes selection was important to be sure that there was no accidental bias in the analysis due to the fact that maybe all replicates of one specific condition were processed within the same batch. The following protocol was applied to both frozen liquid supernatants and to frozen debrit tubes. We let the chosen samples thaw in ice and we prepared for 80% MeOH metabolite extraction. We chose this method with the help of the GC-MS expert of our department, Dr. Andrew Quinn, to specifically target putative LA byproducts. Aside, we prepared the Internal Standard solution (ISTD). The ISTD is a compound that is certain to not be present in the medium but that it has chemical properties similar to some target compound. We chose heptadecanoic acid as ISTD because it is known to be a molecule not found in microbial and in general biological samples and because it has a chemical structure similar to LA, so it should be processed the same way as one of our target compounds. The use of an ISTD is necessary to have an internal control of the accuracy of the extraction, since we already know ISTD concentration. Additionally, it is used as a way to compare detected compounds abundances within the same sample and across different samples.

After sample defrosted, we split them into two 150 μl aliquots in new clean 2 ml Eppendorf tubes. We added 600 μl of cold 99%MeOH to each tube and 10 μl of 2.4 mM of ISTD (final MeOH concentration was 80%). We chose ISTD concentration to have approximately the same amount of ISTD as the amount of undegraded LA in the bacteria-free C- 0.75%. Then, we briefly vortexed the tubes and we stored them at -20°C for 90 minutes. We centrifuged the tubes at 4°C, 13000 rpm for 5 minutes, we transferred the supernatants in new 2 ml Eppendorf tubes and dried them overnight in the vacuum concentrator.

2.7.3 TBDMS/TMS metabolite derivatization

We create a stock 20 mg/mL methoxyamine HCl in pyridine amd vortexed it until fully dissolved. We dissolved sample in 50 µL solvent, vortex, briefly centrifuged, and transfered to GC vial. We heated the samples at 33°C for 1.5 hr and then either we added 50 µL MTBSFTA and heated for 1.5 hr at 65°C or we added 50 µL MSTFA and heated for 2 hr at 35°C. Vials should be briefly vortexed after adding derivatizing reagent. Finally, we put the samples in the GC-MS autosamples and we set MS injection conditions.

2.8 GC-MS run protocol

Samples were analysed on an Agilent 8890/5977B series GC-MSD equipped with an autosampler that injected 1 μL of sample onto a VF-5MS (30m x 0.25 mm x 0.25 um) column. The samples were injected with a split ratio of 15:1, helium flow rate of 1 ml/min and inlet temperature of 280°C. The Oven was held for 2 min at 125°C, raised at 3°C/min to 150°C, 5°C/min to 225°C, and 15°C/min to 300°C and held for 1.3 min. The MSD was run in scan mode from 50-500 Da at a frequency of 3.2 scan/s. Chromatograms were deconvoluted and metabolites were identified using the Agilent Masshunter software with the NIST MS library. Masshunter qualitative and quantitative software programs were used in order to export the relevant data, which could then be normalized and statistically assessed using custom R scripts.

2.9 Modelling approach

2.9.1 Equations and fitting approach

We used a mathematical modelling approach in order to fit the data from the experiments and give predictions regarding whether species are expecting to engage in competition, facilitation, and to coexist over long term serial transfers.

In Model 1, bacterial species abundance over time B depended on the concentration of linoleic acid C according to its consumption through a Monod uptake, with maximum growth rate r, half-saturation constant K, and yield Y. Linoleic acid also induced mortality depending on its concentration. We assumed that toxicity of the environment increased linearly over time and was proportional to the linoleic acid concentration, leading to the linear expression $(\beta + \gamma t)C$. The linoleic acid concentration C over time varied only due to the consumption of bacteria. The equations for the variation of bacterial abundance and linoleic acid concentration in a monoculture were:

$$\frac{\mathrm{d}B}{\mathrm{d}t} = \frac{rCB}{C+K} - (\beta+\gamma t)CB \tag{2.1}$$

$$\frac{\mathrm{d}C}{\mathrm{d}t} = -\frac{1}{Y}\frac{rCB}{C+K} \tag{2.2}$$

The equations for two species B_1 and B_2 in coculture in linoleic acid are given in the Supplementary Material A.

We used this model to fit the growth of At and Ct in monoculture in a range of concentration of linoleic acid (0.05%, 0.1%, 0.5% and 0.075%) and compared the prediction made from this estimation for the coculture of the species and their long-term coexistence over transfers, to the experimental data. In that first preliminary model, the estimated parameters were r_{C1}, r_{C2}, Y_{C1}, Y_{C2}, K_{C1}, K_{C1} and the toxicity parameters for At β_2 and γ_2, as we set β_1 and γ_1 to zero for Ct.

We then used Model 2 accounting for the production of ROS by linoleic acid oxidation. The equations for the monoculture growth became:

$$\frac{\mathrm{d}B}{\mathrm{d}t} = \frac{rCB}{C+K} - \beta RC \tag{2.3}$$

$$\frac{\mathrm{d}C}{\mathrm{d}t} = -\frac{1}{Y}\frac{rCB}{C+K} - \frac{1}{m}(d+eR)C \tag{2.4}$$

$$\frac{\mathrm{d}R}{\mathrm{d}t} = (d+eR)C - lR - \alpha BR \tag{2.5}$$

Because we had data on the spontaneous oxidation of LA in cell-free media, we could first estimate the parameters d, e, m, and l using ROS proxy in different linoleic acid concentrations. We then used these fixed parameters in the monoculture estimation to estimate the parameters of growth, toxicity, and detoxification for single species.

All parameter estimations were obtained using the modFit function from FME package (version 1.3.6.1) in R version 4.1.0.

2.9.2 Comparing the predictions from the monoculture estimation to the cocultures and transfers

For both models, we used the parameters obtained from the estimation of the monoculture data to compare the predicted coculture dynamics, to the actual coculture data, using (SI0). We also performed simulation of the serial transfers with varying dilution rates and linoleic acid initial concentrations in order to predict the likelihood of coexistence of the two species over time. The transfer parameter sweeps are coded in C++.

In Model 2, we mimicked the addition of an antioxidant to the media by putting initial ROS concentration to zero, as well as parameters d, e, and l and compared the predicted dynamics to the actual data using TBHQ.

2.10 Author contributions

Rita Di Martino and Sara Mitri designed research. Rita Di Martino performed the majority of the experiments (experiments described in section 3.2.6 were performed by Katia Annen and Diego Rojas-Gatjens). LC-MS protocol was designed by Laure Menin (EPFL) and LC-MS experiment was performed by Laure Menin (EPFL). LC-MS data were processed by Rita Di Martino. Andrew Quinn designed the protocol for metabolite extraction and GC-MS. Rita Di Martino performed metabolite extraction, GC-MS runs and all other experiments described in this chapter; GC-MS data were processed by Rita Di Martino and Andrew Quinn. Aurore Picot built and analyzed the models. Rita Di Martino and Sara Mitri helped Aurore Picot in implementing the models according to lab experiment results through scientific discussion. Rita Di Martino and Sara Mitri analyzed the experimental data described in this thesis.

Chapter 3

Characterizing nutrients and toxic compounds in MWF

3.1 Introduction

Our overall goal is to determine how medium composition, i.e. the abundance of nutrients or toxic compounds, affects the pattern of interactions in a bacterial community. MWF is a great setting to address this question because its composition represents a mix of both nutrients and toxic compounds. As mentioned in Chapter 1, we know that the sign of interactions among pairwise combinations of the four species described by Piccardi et al shifts from more positive to more negative as toxicity decreases (Fig. 1.5, [87]).

Our hypothesis is that these positive interactions are the result of the toxicity of the environment, in compliance with the SGH. If the environment is less toxic, then we observe competition between the species. However, using MWF, which has a highly complex chemical make-up, it is difficult to decipher exactly how chemical composition affects these interactions. Moreover, we could not get the chemical composition of MWF from the manufacturer, so we do not know on which compounds bacteria are feeding, which ones are responsible for the death of some species or which species can degrade which toxic compound.

To solve the complexity issues, our first goal was to identify compounds that are representative of general MWF composition and to test their effect on the four bacterial species in monoculture. With this approach, we aimed to discriminate which compounds are nutrients and which ones are toxic and to detect if these patterns were consistent across all four species or if there were species-dependent effects.

We then focused on a specific compound and we explored how its concentration shaped the network of interactions. Our hypothesis was that the positive interactions in MWF are the result of facilitation, where one species detoxifies it to the benefit or another. For this reason, we expected positive interactions to be predominant in synthetic media with MWF-derived compounds that are toxic for some species but nutrients for some others, as seen in the real MWF. On the contrary, we expected that if the compound acted as a nutrient for all the species, it would remove these positive interactions and lead to competition.

3.2 Results

3.2.1 Selection of the compounds for the synthetic MWF media

To understand the role of nutrients and toxins in interactions in our system, we needed to compile a list of compounds that is representative of the MWF. Information provided by previous work by the developers of the consortium included a list of six compounds of the MWF: monoethanolamine, triethanolamine, citric acid, formaldehyde, benzotriazole and morpholine [35, 34]. In collaboration with Jasquelin Peña (UNIL, Lausanne) and Laure Menin (EPFL, Lausanne), we performed respectively FTIR and both untargeted LC-MS and GC-MS analysis on the MWF to broaden the list of MWF compounds. We encountered some difficulties in establishing a protocol for LC-MS and GC-MS analysis that would result in reproducible data, because the MWF medium is an emulsion of oil in water and it caused fluctuations in the results. Once a protocol had been established, we obtained data as shown in Fig. 3.1A: a typical LC-MS run results in a spectrum in which each peak represents the signal created when a compound elutes from the LC column into the MS detector. The x-axis shows the retention time (RT), and the y-axis shows the intensity of the signal (abundance). Every eluted compound is then ionized and broken apart into charged fragments, each of them characterized by a mass to charge ratio (m/z, Fig. 3.1B). It is possible to identify the molecular formula of the original eluted compound by inferring the m/z of each of its charged fragments and the retention time from the LC column against a database. Interpreting the LC-MS and GC-MS data was particularly challenging because we had no specific database for data mining of common MWF components. This means that we had no way to filter which was the real compound detected in our MWF sample among the very long list of compounds sharing the same molecular formula. For example, in Fig. 3.1B the peak of m/z 120.056 corresponds to the molecular formula C6H5N3, but there are 234 compounds that share this formula (PubChem).

For this reason, we decided to change our approach and we started to look for the presence of peaks of specific compounds chosen by us instead of trying to identify the unknown peaks in the MS spectra. We built our own in-house MWF database (see Supplementary **??**). This database was compiled with compounds that are frequently found in MWF [35, 34, 18, 33] and it included information for each compound, such as name, molecular formula, absolute molecular weight and relative molecular weight, depending on the ionization performed. We ran this database against the raw MS data, and found some matching compounds. We selected some of these compounds based on their availability, cost and physical properties. Following this approach, we converged to a list of ten compounds that are representative of MWF (Fig. 3.2).

3.2.2 Effect of the selected compounds on bacteria

Our next objective was to characterize each compound as a nutrient or as a toxic compound according to the effect they had on each bacterial species. Ideally, we wanted to identify at least one nutrient and one toxic compound per species. We tested different concentrations of these compounds in monoculture assays to assess the effects of each compounds on the single species. Concentrations were chosen differently depending on the compound (see Chapter 2 for specific protocols/quantities). Previous studies show that both Gram-positive and Gram-negative bacteria are susceptible to specific concentrations of benzotriazole and formaldehyde [1, 113], so we chose concentrations above and below these thresholds. Petroleum sulfonate

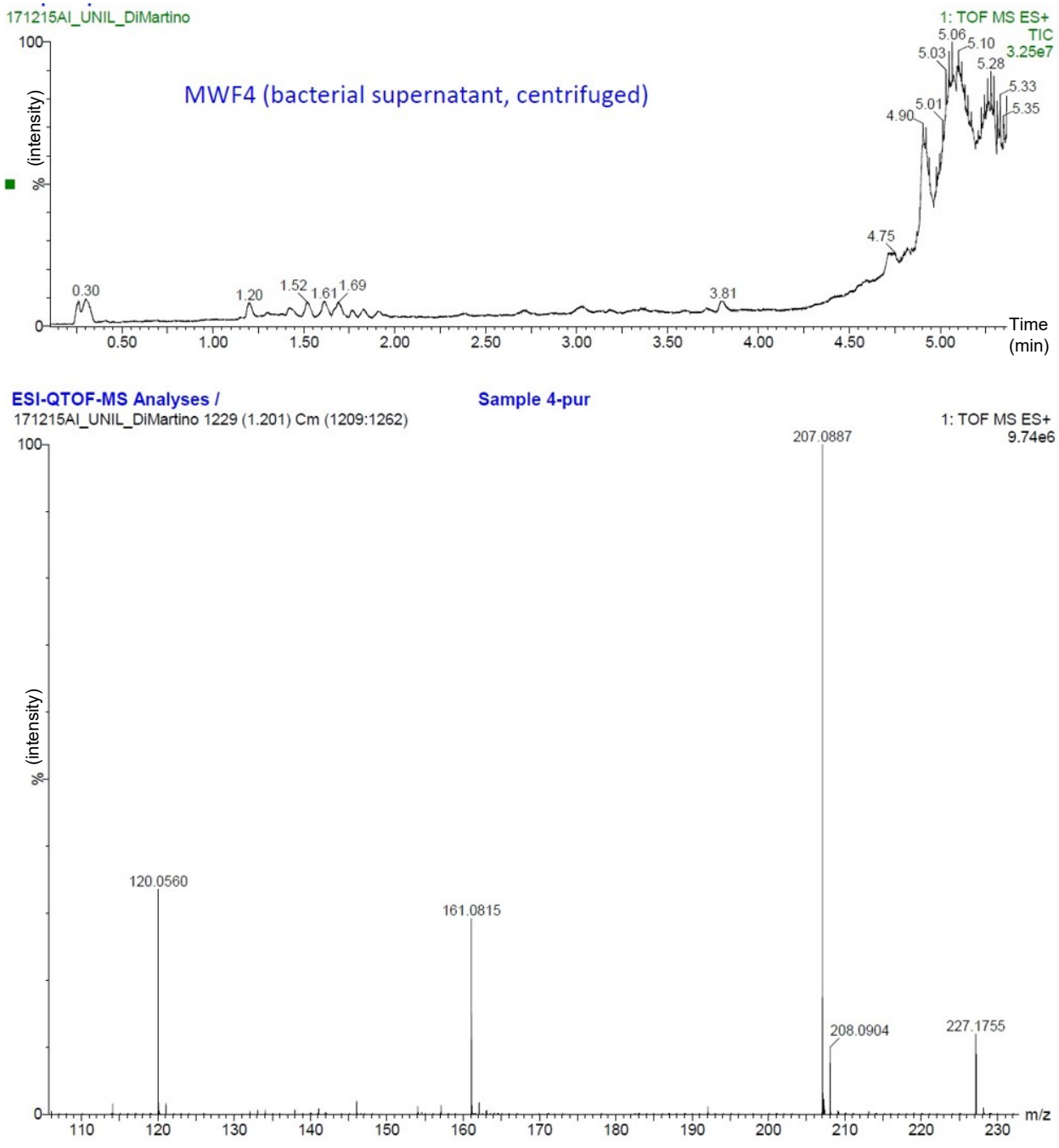


Figure 3.1 – LC-MS analysis of MWF medium. (A) In the chromatogram each peak corresponds to a single eluted compound over time. (B) Mass spectrum of the compound eluted at min 1.20 selected as an example. Each peak is a charged fragment of the original eluted compound and the specific composition of m/z and intensity (%) of each charged fragment allows the identification of the original eluted compound.

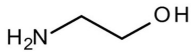
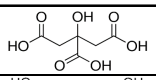
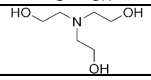
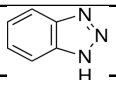
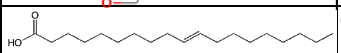
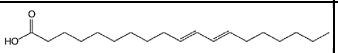
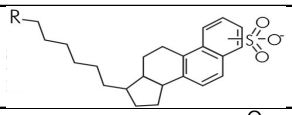
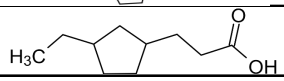
NAME	FORMULA	PHYSICAL PROPERTIES	STRUCTURE	ANALYSIS
Monoethanolamine	C2H7NO	Soluble in water		Patent
Citric acid	C6H8O7	Soluble in water		Patent
Triethanolamine	C6H15NO3	Soluble in water		Patent
Formaldehyde	CH2O	Soluble in water		Patent
Benzotriazole	C6H5N3	Soluble in water		Patent
Morpholine	C4H9NO	Miscible in water		Patent, GC-MS, LC-MS pos
Oleic acid	C18H34O2	Water solubility 0.12 mg/l		GC-MS
Linoleic acid	C18H32O2	Water solubility 1.59 mg/L		Literature
Petroleum sulfonate *	NA	Soluble in water		Literature
Naphthenic petroleum oil *	NA	Water solubility <50 mg/L		Literature

Figure 3.2 – List of the ten compounds representative of MWF. * compounds are repetition of the subunit showed in the "Structure" column, but it was not possible to know the real molecular formula or final structure. "Analysis" shows what alerted us to this compound in relation to MWF. The patent refers to [35].

and naphthenic petroleum oil concentrations were chosen to be within and above the concentrations that are commonly found in MWF ([18, 33]. Monoethanolamine, triethanolamine, citric acid and morpholine concentrations were chosen to be above and below the quantity already tested by the developers of the consortium [35, 34].

To assess the growth of each species on these compounds, we set up monoculture assays where each tested compound was the only carbon source added to a defined medium (MM, see Chapter 2 for medium composition). After an overnight growth in rich medium (TSB), bacteria were diluted to an OD_{600} of 0.05 and re-incubated in fresh rich medium (TSB) for three hours to reach exponential phase. Cells were harvested, washed in PBS and then inoculated in the compound-added defined medium at a starting OD_{600} of 0.1 (10^7-10^8 CFU/ml). We performed these experiments starting from high cell density in order to detect both growth and death of the bacteria as an effect of the tested compounds. As a control, the same quantity of bacteria was inoculated in the defined medium MM without any carbon source added in it. In this condition, bacteria are expected to survive, but not grow. Each condition was repeated in triplicates and bacteria were incubated at 28°C in shaking conditions for seven to ten days. Bacterial abundance was assessed through CFU plating every 24 hours and, if possible, OD_{600} measurements multiple times per day. It was not always possible to rely on OD_{600} measurement because some of the carbon sources turn the MM very turbid upon dissolution. CFU counting was used to plot growth curves of CFU/ml over time and we calculated the area under the curve (AUC) to have a better comparison between the different tested conditions and the control. We calculated the ratio between the AUC of each replicate per condition and the mean of the AUC of the

three control replicates, and used these data to build heatmaps showing the effect of each compound on each of the four species (Fig. 3.3).

3.2.3 Bacterial response to individual compounds: high initial population size

Overall, we could group the compounds in three categories depending on the effect they had on bacteria: nutrients, toxic compounds and neutral compounds (compounds that did not affect bacteria in any measurable way). Citric acid and monoethanolamine were the two compounds that acted as nutrients, and in most cases their effect was concentration dependent. Focusing on At in the 20 mM citric acid condition, we saw a strong increase in bacterial abundance in the late stages of the assays, as reflected by the orange shade in the heatmap, but the difference was not statistically significant. Triethanolamine and naphthenic petroleum oil were found to be neutral, whereby neither growth nor death was detected at any tested concentration. Finally, morpholine, benzotriazole, formaldehyde and petroleum sulfonate were toxic to bacteria. More specifically, morpholine and benzotriazole induced a decrease in the bacterial population, while petroleum sulfonate had a more dramatic effect, leading to the extinction of the entire Ct and Ms populations.

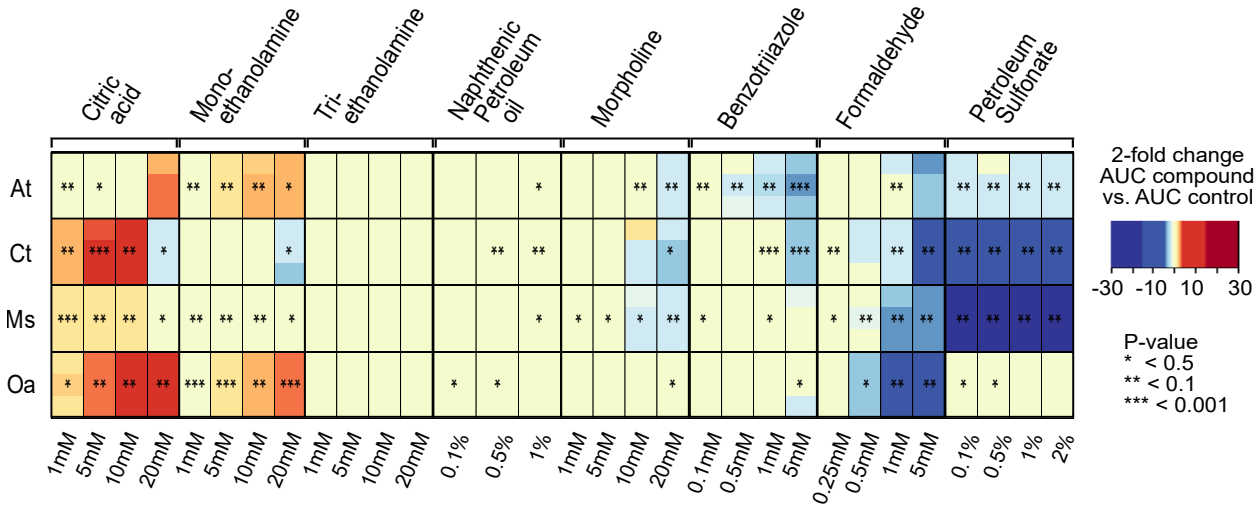


Figure 3.3 – Growth of At, Ct, Ms, and Oa in presence of the compounds at high initial population size. We tested a range of concentrations for each compound in triplicates (each replicate is shown as one of three rectangles in a box). The tested compound is the only carbon source in the medium. Heatmaps show the fold change between the area under the curve (AUC) of each sample replicate and the AUC of the mean of the control replicates where no compound was added. Blue shades represent negative fold change (bacteria died) and orange shades represent positive fold change (bacteria grew). Statistical analysis was performed (T-test to compare the AUC of the three replicates of every compound-supplemented condition vs. the AUC of the three replicates of MM condition with no compound supplemented, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

3.2.4 Death and recovery of bacteria in formaldehyde

Formaldehyde had a negative effect on the four species, and this effect was stronger as the concentration increased. However, at some specific conditions - At in 2 mM, Ct in 1 mM, and Oa in 1 mM - there was an initial decrease in bacterial abundance followed by a recovery (Fig. 3.4, growth curves left side of the dashed line). We considered two possible hypotheses to explain it. The first is that a subpopulation of bacteria

managed to adapt to formaldehyde and then use it as a carbon source to support the following bacterial division. The second hypothesis is based on the chemistry of formaldehyde as a volatile compound. We assumed that its gradual evaporation was reducing the concentration in the medium below the susceptibility threshold, allowing bacterial growth.

We tested these hypotheses by transferring bacteria that were incubated for 14 days in formaldehyde-supplemented media into fresh formaldehyde-supplemented media. This procedure was repeated for all four strains at all tested formaldehyde concentrations. If the first hypothesis is the correct one, we would expect bacteria to grow immediately since they would have adapted to feed on a new available batch of formaldehyde. On the other hand, a decrease in abundance of the bacteria that had already encountered formaldehyde before followed by growth would confirm the toxicity of formaldehyde at high concentration and validate hypothesis two.

Results for At in 2mM of fresh formaldehyde showed an initial decrease in abundance followed by an increase, similarly to the result obtained in the initial experiment (Fig. 3.4, growth curves right side of the dashed line). The difference between this result and the one previously obtained was a shorter interval of time before bacterial growth resumed after the initial drop: in this case, after 48 hours of incubation, compared to the 72 hours of the first experiment. We observed a similar result for Oa in 1 mM, in which growth was observed already after 48 hours of incubation, as opposed to the previous experiment in which no growth was registered for more than seven days. Finally, Ct's abundance remained constant in 1 mM, as opposed to the drop in the first 24 hours of incubation observed in the previous experiment. Overall, these results suggested an adaptation of the bacteria to the challenging formaldehyde environment, but further investigation is necessary to evaluate the impact of formaldehyde evaporation that must be occurring.

We encountered some difficulties during this second experiment with Ms and Oa. Regarding Ms, there was a drop in bacterial abundance even in our control condition 24 hours after the transfer of the full bacterial population into fresh formaldehyde-supplemented media. Our control was bacteria incubated in minimal medium without formaldehyde (MM curve, Fig. 3.4C) and it was expected to remain constant over time. We could not explain this anomaly, so we decided to put aside this subset of data and to focus on the results obtained with the other strains for choosing between the first and the second hypothesis. Regarding Oa, in condition 2 mM we could not obtain a compact pellet prior to the transfer in the fresh medium, so we lost two replicates out of three.

3.2.5 Bacterial response to individual compounds: low initial population size

We performed the experiments reported above starting from high cell density (OD of 0.1, 10^7-10^8 CFU/ml) in order to detect both growth and death of the bacteria as an effect of the tested compounds. One problem with this approach is that a lack of growth could also be because the population size was too high to increase at the selected compound concentrations. This could have been the case for naphthenic petroleum oil and triethanolamine, in which there was neither growth nor death at any concentration. For example, 100-fold fewer cells (from 10^8 CFU/ml to 10^6 CFU/ml) in the 20 mM triethanolamine condition would increase its concentration from 1.2×10^{11} molecules per cell to 1.2×10^{13} molecules per cell, and this variation could trigger bacterial growth.

To test whether initial population size affected bacterial response to the compounds, we repeated the

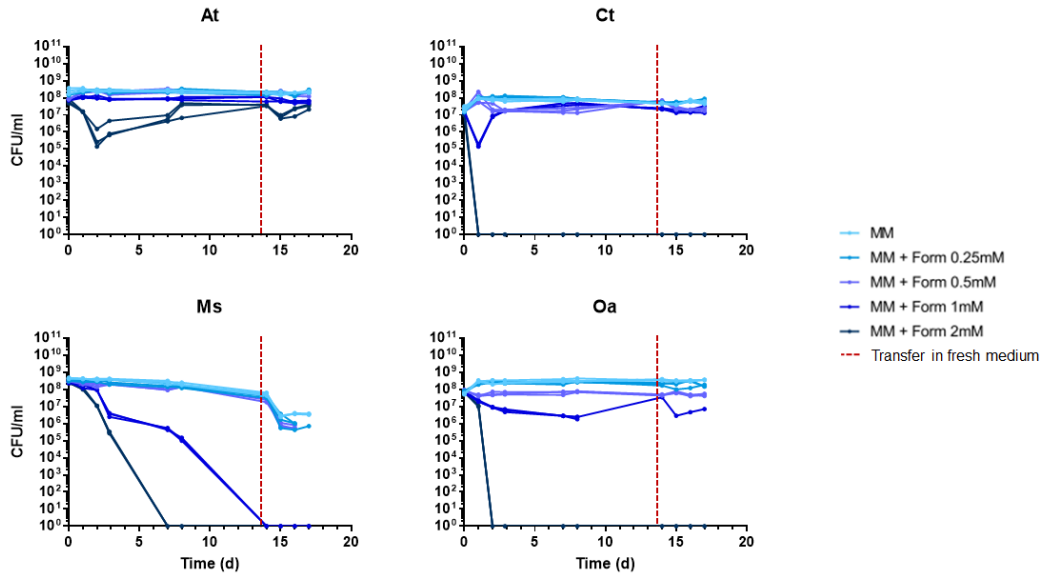


Figure 3.4 – Growth curves of At, Ct, Ms and Oa in formaldehyde. Y-axis represents population size expressed as CFU/ml, x-axis represents time expressed in days. Different colors represent different tested formaldehyde concentrations (three technical replicates per condition, graphs show the results of one biologically independent experiment). Red dashed line marks the transfer of the entire population in fresh formaldehyde media.

same assay using a 100-fold lower initial population size (10^5-10^6 CFU/ml). Our hypothesis was that a smaller population size would increase the magnitude but not the sign of an effect, meaning that a lower quantity of cells would grow or die faster at the same concentration.

We set up a big screen of four different compounds at four different concentrations. Two of these compounds had already been tested before (petroleum sulfonate and naphthenic petroleum oil), while the other two had not (linoleic acid and oleic acid). Given the previous results, we added a lower concentration of petroleum sulfonate to check if we could reduce its toxicity. On the other hand, we added a higher concentration of naphthenic petroleum oil to check if this new condition could promote bacterial growth. The concentrations of linoleic acid and oleic acid were chosen according to information in the literature on their average abundance in MWF [33], and we added extra concentrations below and above those. In total, we prepared 17 different media (four compounds at four different concentrations each, plus the control of the minimal medium with no carbon source). Due to the size of the experiment, we prepared just one replicate per strain per condition, allowing us to test a wider range of conditions at the same time. As before, each tested compound was the only carbon source added to the defined medium. Cell cultures were prepared as described before (see Chapter 2), except for the starting quantity of cells, that was 100-fold lower than the first experiment. An inoculum of 10^5-10^6 CFU/ml was added to the compound-supplemented media and in the MM medium without any carbon source as a control. We again plotted growth curves of CFU/ml over time and calculated the area under each curve (AUC), as shown in Fig. 3.5. Since we tested only one replicate per species per condition, it was not possible to perform statistical tests.

Effect of the initial population size. The results of the assays in naphthenic petroleum oil and in petroleum sulfonate showed that a compound can affect bacterial growth differently depending on the ini-

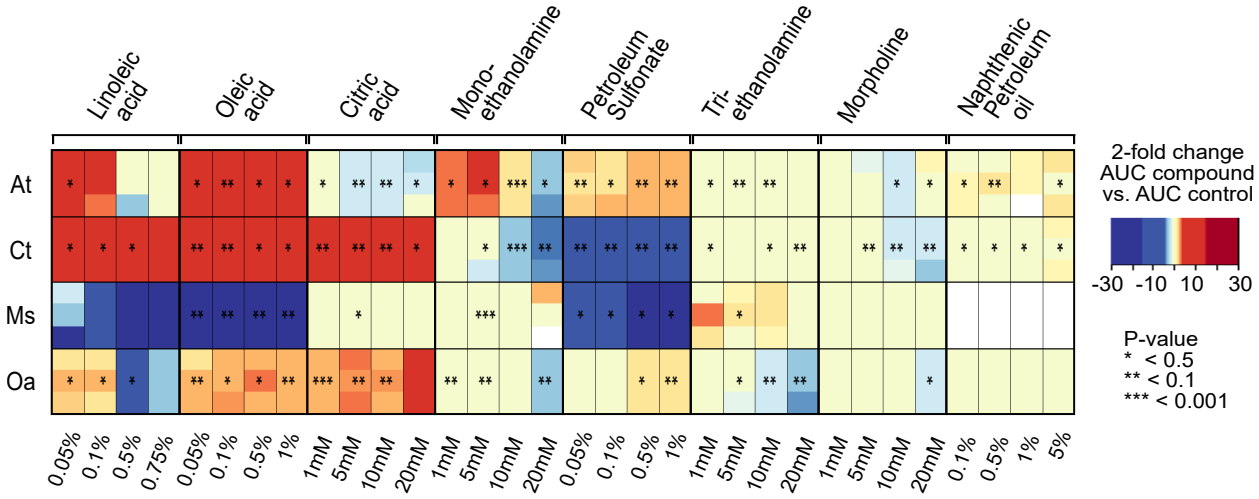


Figure 3.5 – Growth of At, Ct, Ms, and Oa in presence of the compounds at low initial population size. Blue shades represent negative fold change (bacteria died) and orange shades represent positive fold change (bacteria grew). We tested a range of concentrations for each compound in single replicate per species. The tested compound is the only carbon source in the medium. Heatmaps show the fold change between the area under the curve (AUC) of each sample replicate and the AUC of the control replicate where no compound was added. Statistical analysis was performed (T-test to compare the AUC of the three replicates of every compound-supplemented condition vs. the AUC of the three replicates of MM condition with no compound supplemented, * = p< 0.05, ** = p< 0.01, *** = p< 0.001).

tial population size (Fig. 3.6 A-B). We were expecting that a smaller population size would increase the magnitude but not the sign of an effect, meaning that fewer cells would grow or die faster at the same concentration. But some species switched their response in some specific conditions. For example, only when their population size was low, Ct grew in naphthenic petroleum oil, and Oa grew at high petroleum sulfonate concentrations. (Fig. 3.6 A-B). For this reason, we decided to further investigate the relationship between initial population size and response to compounds. With the help of Katia Annen, a trainee from the Ecole supérieure de la Santé of Lausanne, we repeated the assays of some compounds of interest that we tested before, this time using the lower initial population size.

In comparison to the first experiment, it seemed that At, Ct and Oa were now affected negatively by monoethanolamine at high concentrations (Fig. 3.6 panel B). The 100-fold decrease of initial population size, coupled with the same compound concentrations tested before, determined a 100-fold increase of number of molecules available per each cell. Our hypothesis was that this quantity of molecules per cell was too high to be tolerated by bacteria, and thus impaired their growth. Surprisingly, Ct grew in high concentrations of citric acid, while Ms was not particularly affected by citric acid at any tested concentration. Similar scenario encountered for Monoethanolamine occurred for At and Ms in naphthenic petroleum oil (Fig. 3.6 panel B). On the contrary, the lower starting population size allowed Ct to grow in all tested concentrations. Finally, both At and Oa grew in petroleum sulfonate compared to the previous experiment (Fig. 3.6, comparison between panel A and panel B).

Overall, we observed that the variation in initial population size changed the effect of the compounds on bacteria in ways that can be unpredictable. Since we were able to follow both bacterial growth and death at this lower starting population size, we maintained this condition for all future experiments.

Effect of the compounds: focus on linoleic acid (LA). The effect of the new tested compounds on

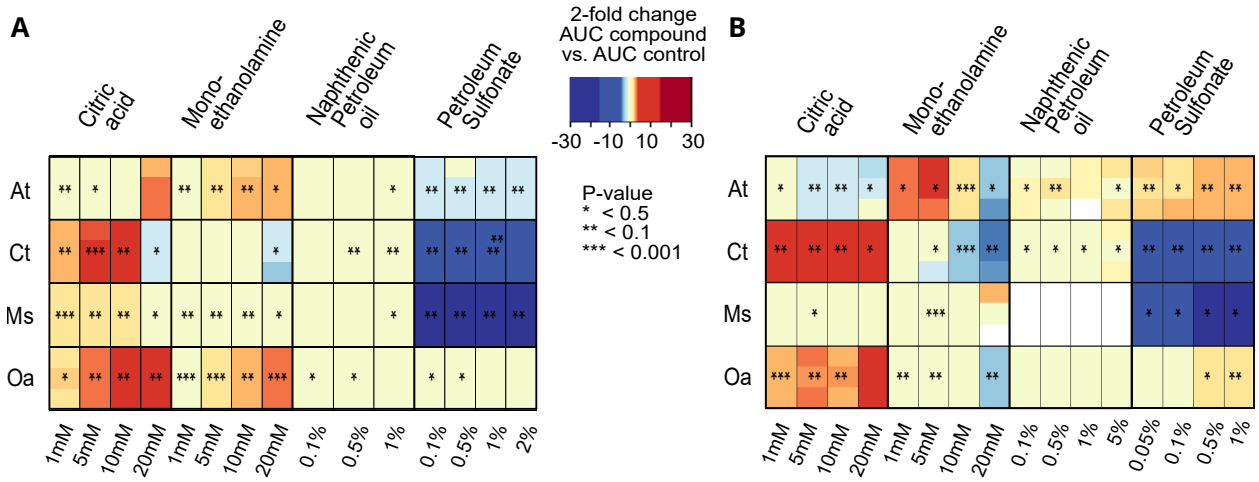


Figure 3.6 – Comparison of the growth of At, Ct, Ms, and Oa in presence of the compounds at different starting population sizes. Panel A shows the growth at high starting population size (10^7-10^8 CFU/ml), panel B low starting population size (10^5-10^6 CFU/ml). We tested a range of concentrations for each compound in triplicates. The tested compound is the only carbon source in the medium. Heatmaps show the fold change between the area under the curve (AUC) of each sample replicate and the AUC of the mean of the control replicates where no compound was added. Blue shades represent negative fold change (bacteria died) and orange shades represent positive fold change (bacteria grew). Statistical analysis was performed (T-test to compare the AUC of the three replicates of every compound-supplemented condition vs. the AUC of the three replicates of MM condition with no compound supplemented, * = $p< 0.05$, ** = $p< 0.01$, *** = $p< 0.001$).

bacteria was very species-specific. Linoleic acid (LA) was the compound that spiked our interest the most. We found that LA was a nutrient for Ct at any concentration, toxic for Ms at any concentration and acted as a nutrient for At and Oa only at low concentrations but was toxic at higher ones (Fig 3.5). The concentration-dependent effect of LA on a subset of species made it a good candidate to build our controlled system to explore the SGH in bacteria, so we decided to use LA as single carbon source for our defined medium. The effect of the different level of toxicity provided by increasing the concentration of LA on the interaction network and coexistence between species will be extensively discussed in Chapter 4 and Chapter 5.

3.2.6 Bacterial growth in Minimal Medium (MM)

Before we move on to the core of this PhD work, there is another topic that I would like to briefly discuss. When we tested the compounds at the lower initial population size, we noticed that this variation affected also growth in our control Minimal Medium (MM) in which no compound was added. Indeed, Ct and Oa grew by two orders of magnitude in MM, while Ms decreased over time. None of these situations was ever observed in the 100-fold higher population size, in which none of the species varied in their abundance over time. Our hypothesis was that cells were probably still retaining molecules of the rich medium in which they grew before and that they were using this reservoir to grow later in the MM. Compared to the stable situation at high population size, we thought that probably this reservoir could manifest only at lower population sizes. To test this hypothesis, we incubated each species in monoculture in MM at high initial population size (10^7-10^8 CFU/ml). After 24 and 48 hours, we transferred an aliquot of these cultures into fresh MM to have a 100-fold lower bacterial population (10^5-10^6 CFU/ml) and we followed bacterial abundance over

time. We expected to have no growth after the transfer due to the consumption of the putative reservoir during the former incubation in MM. Surprisingly, Ct and Oa grew as well as before, and neither the 24 nor the 48 hours incubation in MM affected their growth. Ms decreased over time after both 24 and 48 hours of incubation in MM similarly to what showed before (Fig. 3.7).

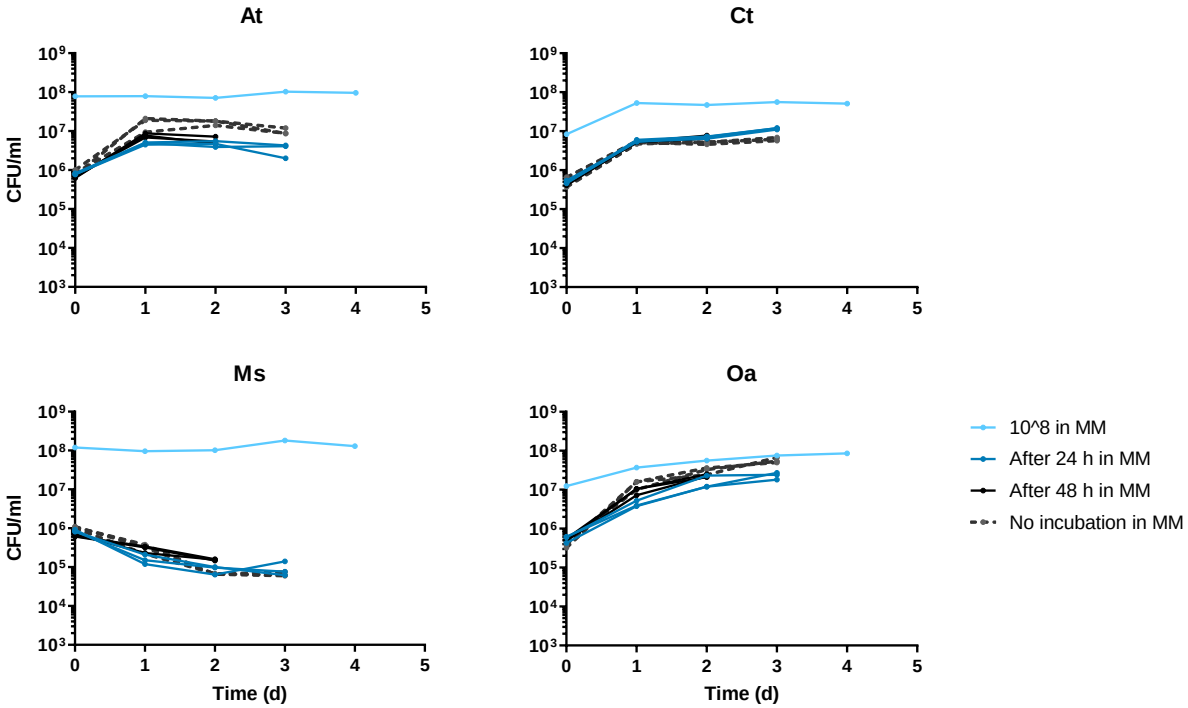


Figure 3.7 – Growth of At, Ct, Ms, and Oa in MM after being transferred from a previous incubation in MM. Y-axis represents population size expressed as CFU/ml, x-axis represents time expressed in days. Different colors represent different tested conditions (three technical replicates per condition, graphs show the results of one biologically independent experiment). We incubated each species in monoculture in MM at 10^8 CFU/ml (light blue curves). We transferred a 1:100 dilution into fresh MM after 24 hours (blue curve) and 48 hours (black solid curve). Neither the 24 nor the 48 hours incubation in MM affected the growth. At, Ct and Oa in both conditions grew similarly to the condition in which no former incubation in MM was performed before inoculation of the cells in MM (dashed line). Ms decreased over time after both 24 and 48 hours of incubation in MM similarly to the condition in which no former incubation in MM was performed before inoculation of the cells in MM. Light blue curves show that starting from a higher population size there is neither growth nor decrease in MM.

This result led us to exclude the reservoir hypothesis and rather focus on something that could be present in the MM. We prepared three different media: the regular Minimal Medium (MM), a variant of MM without EDTA and NTA as they were the only carbon-containing molecules (MM-), and a medium with only salts and phosphate (M9), which acted the basis of the MM. We expected bacteria to grow only in the MM medium but not in MM- and M9 media. We incubated the four species in monocultures in these three media at two different starting population sizes (10^5-10^6 and 10^3-10^4 CFU/ml) to enhance even more the effect of the media on bacteria.

To our surprise, Ct and Oa grew in all three media up to the same final population size of 10^7-10^8 CFU/ml regardless of the initial population size (Fig. 3.8C, D). At also grew in all three media, but slightly worse in M9 (Fig. 3.8A). Ms did not decrease in the MM- regardless of the initial population size (Fig.

3.8C, solid and dashed green curves), while it decreased in a similar way in both MM and M9 media.

Regarding Ms, since the MM- medium without EDTA and NTA didn't affect population size, we concluded that the higher number of molecules of EDTA and NTA per cell was impairing Ms' survival. Moreover, we believed that Ms decreased in M9 because this medium is too poor (lacking in metals, for example) to allow for the survival of Ms.

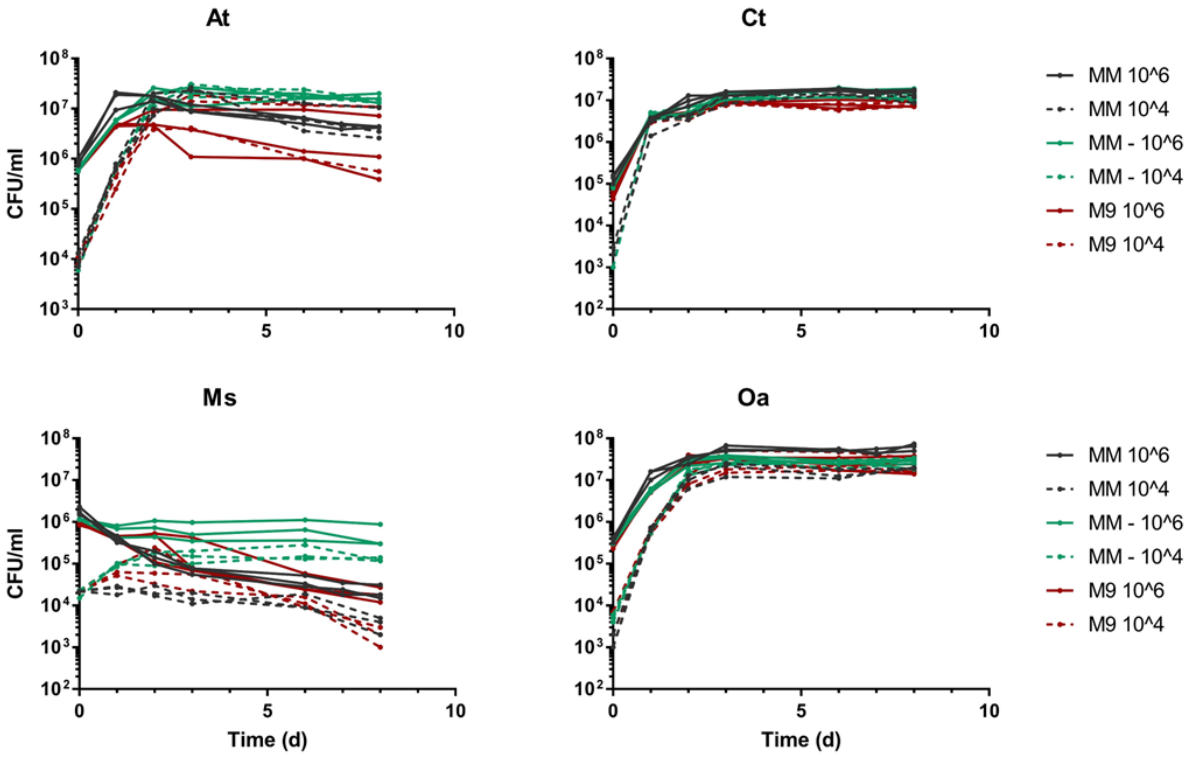


Figure 3.8 – Growth of At, Ct, Ms and Oa in three different media. Y-axis represents population size expressed as CFU/ml, x-axis represents time expressed in days. Different colors represent different tested concentrations (three technical replicates per condition, graphs show the results of one biologically independent experiment). We tested MM (black curves), MM (MM without EDTA and NTA, green curves) and M9 (salt, phosphate and ammonium chloride, red curves). Two different population sizes were tested per each medium (10^5-10^6 CFU/ml solid curves, 10^3-10^4 CFU/ml dashed curves).

Since three of the four species grew in all conditions, we concluded that there must be some contaminants in the sterile deionized water that we use for all media preparation. We assumed that the concentration of these putative contaminants is low enough to show its effect only at lower bacteria population sizes. With the help of Diego Rojas-Gätjens, a visiting undergraduate student, we performed additional experiments to test our assumption. We focused only on At and Oa to have a quicker grasp on the results on the following points:

— What would happen if we decrease even more the initial population size? Would bacteria still grow up to 10^7-10^8 CFU/ml?

— If the contaminants are present in the water, could we eliminate this additional growth by changing the water source?

— What if the contaminants are actually present on the walls of the glass tubes as post-washing

residues? Would we eliminate growth in MM if we grew bacteria in single-use plastic tubes instead?

In the first experiment, we decreased even more the initial population size, starting from as low as 10^2 CFU/ml. We prepared cell cultures as described in Chapter 2, we grew bacteria for 5 days at 28°C, 200 rpm and we followed bacterial abundance through CFU plating. We found that both At and Ct reached a final population size of about 10^7 CFU/ml regardless of the initial population size (Fig. 3.9). This result was consistent with what we observed in Fig. 3.8 and reinforced our hypothesis that lower initial population size responded to the presence of the unknown contaminants and it resulted in bacterial growth of several orders of magnitude.

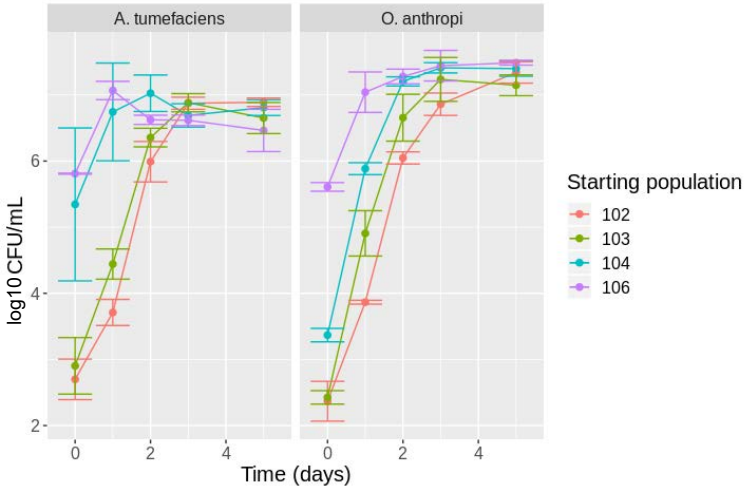


Figure 3.9 – Growth of A.tumefaciens (At) and O.anthropi (Oa) in MM at different initial population sizes. Time is on the x-axis, log value of CFU/ml on the y-axis. Different colors represent different initial population sizes (10^2, 10^3, 10^4, 10^6),lines represent the mean of the three technical replicates of that condition, error bars represent the standard deviation from the mean (three technical replicates per condition, graphs show the results of one biologically independent experiment).

In our second experiment, we changed the water source that we used for MM preparation and we purchased 1l of water ROTIPURAN®Ultra (Roth), an extra highpure water used for sample preparation in ultra trace analysis. We compared the growth of At and Oa in MM prepared with our standard ddH_2O and with ROTIPURAN®Ultra water. We tested two different initial population size, 10^4 CFU/ml and 10^6 CFU/ml to test if we could observe a initial-population-size-dependent effect in ROTIPURAN®Ultra as we saw already in standard ddH_2O (Figs. 3.9 and 3.8). We decided to test within this experiment also if there were effects depending on the type of growth tube that we used, so we performed the above described experiment in both 14 ml Falcon® tubes and in the glass tubes that we used to perform all the growth assays described in this Chapter (and in the following ones too). Unexpectedly, we found that both At and Oa could grow as well in ROTIPURAN®Ultra MM as in standard ddH_2O MM, regardless of the initial population size (Fig. 3.10). This result meant that it is very hard to eliminate basal growth effects, even when using certified components that should contain the least amount of contaminants.

Regarding the putative material-tube effect, the observed bacterial growth in Falcon® tubes was lower than the growth in the glass tubes in the corresponding conditions (Fig. 3.10, comparison between panel A and B). However, this reduction occurred in rich media as well (data not shown), so we concluded that the

lower growth was not imputed to the lack of contaminants present on the glass tube walls, but rather to the different liquid:gas ratio in the Falcon® tubes compared to the glass ones.

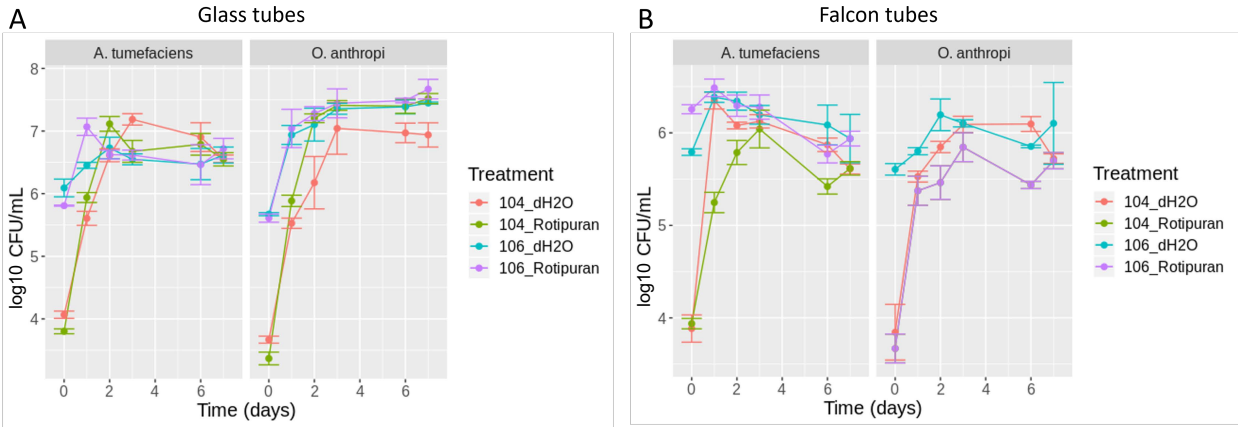


Figure 3.10 – Growth of A.tumefaciens (At) and O.anthropi (Oa) in MM prepared with different water sources. Time is on the x-axis, log value of CFU/ml on the y-axis. Different colors represent different water source (ROTIPURAN®Ultra or ddH2o) and initial population size (10^4 or 10^6), lines represent the mean of the three technical replicates of that condition, error bars represent the standard deviation from the mean (three technical replicates per condition, graphs show the results of one biologically independent experiment). A) Growth of aforementioned conditions in glass tubes. B) Growth of aforementioned conditions in Falcon® tubes.

Overall, the variation in initial population size affected bacterial response in different ways, even in defined conditions in which no effect was expected. Since this effect is due to something that is present in both media containing carbon sources and in the MM control, we take it into account when comparing between these conditions. For this reason, we are confident that the effects that we detected after bacterial incubation in different compounds (growth, death or neither of them) is really induced by the specific compound.

3.3 Summary

After this first set of experiments, we grouped the compounds into three different categories according to statistically significant effects that they had on bacteria: “nutrients” for compounds that promoted bacterial growth, “toxins” for compounds that reduced the population size and neutral compounds that did not affect in the population size in either direction (Fig. 3.11). The monoculture approach we took was designed to characterize the effect of a single compound on each species. These compounds were representative of the components of MWF, and we were able to identify a few compounds whose effects on bacteria changed depending on compound concentration. Among those, linoleic acid (LA) seemed to be one of the most promising to study the role of the environment in shaping interactions and coexistence within a community and to test the SGH. In all the experiments discussed in the upcoming chapters of this thesis, LA was the only carbon source present in the synthetic media, as its increasing concentration provided increasing toxicity.

	At			Ct			Ms			Oa		
Compound	Nutrient	Neutral	Toxic	Nutrient	Neutral	Toxic	Nutrient	Neutral	Toxic	Nutrient	Neutral	Toxic
Monoethanolamine	b		b		b	b	b	b		b		b
Citric acid				a		a						
Triethanolamine												
Formaldehyde		a	a									
Benzotriazole					a	a						
Morpholine		a	a		a	a		a	a			
Oleic acid												
Linoleic acid	a		a							a		a
Petroleum sulfonate	a/b	a/b	a/b							a/b	a/b	
Naphthenic petroleum oil		b	b	b	b			b	b			

Figure 3.11 – Effect of the ten compounds on At, Ct, Ms and Oa. Grey squares mark the effect of the compound on each species. When more than one square per compound is marked, it means that the effect changes according to concentration (a), initial population size (b), or both (a/b).

3.4 Author contributions

Rita Di Martino and Sara Mitri designed research; Rita Di Martino performed the majority of the experiments (experiments described in section 3.2.6 were performed by Katia Annen and Diego Rojas-Gatjens). LC-MS protocol was designed by Laure Menin (EPFL) and LC-MS experiment was performed by Laure Menin (EPFL). LC-MS data were processed by Rita Di Martino; Rita Di Martino and Sara Mitri analyzed the data described in this chapter.

Chapter 4

Interactions between bacteria in a controlled nutrient-toxin gradient

4.1 Introduction

In Chapter 3, we were looking for compounds that had different effects on different species. Linoleic acid (LA) was a good candidate, as it had concentration-dependent effects on most tested species (Fig. 3.5). Our original goal was to analyze how a gradient of nutrient and toxicity could affect the interactions between two species in co-culture and if the results would be in line with the Stress Gradient Hypothesis (SGH). Having chosen the environment in which such a gradient exists then, our next questions are: how do two species interact when in co-culture at different LA concentrations? Can we predict the interaction in co-cultures based on our knowledge of how they grow in monoculture?

We focused on two different concentrations of LA to build a gradient of nutrient and toxicity for our species: 0.1% LA as the low concentration and 0.75% as the high concentration. At low LA concentration (0.1%), LA acts as a nutrient for most species in monoculture, so our hypothesis was that two different species would compete in a co-culture at this condition, resulting in negative interactions. On the other hand, at high LA concentration (0.75%), LA is a nutrient only for Ct, while it was toxic for the other species. Consequently, our hypothesis was that a co-culture of Ct with another species could lead to the rescue of the partner species, because Ct would reduce LA concentration as its population size increased, thus bringing LA in a range tolerated by the partner species. The net interaction at this higher toxic environmental condition would be positive.

Since the first stages of the planning of the co-culture experiments, we decided to couple the wet lab experiments with mathematical models. We wanted to generate quantitative predictions on the hypothesis described above and to experimentally test them in the lab. The mathematical models were built and analyzed by Dr. Aurore Picot, a former lab member. In this chapter, I will only briefly describe the main features of the models that she developed, but further details can be found in Chapter 2.

4.2 Results

4.2.1 Choosing the co-culture of interest: focus on At and Ct

We selected Ct as the focal species since it could grow in mono-culture at both 0.1% and 0.75% LA (Fig. 4.1, panel B), meaning that it could potentially compete at low LA concentration and rescue a sensitive partner in high LA concentration. Although LA had a similar effect on Oa and At, we selected At as a partner species and focused our attention on the interaction between At and Ct.

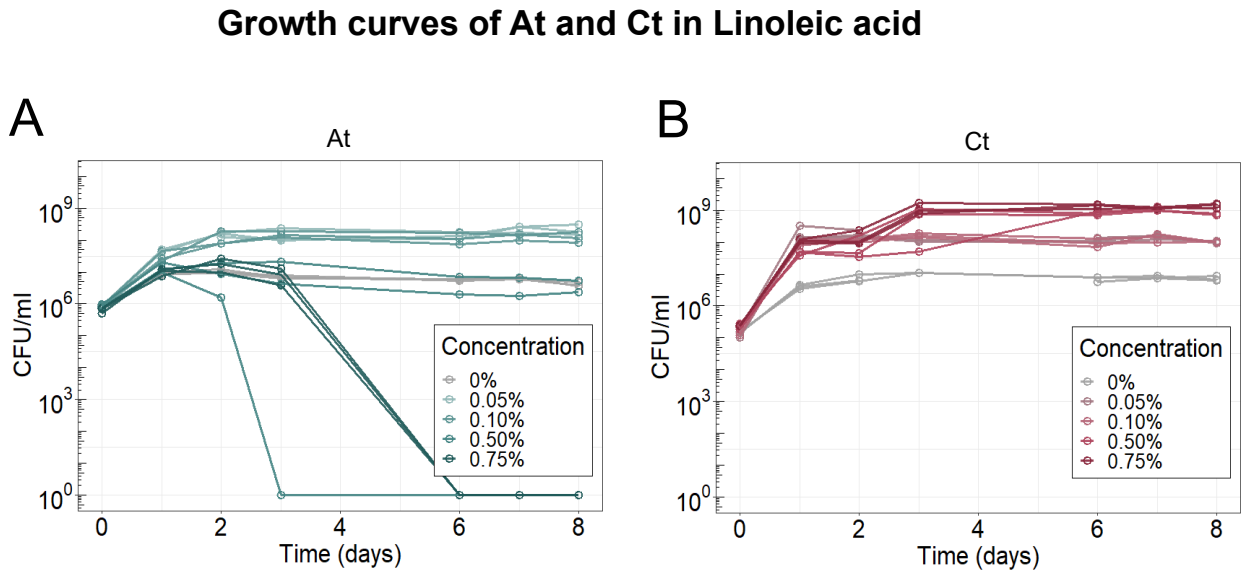


Figure 4.1 – Growth of At and Ct in monoculture over a gradient of LA. Y-axis represents population size expressed as CFU/ml, x-axis represents time expressed in days. At is in green, Ct is in red, darker shades represent increasing LA concentrations (three technical replicates per condition, graphs show the results of one biologically independent experiment). At can growth only at lower La concentration and it dies at higher LA concentrations after an initial phase of growth (panel A). Ct increases its growth capacity following the increasing LA concentration (panel B).

4.2.2 Model 1: LA is the exclusive source of both nutrients and toxicity

We used the monoculture data of both At and Ct at all tested LA concentrations to develop the first version of the mathematical model, that from now on I will refer to as Model 1. The key parameters of Model 1 were the growth rate and the death rate and they were chosen to best fit the experimental data. To capture the response of At to LA concentration in the model, LA acted as a nutrient source that could also add toxicity in the system and cause death at increasing concentrations (see Chapter 2. More specifically, Model 1 assumed that the toxicity carried by LA was not present in the system since the beginning, but that it increased linearly over time and it was proportional to the linoleic acid concentration (higher LA concentration, higher accumulation of toxicity). We supposed that toxicity was accumulating over time by analyzing the growth curve of At monoculture 0.75%: as we can see in Fig. 4.2, At could grow at early stages of the monoculture, but it died later on. This behaviour suggested that some toxicity may be accumulating over time and that eventually it crossed a threshold that made the environment unsustainable for At. We then used Model 1 to predict how At and Ct were expected to interact if co-cultured at different

LA concentrations (Fig. 4.2, column ”Model”).

4.2.3 Linoleic acid concentration determines interaction sign

The model predicted that increasing the concentration of LA in a co-culture of the two species could change the interaction sign from negative to positive. More specifically, at low concentration, both species competed for the sole nutrient source, LA. As LA concentration was high enough to kill At, however, we expected to observe facilitation, as Ct consumed LA and reduced its concentration, making the environment less toxic for At. We tested this prediction in the lab by growing At and Ct in mono- and co-culture at 0.1% and 0.75% LA. The results were in line with the predictions of the model: at low LA concentration (0.1%, Fig. 4.2, upper row ”LA 0.1%”), At grew significantly worse in the co-culture compared to mono-culture (AUC in co-culture $5x10^6 \pm$ standard deviation, mono-culture $5x10^9 \pm$, t-test $P < 0.001$), showing that there was competition for LA. Instead, at high LA concentration (0.75%, Fig. 4.2, bottom row ”LA 0.75%”), the presence of Ct in the co-culture rescued At, allowing it to survive and grow as opposed to At in monoculture at 0.75% LA. The growth of Ct was not significantly affected by At's presence in either condition (t-test with Benjamini-Hochber correctiong, Ct 0.1% vs. At-Ct 0.1% p = 0.9782, Ct 0.75% vs. At-Ct 0.75% p = 0.4214).

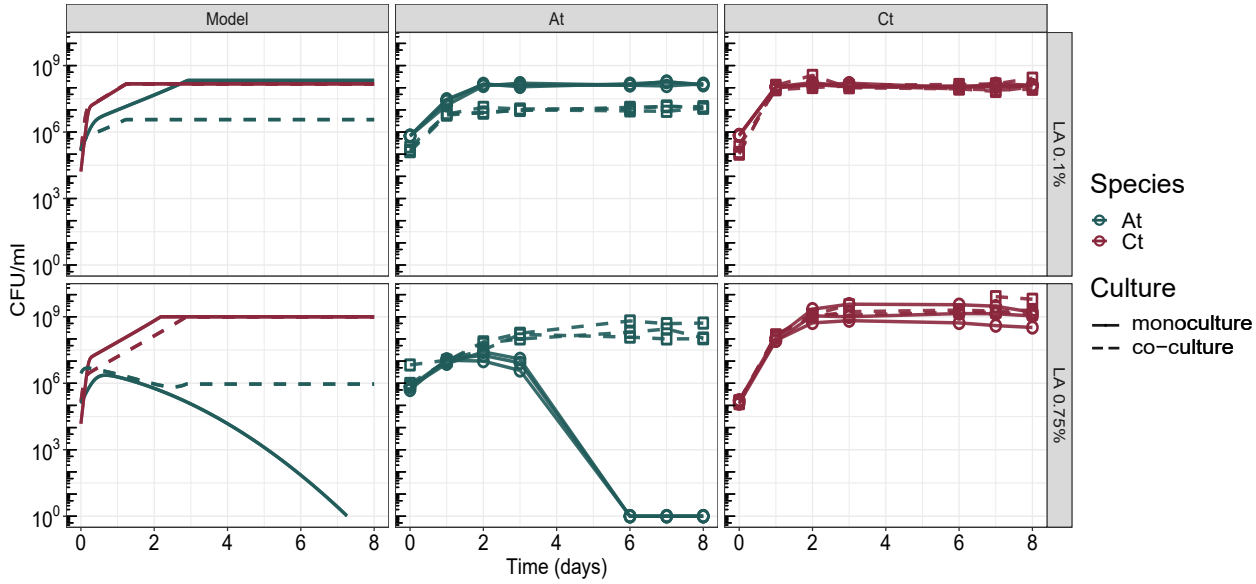


Figure 4.2 – Growth of At and Ct in mono- and co-cultures in LA 0.1& and LA 0.75%. Y-axis represents population size expressed as CFU/ml, x-axis represents time expressed in days. Rows show different medium conditions: upper row LA 0.1%, bottom row LA 0.75%. Columns show different types of culture: first column shows only model data resulting from Model 1 (monocultures and co-cultures of both At and Ct), second column shows only At data (monoculture and co-culture), third column shows only Ct data (monoculture and co-culture). At is in green, Ct is in red (three technical replicates per condition, graphs show the results of one biologically independent experiment). Solid lines are monoculture data, dashed lines are co-culture data. At LA 0.1%, both At and Ct monocultures survive, and At growth in the co-culture is impaired, while Ct growth is uneffected (p = 0.9782). At LA 0.75%, At monoculture dies after three days of slight growth (hump-shaped curves), while Ct monoculture grows. At in rescued by Ct in the co-culture, while Ct growth is uneffected (p = 0.4214). Model data extrapolated from Model 1 capture quite good the experimental data at LA 0.1% but not as much in LA 0.75%.

Although these results matched the model's predictions qualitatively, At's growth in co-culture was greatly underestimated by the model (Fig. 4.2, column "Model"). Furthermore, the model did not correctly predict the hump-shaped growth of At monoculture at 0.75% LA, even though it assumed the accumulation of toxicity. This suggests that estimating model parameters where both growth and death are caused by a single compound is challenging.

4.2.4 Metabolic analysis of spent supernatants

We next focused on exploring the mechanism behind the toxicity at high LA concentration. As mentioned previously, our first hypothesis was that the high concentration of LA itself was responsible for the death of At in monoculture and that the rescue in co-culture was possible because Ct reduced LA concentration by consuming it. We tested this hypothesis by performing metabolomics analyses on the spent media in different culture conditions. We collected the spent supernatants of At and Ct in mono-culture, the co-culture of At and Ct, and the bacteria-free samples at the end of the growth assay in all tested LA concentrations (0%, 0.1% and 0.75%). Our expectation was to identify compounds that correlated positively with At's death at high LA concentration (in the At monoculture samples), as well as with At's survival at low LA concentration (in the At-Ct co-culture samples). In collaboration with the GC-MS expert of our department, Dr. Andrew Quinn, we developed a protocol to extract LA and its putative byproducts as target metabolites from the collected spent supernatants and to further process it for GC-MS analysis (see Chapter 2). We used the Biocatalysis/Biodegradation Database developed by eawag to obtain a prediction of the metabolic pathways steps that LA could undergo to by microbial degradation. We assembled all the pathways ramifications and we extracted all the compounds to include them in our list of putative LA byproducts to hopefully identify by GS-MS (Fig.SI5).

We wanted to quantify how much LA was still present at the end of the growth assay and to compare LA abundance among the different culture conditions. We expected that the quantification and comparison of LA across conditions would have given us a clear picture of the LA-dependent dynamics.

To our disappointment, this was not possible. We were not able to detect the specific signal of LA in any of our samples, not even in the bacteria-free ones, in which no biological degradation was possible due to the absence of cells. Instead of a single LA signal, we detected multiple signals corresponding to multiple putative byproducts of LA degradation across all samples, including in the bacteria-free controls (Fig. 4.3). Our interpretation of this result was that LA underwent spontaneous degradation through oxidation due to exposure to light, oxygen and metals [4, 55]. As a consequence of this spontaneous oxidation, a wide panel of byproducts were released that we were able to identify ([95], see Supplementary File "GC-MS compounds")

This unexpected result motivated us to revisit our initial hypothesis. Since no LA was present at the end of the growth assay in any of the culture conditions, but it was rather transformed into several byproducts, we considered two different transformation processes:

1. the first one was the biological degradation performed by bacteria that attacked the LA molecule and split it into byproducts as part of the consumption process. This process requires the presence of bacteria in the sample and would not occur in the bacteria-free samples.

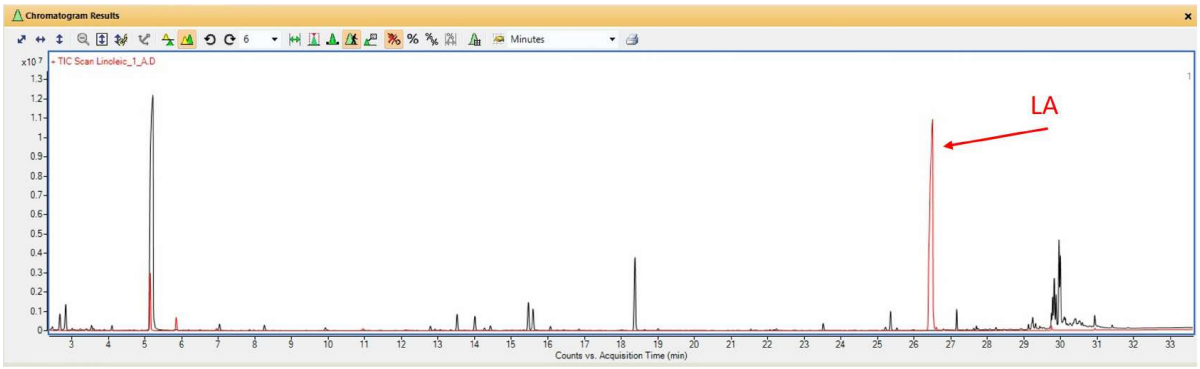


Figure 4.3 – Result of GC-MS run of two different type of samples. In red there is fresh LA 0.75% medium prepared on the same day on which metabolite extraction was performed. In black there is one replicate of bacteria-free sample in LA 0.75% collected at the end of the 8 days growth assay and frozen at −80°C. Red arrow points at the signal corresponding to LA. This signal was present only in the fresh LA 0.75% sample and it was not found in the collected bacteria-free sample LA 0.75%.

2. the second one was the independent spontaneous oxidation, an underlying process that was happening in all samples regardless of the presence of bacteria and rather additionally to the presence of bacteria. Since the bacteria-free samples were not exempt from light and oxygen exposure. This second process could explain why we could not even detect LA in the samples untouched by bacteria.

Both processes would result in the accumulation of several byproducts. At this point, we reformulated our hypothesis and we speculated that maybe the accumulation of one specific byproduct was responsible for the toxicity in the system. Our new hypothesis predicts that we would find this hypothetical toxic byproduct at high concentration in both At in mono-culture and bacteria-free samples, as it accumulated as LA spontaneously oxidized. On the other hand, we expected the same byproduct to be absent or at least less abundant in Ct's mono-culture and the co-culture of At and Ct, as it could be consumed by Ct. For the second scenario, we assumed that Ct was capable of degrading the hypothetical toxic byproduct, regardless of whether its release in the system was due to both the spontaneous oxidation or biological degradation: as soon as this compound appeared in the system, Ct would reduce it through consumption or degradation.

With this in mind, we went back to the GC-MS data to try to identify a pattern of variation of abundance of specific byproduct(s) across culture conditions that could support our new hypothesis. Additionally, we were curious to see if we could detect distinct patterns of degradation and/or accumulation of compounds among the mono- and co-cultures that could hint to species-specific consumption of LA byproducts or even cross-feeding. For example, we were wondering if we could identify a specific byproduct that would be present in Ct's mono-cultures (both at 0.1% and 0.75% LA), but absent in the co-cultures: this situation would have implied that At had consumed that byproduct, as cross-feeding between Ct and At might also explain their positive interaction.

Unfortunately, this analysis was unsuccessful. Despite our efforts and the help of Dr. Andrew Quinn in analyzing our GC-MS data, we had to conclude that it was not possible to identify any patterns that could

be linked to a specific culture condition, neither for the type/number of identified byproducts, nor for their detected abundances. We found no stable pattern of signals across condition, not even across different LA concentrations, as if the starting LA concentration had no impact on byproduct abundance. Instead, our data revealed rather scattered signals that were very hard to decipher. We could exclude that this unpredictable and inexplicable variability was due to sporadic mistakes during the different steps of metabolite extraction and running the GC-MS because samples were processed by randomly assigning them to different batches over different days. There are multiple ways to perform quality checks and to verify if there is a batch effect in GC-MS data. One possibility is to add a known amount of a compound that is surely absent in the medium. This compound acts as an internal standard (ISTD) and is useful to check the quality of each sample, by comparing the quality of samples across the same batch and different batches, and to make quantitative comparisons between different compounds within the same sample and across different samples (see Chapter 2 for more detailed explanation). When we performed the ISTD-based quality check, we found that in some cases there was indeed a batch effect. However, this partial batch-effect was not enough to explain the confusion across our data. We put a lot of effort into the processing of these GC-MS data, but eventually we concluded that there was something intrinsically wrong in our samples that prevented a clean metabolite extraction. As we continued to investigate the origin of toxicity in our system, it became clear what was the source of disturbance in our samples. This will be addressed in the following paragraphs of this chapter.

To conclude this section, our GC-MS data revealed no pattern linked to the presence/absence of specific metabolites/LA byproducts that could explain the toxicity or the interactions across the nutrient-toxin gradient. Even if the quality of our data did not provide any evidence for cross-feeding between Ct and At, this does not mean that we can exclude this possibility. We simply conclude that our approach was not successful in detecting metabolic exchanges between bacteria in the form of release and consumption of specific LA degradation byproducts.

4.2.5 ROS accumulates upon oxidation of LA and causes death of At unless Ct is present

We moved on from the GC-MS data and tried to think of other ways to understand the source of toxicity. The very relevant result that we gained from the GC-MS analysis was that LA was undergoing spontaneous oxidation due to light and oxygen exposure, regardless of the presence of bacteria. Based on the literature, we hypothesized that spontaneous oxidation of LA might release reactive oxygen species (ROS) in addition to the byproducts that we detected by GC-MS [88, 84, 16, 77]. Accordingly, we used a colorimetric assay to test for the presence of ROS in our system. The chosen assay was the Thiobarbituric Acid Reactive Substances (TBARS) assay to indirectly assess the presence of ROS-induced oxidative stress, as described in [4]. We focused on the LA 0.75% medium and performed the TBARS assay over an eight-day long growth experiment on At and Ct monocultures, the co-culture of At and Ct, and the bacteria-free samples. In parallel to the TBARS assay, we quantified CFUs to follow bacterial abundance.

Indeed, we found that ROS accumulated over time in both the monoculture of At and the bacteria-free samples (Fig. 4.4 panels E-F, dotted and diamond lines). The accumulation of ROS in the bacteria-free samples supported the idea of a chemical reaction leading to ROS production, such as spontaneous oxidation, that did not depend on bacterial presence. The time-course of ROS accumulation in At monoculture also

matched the death of At, supporting our initial idea that At in monoculture can endure toxicity only up to a still not quantified threshold (comparison of CFU and ROS values, Fig. 4.4, panels B and E).

Remarkably, ROS were significantly less abundant in Ct monoculture and in the co-culture than in the mono-culture of At and the bacteria-free samples (Fig. 4.4, panels E and F. Tukey multiple pairwise-comparisons, At vs. Ct p = 0.00016, At vs. At-Ct p = 0.00016, C- vs. Ct p = 5.01×10^{-7}, C- vs. At-Ct p = 5.01×10^{-7}). The lack of ROS accumulation whenever Ct was present led us to hypothesize that Ct neutralizes ROS, reducing environmental toxicity and rescuing At in co-culture, allowing it to survive and grow. These results also showed that toxicity was not caused by the increase in LA concentration itself, but rather by the accumulation of ROS that was greater at high LA concentration. At 0.1% LA, the TBARS assay revealed that ROS abundance is lower than in 0.75% LA (Tukey multiple pairwise-comparisons, At 0.1% vs. At 0.75% p = 0.00059, Ct 0.1% vs. Ct 0.75% p = 0.0024, At-Ct 0.1% vs. At-Ct 0.75% p = 0.0016, C-0.1% vs. C-0.75% p = 0.0011, see Supplementary Fig. SI1, panels A to F).

Incidentally, the presence of ROS also explains why we had such a strong disturbance in our GC-MS data: reactive species can interfere with the chemical reactions of the derivatization during the steps of metabolite extraction, preventing an accurate sample preparation [55].

Following these results, we adapted our model accordingly, leaving LA as a nutrient exclusively and adding ROS as an additional toxic compound that was generated through spontaneous LA oxidation (Fig. 4.4C). I will refer to this updated version of the model as Model 2. The predictions of Model 2 fit our data significantly better, even capturing the hump-shape of the At monoculture curve at 0.75% LA (Fig. 4.2, panels A and C).

4.2.6 Antioxidant rescues At and reverses the interaction between At and Ct in co-culture

We used Model 2 to explore what would happen if we removed the toxicity by setting the production of ROS to 0 and leaving LA strictly as a nutrient. This led to two predictions: in the absence of toxicity, (i) at 0.75% LA, At should survive even in monoculture and reach a higher population size compared to 0.1% LA, and (ii) we should observe competition between At and Ct even at 0.75% LA (Fig 4.5, panel A). To test these predictions, we first added an antioxidant molecule to our the bacteria-free 0.75% LA to verify whether its presence would decrease ROS concentration. We chose to use tert-butylhydroquinone (TBHQ) for its antioxidant properties [39, 90] at final concentration of 15μM dissolved in DMSO. We chose a concentration that we verified would not inhibit bacterial growth. In addition to the bacteria-free samples, we also added TBHQ to At and Ct monocultures, and the co-culture of At and Ct. We supplemented TBHQ at the beginning of the growth assay and every 24 hours to have a regular input of fresh antioxidant, mimicking continuous ROS neutralization, as we supposed was done by Ct. In parallel, we added to an equal set of new sample only DMSO without TBHQ, to be sure that whichever effect that we would have detected would have been due to TBHQ action itself and not to the solvent that TBHQ was dissolved in.

We found that TBHQ successfully decreased ROS concentration in all tested culture conditions compared to their value in standard 0.75% LA (comparison between Fig. 4.4 panels E-F and Fig. 4.5 panels E-F). In support of prediction (i), adding TBHQ rescued At in monoculture, allowing it to reach a significantly higher population size in 0.75% LA + TBHQ compared to 0.1% LA (t-test with Benjamini-Hochber correction, At 0.1% LA vs. At 0.75% LA + TBHQ p = 0.013). We suppose that in this ROS-free condition

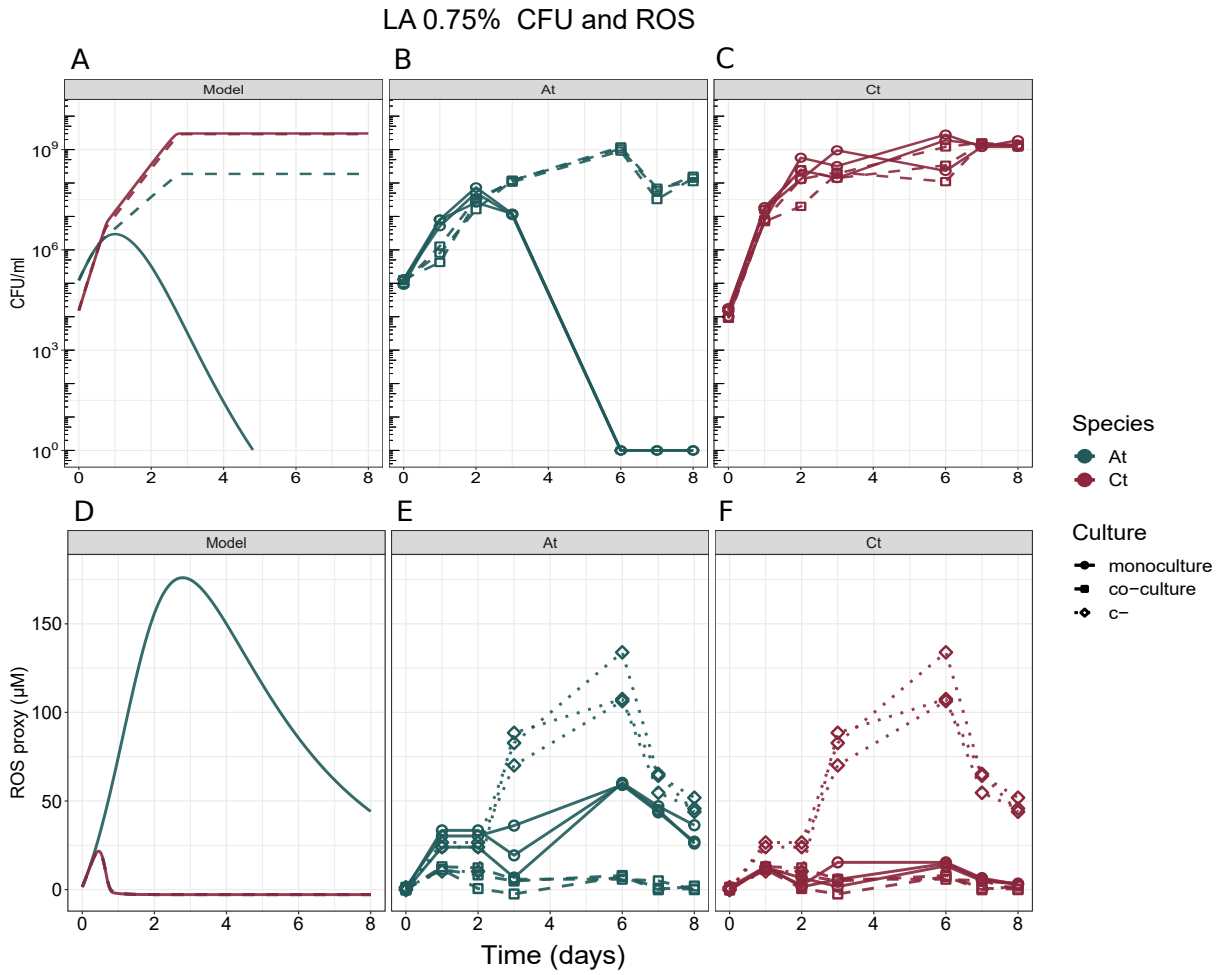


Figure 4.4 – ROS abundance over time and comparison with correspondent bacterial growth. Upper row shows CFU/ml data (y-axis), bottom row shows the variation of a ROS-proxy abundance (y-axis) over time (x-axis). Columns show different types of culture: first column shows only model data resulting from Model 2 (monocultures and co-cultures of both At and Ct, panels A and D), second column shows only At data (monoculture and co-culture, panels B and E), third column shows only Ct data (monoculture and co-culture, panels C and F). At is in green, Ct is in red (three technical replicates per condition, graphs show the results of one biologically independent experiment). Squares-solid lines are monoculture data, round-dashed lines are co-culture data, diamond-dotted lines are bacteria-free data (c-, data available only for bottom row on ROS, since the CFU data of c- samples was always 0. Please note that c- data are the same for both C and D plots, since they were controls for the same respective experiment). There is correspondence between At death and high ROS abundance (panels B and E). ROS concentration is always low in presence of Ct (panel F): this corresponds to Ct growth in monoculture and in co-culture (panel C). ROS removal in co-culture allowed rescue and growth of At too (panels B and E).

At could exploit the greater availability of LA as a nutrient. And as per prediction (ii), in 0.75% LA + TBHQ At grew significantly worse in the presence of Ct than in monoculture, meaning that interaction between At and Ct switched from facilitation to competition in the absence of toxicity. DMSO data showed that the solvent itself did not have any effect neither on ROS abundance variation over time nor on At potential survival (t-test with Benjamini-Hochber correction, C- LA 0.75% vs. C- LA 0.75% + TBHQ p = 0.9782, see Supplementary A Fig. SI3 and Fig. SI4).

Overall, both model predictions were confirmed, demonstrating that ROS were responsible for the presence of toxicity in our LA system and that we could shape the interaction between At and Ct by manipulating the level of toxicity in the environment.

4.3 Summary

We tested the SGH in a simple model system, where changing the concentration of a single compound (linoleic acid, LA) switched the interaction between two bacterial species from competition to facilitation. We found that the LA gradient we created translated to a gradient of nutrients as well as toxicity, as ROS were released upon LA oxidation and ROS production increased with LA concentration.

The switch in interaction sign happened because the bacteria exhibited two interaction types whose net effect added up differently depending on LA concentration: at low concentration, competition for the single nutrient was prevalent, while at high concentration, facilitation dominated because of the increase in toxicity upon ROS accumulation, despite the underlying presence of competition. Once we had understood the molecular mechanism behind toxicity, we were able to manipulate it by adding an antioxidant to the medium. The presence of the antioxidant neutralized the toxic ROS in all culture conditions, mimicking the presence of Ct. The toxicity removal allowed a greater growth of At in monoculture compared to its abundance in the co-culture under the same medium condition, switching the interaction sign back from positive to negative and revealing that the two species still compete at high LA concentration. This result demonstrates that toxicity can be crucial in determining whether the net observed interaction is facilitative or competitive.

The understanding of the ROS mechanism in providing toxicity also allowed us to greatly improve the accuracy and predictive efficiency of our mathematical models: the use of both Model 1 and Model 2 in predicting how we can transition from an interaction network to understanding the conditions for coexistence will be discussed in Chapter 5. We discuss the implications of this work in more detail in Chapter 6.

4.4 Author contributions

Rita Di Martino and Sara Mitri designed research. Andrew Quinn designed the protocol for metabolite extraction and GC-MS. Rita Di Martino performed metabolite extraction, GC-MS runs and all other experiments described in this chapter; GC-MS data were processed by Rita Di Martino and Andrew Quinn. Aurore Picot built and analyzed the models. Rita Di Martino and Sara Mitri helped Aurore Picot in implementing the models according to lab experiment results through scientific discussion. Rita Di Martino and Sara Mitri analyzed the experimental data described in this chapter.

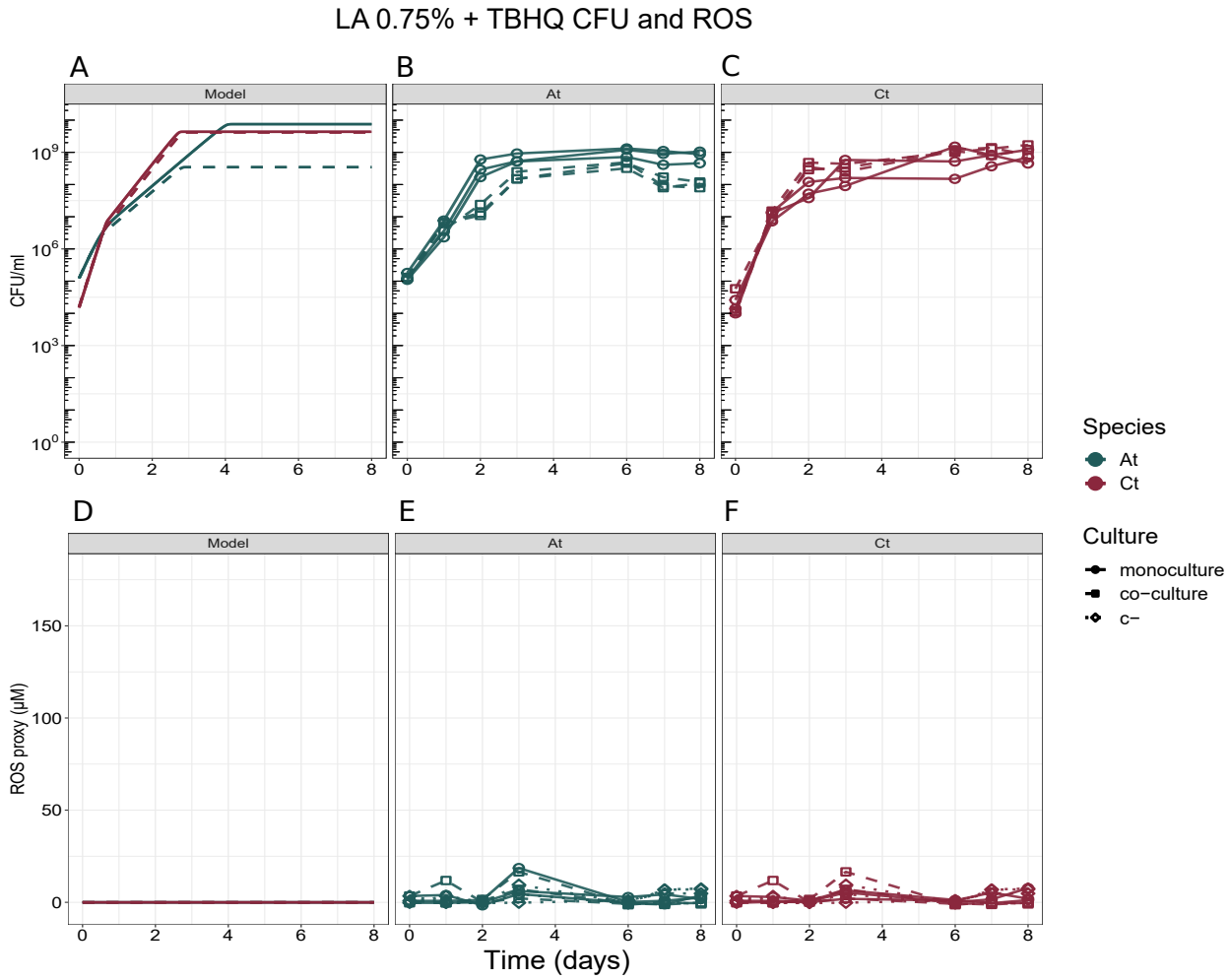


Figure 4.5 – ROS abundance over time and comparison with correspondent bacterial growth in presence of TBHQ. Upper row shows CFU/ml data (y-axis), bottom row shows the variation of a ROS-proxy abundance (y-axis) over time (x-axis). At is in green, Ct is in red (three technical replicates per condition, graphs show the results of one biologically independent experiment). Columns show different types of culture: first column shows only model data resulting from Model 2 (monocultures and co-cultures of both At and Ct, panels A and D), second column shows only At data (monoculture and co-culture, panels B and E), third column shows only Ct data (monoculture and co-culture, panels C and F). Squares-solid lines are monoculture data, round-dashed lines are co-culture data, diamond-dotted lines are bacteria-free data (c-, data available only for bottom row on ROS, since the CFU data of c- samples was always 0. Please note that c- data are the same for both C and D plots, since they were controls for the same respective experiment). The model data are extrapolated from Model 2 predictions when imposing ROS abundance = 0, as if it were cancelled by the presence of a simulated antioxidant. TBHQ supplementation rescue At in the monoculture (panel B), unmasking that actually it could grow more alone without toxicity than the amount that it grew in co-culture with Ct. TBHQ supplementation keeps ROS abundance low in all tested conditions (panels E and F).

Chapter 5

Coexistence between bacteria in a controlled nutrient-toxin gradient

5.1 Introduction

In Chapter 4, we discussed how a gradient of nutrient and toxicity can shape interaction dynamics between two species and we found evidences supporting SGH in our system,. The mechanistic understanding of the role of ROS in inducing toxicity allowed us not only to have better insight of the molecular basis of the observed interactions, but also to improve our mathematical model. We were able to decouple toxicity from LA and we gained more predictive power and accuracy with the transition from Model 1 to Model 2.

So far, we focused on understanding the interaction dynamics depending on nutrient-toxicity gradient. As next step, we decided to expand our horizon and to explore how our findings on gradient-depending interactions could affect the possibilities of coexistence of the two species. Our awareness on the ROS mechanism gave us a solid base to make predictions on coexistence, because often the lack of mechanistic explanation behind facilitation prevents to predict whether species would co-exist, while having explicit mediators of interactions (e.g. metabolites, waste-products or toxic compounds) helps to better predict coexistence ([83]). Moreover, our current knowledge on coexistence focuses on competition as major force and poor is known on the role of facilitation ([32]). We decided to explore coexistence in both Model 1 and Model 2 to then make comparisons between obtained results. As a reminder, in Model 1 toxicity depended on LA concentration, while in Model 2 it emerged on ROS accumulation, and LA acted exclusively as a nutrient. While this difference may seem an implementation detail, we know from early theoretical work that the separation into one resource and one inhibitor can promote coexistence by increasing the number of limiting factors [65, 67, 76]. We know from literature that it is possible to have coexistence on a single compound over serial dilutions under specific metabolic tradeoffs ([100, 74]). The conditions of dilution and resource concentration to obtain coexistence are expected to be specific, thus, increasing number of limiting factors is expected to increase the this theoretically possible coexistence ([76, 59, 94]). Here, the presence of ROS as distinct inhibitor on top of the nutrient LA increased the number of limiting variables. In this chapter, we tested how the explicit presence or absence of ROS in the two models reflected into coexistence probability in the short-term and in the long-term. Additionally, we performed a short transfer experiment to test our models findings in the lab.

5.2 Results

5.2.1 Separation of nutrient and toxic compounds makes short-term coexistence between At and Ct more likely

To explore coexistence probability between At and Ct, we first extended both Model 1 and Model 2 to simulate a transfer experiment. We simulated the growth of both At and Ct in monoculture and At-Ct coculture for 72 hours. After 72 hours, bacteria were diluted into fresh medium and regrown for another cycle of 72 hours. We simulated five transfers and we explored a wide range of LA concentrations and dilution rates to discover for how long the persistence of the two species was possible. We can analyze the results of the simulations in two parts: short-term coexistence (persistence) and long-term coexistence (equilibrium). We found that both Model 1 and Model 2 allowed the possibility of coexistence in the short-term, but Model 2 predicted a much larger parameter range for coexistence (Fig. 5.1, panels A-B). However, long-term stable coexistence was not possible in either of the models (see Supplementary Fig. SI2).

5.2.2 Experimental data support short-term coexistence of At and Ct

Next, we tested the prediction of short-term coexistence experimentally. Similarly to the mathematical model design, we grew both At and Ct monocultures and At-Ct coculture for 72 hours in both 0.1% LA and 0.75% LA. After 72 hours, we transferred a 1% aliquot of each culture into fresh medium and we grew bacteria for another cycle of 72 hours. We performed five transfers as a short-term timeline. At the end of the experiment, we compared the final population size (transfer 5) to the population size achieved after 72 hours of growth prior to transfer 1 to see if over time there were variations in the cell number achieved over the 72 hours growth period. Significant variations in these population sizes could indicate a tendency towards extinction or improvement in growth (depending on the sign of the putative variation). In co-culture in 0.1% LA, we found a significant variation for Ct compared to the beginning of the transfer experiment (t-test with Benjamini-Hochber correction, $p = 0.0029$), but there was no significant change for At (t-test, $p = 0.30$). Moreover, in 0.1% LA, competition was still evident between At and Ct, as At in mono-culture maintained a significantly higher population size than in the presence of Ct (t-test, At 0.1% vs. At-Ct 0.1%, $p = 9.37 * 10^{-}14$) (Fig. 5.1C). Both the increase of Ct in 0.1% LA co-culture and the competition towards At suggested coexistence in this short time scale, but most likely it would not be sustained over time, as assessed by our mathematical model.

In 0.75% LA, we observed the extinction of At monoculture as we expected, but we found no significant variation neither for At nor for Ct in the co-culture. Although we have no measure of ROS concentration over this experiment, we know from former time-point measurements that 72 hours is enough time for ROS to accumulate in the system, even if it is not yet abundant enough to kill At. However, the presence of ROS in the system at this early stage is enough to fit the conditions assumed by Model 2.

Overall, our experimental data shows that coexistence between At and Ct is possible in the short-term at both low and high LA concentrations despite the presence of negative interactions. More specifically, our results support the validity of Model 2, since it was the model that predicted more conditions under which short-term coexistence at high LA concentration was possible.

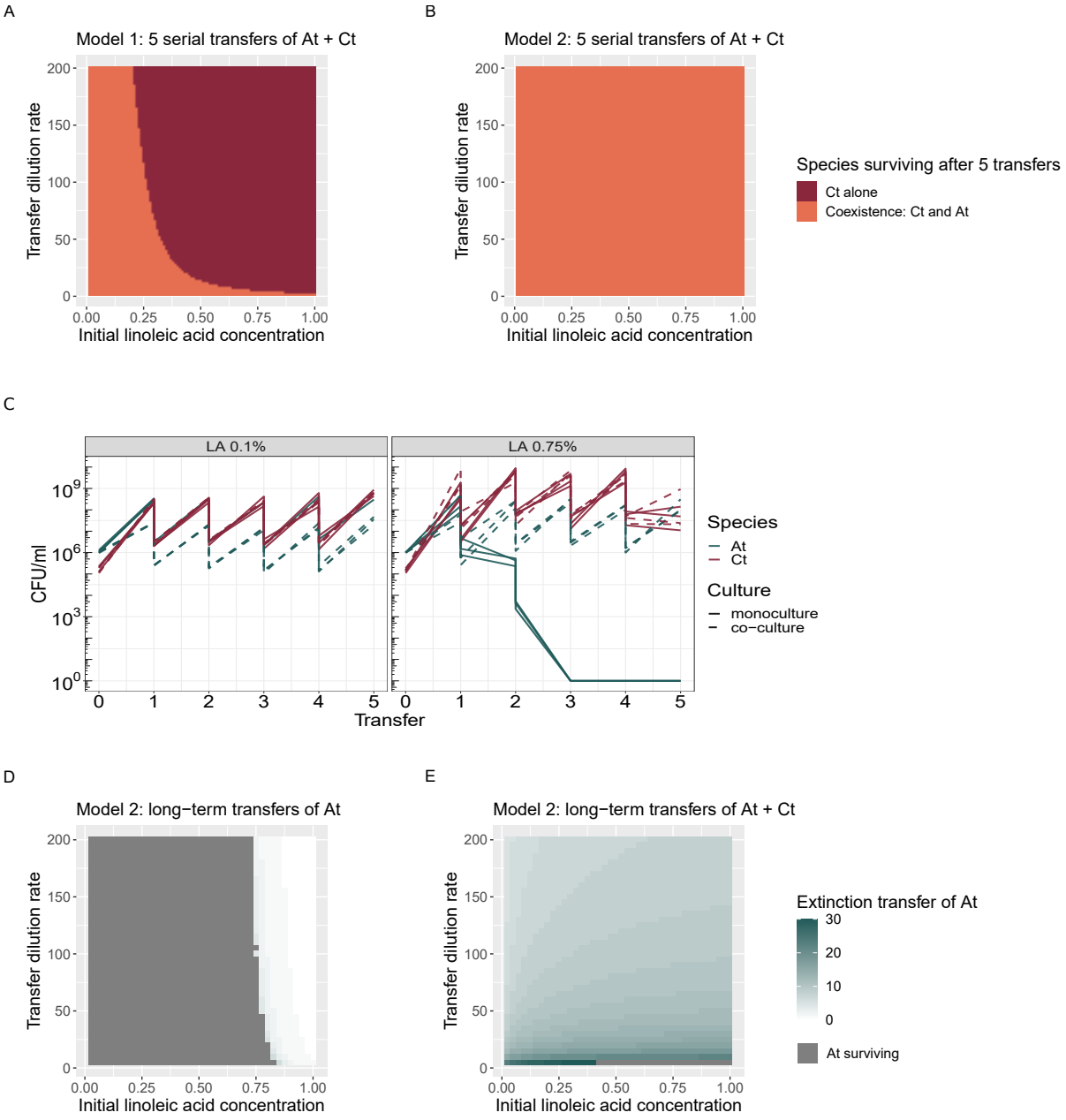


Figure 5.1 – Co-existence experiments and models. Prediction of short-term coexistence between At and Ct according to Model 1 (panel A) and Model 2 (panel B): both models allow co-existence, but the parameter space in which this is possible is larger in Model 2 (panel B, more orange area representing coexistence compared to red area of Ct surviving alone. 5-Transfer experiment of At and Ct in mono- and co-culture at both 0.1% LA and 0.75% LA (panel C). Y-axis represents population size expressed as CFU/ml, x-axis represents the number of transfers. At is in green, Ct is in red (three technical replicates per condition, graphs show the results of one biologically independent experiment). At monoculture goes extinct as expected in 0.75% LA, but co-culture is maintained at both LA concentrations as predicted best by Model 2. Model 2 predicts a large space for long-term survival of At mono-culture at low LA concentration (panel D), but Ct prevails at the same concentration in the co-culture (panel E). At high LA concentration, At monoculture dies as expected, while in the co-culture it goes extinct after a higher number of transfers than compared to the number of transfers that led to extinction in monoculture, meaning that eventually there was facilitation of Ct towards At.

5.2.3 The Stress-Gradient Hypothesis holds in simulations predicting long-term dynamics

Lastly, we focused only on Model 2 and we simulated the outcome of At monoculture and At-Ct co-culture in the long term. We wanted to classify the net effect of Ct on At across these gradients. To do so, we measured the time (i.e. number of transfer) at which At went extinct in monoculture and compared it to its extinction time when in co-culture with Ct. If At survived longer in the presence of Ct, then the net effect of Ct towards At was positive (facilitation). Vice versa, if At went extinct earlier in the presence of Ct, then the effect of Ct towards At was negative and competition was the prevailing interaction. We found that At monoculture always survived in the long-term at low initial LA concentrations (Fig. 5.1, panel D). At the same LA concentration, At went extinct when in co-culture with Ct, so we concluded that the absence of coexistence must be due to competitive exclusion by Ct (Fig. 5.1, panel E).

We obtained different results at high LA concentration. As expected, At did not survive in monoculture because of the accumulation of ROS toxicity included in Model 2. In the co-culture, At eventually it went extinct in this condition too, but the number of transfers required for it to go extinct is higher in the presence of Ct compared to At monoculture. This result means that the interaction of Ct towards At is facilitative, since Ct improves At survival in the long-term at high LA concentration.

Overall, the outcome of our simulations fits with the SGH in the long-term: competitive exclusion was the dominant interaction at low LA concentration, while facilitation (as per lengthening At survival) was observed at high LA concentration.

5.2.4 Removing ROS toxicity or Ct detoxification capacity does not change At survival in the long-term

In Chapter 4, we used an antioxidant compound to neutralize ROS toxicity and we found that this led to reversing the interaction from facilitation to competition at high LA concentration. In that part of the thesis, we also imposed the parameter for ROS toxicity equal to 0 in Model 2: our goal was to mimic the absence of ROS, not as if it were due to Ct detoxification, but as if it were removed by an external antioxidant as well, and to evaluate how that would have affected interaction dynamics. Here, we applied the same idea to the model transfer experiment to see if the removal of toxicity would affect the previously observed coexistence pattern. We found that setting ROS to 0 allowed At to survive in monoculture at any condition, as opposed to what we previously observed (comparison between Fig. 5.1 panel D and Fig. 5.2 panel A). However, the removal of toxicity did not affect the outcome of the At-Ct co-culture: since in this new condition we only expect competition for the nutrient source and no need for detoxification since ROS = 0, At went extinct and only Ct persisted as previously observed (comparison between Fig. 5.1 panel E and Fig. 5.2 panel B).

Lastly, we tested what would happen if Ct were unable to detoxify ROS. We set the detoxification parameter equal to 0, but left Ct's death parameter unchanged: this decision implied that ROS would accumulate over time in the system even in the presence of Ct, but the only sensitive species would still be At, since it is the one that has the death parameter related to ROS concentration, while Ct did not. The At monoculture result was exactly the same as the one observed before, since there were no modification in At parameters that could cause a change of result: when LA concentration is low, At could still cope with ROS toxicity, but it died at higher concentrations (comparison between Fig. 5.1 panel D and Fig. 5.2 panel C). In At-Ct

co-culture, we found competition between At and Ct at low LA concentration, resulting in an earlier extinction time for At compared to what was observed before (comparison between Fig. 5.1 panel E and Fig. 5.2 panel D). Finally, there was no facilitation effect at high LA concentrations since there was no detoxification by Ct, so At went extinct faster here as well (comparison between Fig. 5.1 panel E and Fig. 5.2 panel D).

5.3 Summary

We applied both Model 1 and Model 2 to predict coexistence possibilities of At and Ct in both short-term and long term. Both models predicted that the two species should coexist in the short term, but the results that we obtained from the experimental validation through transfer series were more consistent with the predictions of Model 2. Finally, we found no stable coexistence in the long-term. However, the SGH still recapitulates our results well, because competitive exclusion occurred at low LA concentrations, while at higher LA concentrations, facilitation explains the delayed extinction of At. The observed facilitation was a result of the detoxification performed by Ct: despite the lack of specific ROS measures over the transfer experiment, we can still assume that Ct was reducing ROS load in the system and that the consequent improvement of environmental conditions extended At's survival.

In Chapter 4, we used an antioxidant compound to neutralize ROS toxicity and here we ran a simulation to reproduce a similar scenario in which ROS is not present anymore. As a follow up of the experimental transfer experiment that we performed and these last theoretical transfer experiments, we believe that it would be interesting to test if we could obtain the same interaction switch if adding the antioxidant during a new transfer experiment. Without these experimental data, we prefer not to speculate whether a putative switch from positive to negative interaction would affect the possibilities of coexistence in the short term: after all, we already verified in the formerly described transfer experiment that short-term coexistence was possible despite the presence of competition. However, we could use the additional experimental data to improve and corroborate what we saw from the long-term modelling in absence of ROS.

5.4 Author contributions

Rita Di Martino and Sara Mitri designed research. Andrew Quinn designed the protocol for metabolite extraction and GC-MS. Rita Di Martino performed metabolite extraction, GC-MS runs and all other experiments described in this chapter; GC-MS data were processed by Rita Di Martino and Andrew Quinn. Aurore Picot built and analyzed the models. Rita Di Martino and Sara Mitri helped Aurore Picot in implementing the models according to lab experiment results through scientific discussion. Rita Di Martino and Sara Mitri analyzed the experimental data described in this chapter.

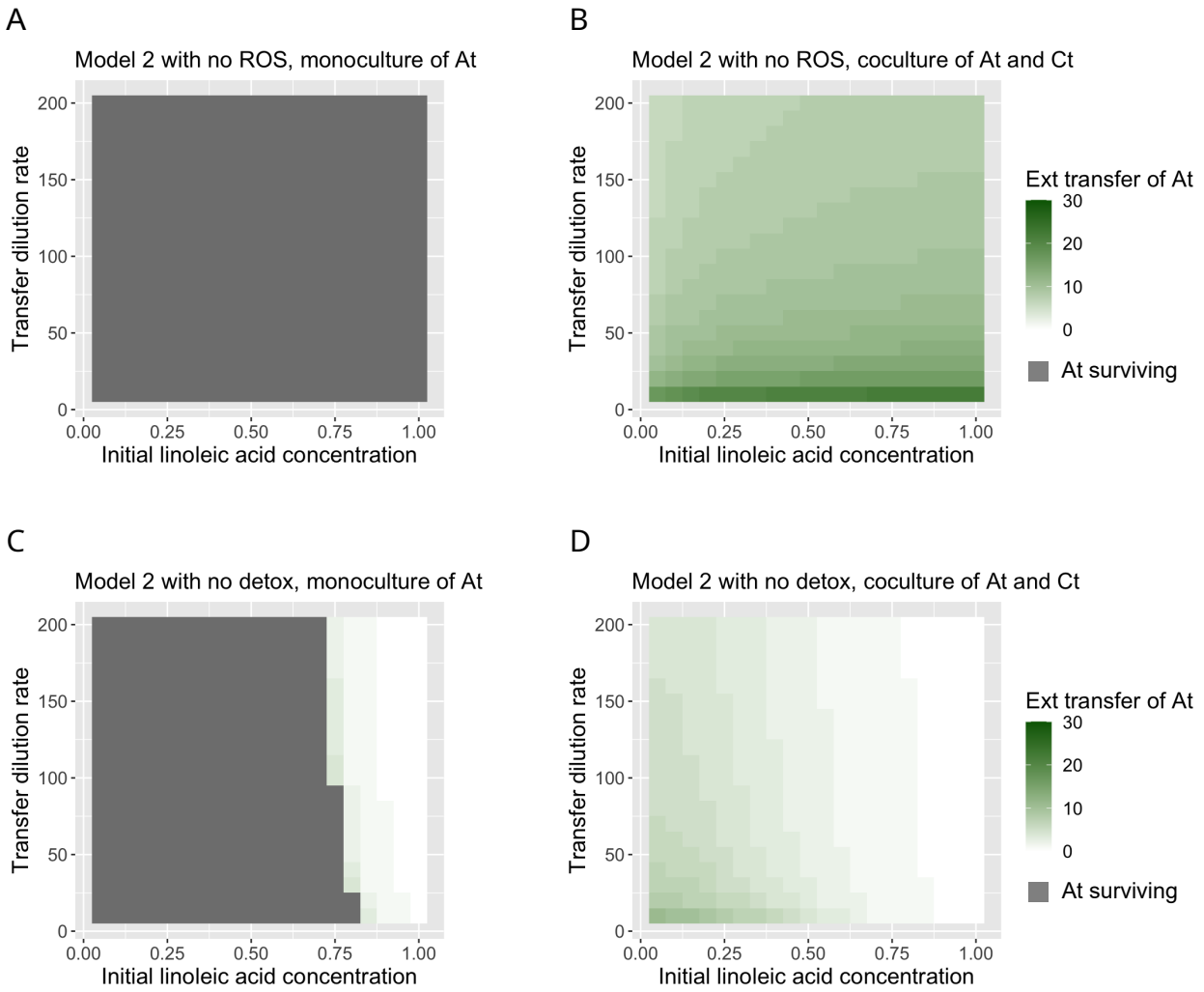


Figure 5.2 – Model 2 predictions on long-term coexistence in absence of ROS (ROS equal to 0, panels A-B) and in absence of Ct detoxifying capacity (panels C-D). Setting ROS equal to 0 allowed At survival in monoculture at any condition, as opposed to what we previously observed in Fig. 5.1 panel D, but Ct is still the only species prevailing in the co-culture (panel B). Setting Ct detoxification capacity equal to 0 gave the same results for At monoculture as seen previously in Fig. 5.1 panel D (panel C). In At-Ct co-culture, competition between At and Ct at low LA concentration results in earlier extinction time for At compared to what observed in Fig. 5.1 panel E (panel D). In At-Ct co-culture at high concentration At goes extinct faster than what seen in Fig. 5.1 panel E because of a lack of facilitation since Ct cannot degrade ROS (panel D, detoxification capacity = 0).

Chapter 6

Discussion

6.1 General summary

The general idea of this PhD work was developed around my curiosity on how the environment influences interactions and coexistence dynamics between microbial community members. Particularly, I was interested in exploring the effect of more or less permissive environmental conditions in promoting the establishment of different types of interaction. The questions that inspired the development of this PhD work were many: how does stress operate in influencing interactions? If we expect to have a SGH scenario (more permissive conditions increase negative interactions, more stressful conditions increase positive interactions), which are the sources of stress in which this scenario can happen? How do bacteria respond if we remove the stress source? Can we use observed interactions to predict longer-term coexistence patterns? And, more on a technical note, how much can we simplify a system without losing its key features?

To answer these questions, we chose a small synthetic community as a model system. The four species of this community had already been described for their ability to grow in toxic MWF waste and to reduce its pollution load [35, 34]. The same community was used by Piccardi et al. with the similar purpose of investigating to which extent interactions are context-dependent, but the complex nature of MWF as a growth environment prevented the authors from understanding the mechanisms ruling the toxicity-induced dynamics [87].

When we started to design this PhD project, we wanted to move past the complexity of MWF, so since the very beginning we decided to invest quite some time in setting up a system that would be simpler and more controlled, yet elaborate enough to give rise to different outcomes depending on how we were tuning environmental features. Our chemical knowledge of MWF was very limited: we knew that its fatty acids and mineral oils could be good substrates for bacterial growth, but we did not know which were the toxic components that provided MWF with biocidal properties and that killed many bacterial species. We examined MWF literature and we selected ten compounds that were representative of numerous MWF compositions [33, 18]. We tested a range of concentrations of every compound individually in monoculture growth assays to assess which compounds had nutrient properties and which ones were toxic. Ideally, we wanted to obtain a clear pattern of nutrient and toxic compounds to then assemble controlled media with defined amounts of both nutrients and toxic compounds to explore how the nutrient-toxic gradient could shape interactions.

The result of the compound screening revealed a much more diverse pattern, with some compounds displaying species-specific and concentration-dependent effects. We found that, among other compounds, linoleic acid (LA) had the dual behaviour of being nutrients or toxic to some species at increasing concentrations. This peculiarity made LA a good candidate for the preparation of a simple system, because we could tune the level of nutrient and toxicity just by varying the concentration of a single compound.

We found that there was a switch of interactions from competition towards facilitation as we increased LA concentration. We were able to identify ROS accumulation upon LA oxidation as the mechanism behind the increase in toxicity at a higher LA concentration. Understanding this mechanism allowed us to figure out why there was a switch in interactions depending on LA concentration: at low concentration, competition for the single nutrient was prevalent, while at high concentration, facilitation occurred because Ct reduced ROS abundance (4.4). However, this pattern happened at high LA concentration only in the presence of toxicity. We artificially removed toxicity from the high LA medium (0.75%) by adding an antioxidant (TBHQ) to neutralize ROS and we found that, at this altered high nutrient condition, interaction sign reverted back to negative because At and Ct competed for the sole nutrient source (as already observed at low LA concentration). All these findings fit with the SGH, supporting that this theory applies to microbial ecology too.

We implemented the ROS toxic component in our mathematical models and we saw that short term coexistence of the two species was possible at both low and high LA concentration. This hypothesis was also experimentally validated by performing a short transfer experiment. However, we still had only one single nutrient for our bacteria to feed on and this condition was not sufficient to grant stable long term coexistence in either of our models. Nonetheless, we found that representing toxicity as an independent entity from the nutrient part delayed the extinction of At, thus extending the coexistence time.

6.2 ROS induces the SGH pattern in microbial communities

Our work is not the first to explore how stress can affect microbial communities and their interactions. Hernandez et al. focused on how decreasing soil moisture at increasing altitude levels impacts the resident soil microbiome [48]. They analysed community composition of soil samples collected at different altitudes and found that the increasing stress induced by the lack of soil moisture caused a reduction in high taxon diversity of the community and the increase in relative abundance of more mutualistic partners. The results of this study showed how the SGH can explain the composition of natural communities over a stress gradient, but it did not explore how the very same community could respond to variation of stress exposure: for example, would a community sampled from a specific point across the stress gradient (point 1) and exposed to a different level of stress (point 2) rearrange its dynamics to be more similar to what happens within a resident community of point 2? Or the original dynamics would be strong enough to resist to the stress variation?

Fetzer et al. explored interaction dynamics and community functioning when several communities where exposed to increasing stress expressed as increasing benzoate composition [29]. These two works are only two examples of how known sources of stress for bacteria, such as low moisture and chemical stress, can be explored not only for their effect on the single cell but also for their broader impact on mul-

tispecies community. When we discovered that the stress source and interaction driver of our LA system was ROS accumulation, we were quite surprised to not find much information about how ROS can shape microbial community, even if ROS are very well characterized for their stress action [53, 103, 55] and it is known that bacteria can cope with them at the extracellular level too, thus allowing the possibility of creating accidental facilitation for surrounding species that would not be able to cope with ROS stress by themselves [20, 3]. With this remark, we would like to highlight that further exploration of the impact of ROS on community dynamics is needed to better understand the circumstances under which this stress source operates in the framework of SGH.

6.3 MWF is a good system to study the impact of toxicity on microbial communities

Two questions that I have been asked multiple times over these past years are: why do you use metal-working fluids (MWF) as a model system? Isn't it terribly hard to work with? The quick answer to the last question is yes, it is, but there is much more to it.

MWF is a human-designed fluid, so it is far from being a "natural" environment, but surely its composition was not decided to provide scientists with a challenging environment to study microbial interactions. Furthermore, the microbial species that colonize MWF spontaneously self-assemble into a community depending on features such as metal-content or their ability to be airborne [80, 72, 7], so microbial community composition is not artificially chosen by its operators. Hence, just because MWF is not something that we can find in nature, it does not mean that it cannot be considered "natural", and as such it is important to study its dynamics.

A considerable part of the hard work of establishing and characterizing a small microbial community that could grow in MWF was accomplished by former studies [35, 34, 36, 104, 87]. More specifically, the analysis performed by Piccardi et al. resulted in our understanding that this specific community was dominated by mostly positive interactions, something quite unusual, since previous work showed that competition is the prevalent interaction among bacterial species within a community [32, 79]. But the extra feature that makes MWF such a peculiar system and that explains why we observed positive interactions is its toxicity. The presence of biocides in MWF makes it a hostile environment: there are some species that can grow by themselves, but many others are not capable of surviving on their own and they can grow if in co-culture with others [87, 7]. The observed positive interactions are most likely due to accidental facilitation caused, for example, by detoxification performed by Ct [87]. It is worth remarking that the focal species in the four species community in MWF is the same focal species of our simpler LA system. We were wondering if Ct was absolving the same task of ROS-neutralizer in MWF as we observed in LA. For this reason, we performed the ROS-detection assay on MWF samples with and without Ct over three days incubation (since we saw that three days were enough to have evidences of ROS accumulation, Fig. 4.4). We could not detect any ROS accumulation over time (no significant variation between MWF samples and MM controls, data not shown), but we considered this possibility since we know that in MWF there are stabilizers that prevent fatty acid oxidation [18].

This phenomenon is of course not restricted solely to MWF, so understanding the nutrient-toxic depen-

dent dynamics among bacteria in this system can give us insight to understand what happens in other environments characterized by the presence of toxicity. For example, a similar facilitation dynamic was observed on mercury-polluted soil: a study showed that the presence of a *Cupriavidus metallidurans* strain could decrease mercury pollution load and lead to an increase in the biomass of nitrogen-cycle microorganisms [12]. Similarly, different antibiotics can target different species, but if the sensitive bacteria co-habit with species that can degrade the antibiotic, then the sensitive species can benefit from this cross-protection/detoxification and survive as well; this pattern was observed in very diverse environments, spanning from soil to polymicrobial infections [109, 96, 106].

Overall, we believe that the study of interaction networks in MWF provides more data on how bacterial interactions are affected by toxicity and shows how some facilitation patterns are not restricted to a specific antibiotic or harsh condition, but we think they are more general. Additionally, MWF proved to be another environment in which the interaction dynamics can be explained by the Stress Gradient Hypothesis (SGH).

6.4 Single-compound based systems can efficiently reproduce essential feature and patterns of their more complex original counterparts

Despite all the benefits of MWF that have been described in the former paragraph, it is indisputable that its complex chemical composition makes it very hard to disentangle the underlying mechanisms that allow interactions and coexistence. For this reason, we developed a single-compound based system using linoleic acid (LA). LA could provide both nutrients and toxicity depending on its concentration. With this setting we were able to assess the impact of more or less environmental stress on interspecies dynamics and compare it to what is predicted by the SGH.

Our setting falls in line with other studies that operated in similar ways to address the same question, but using different stress sources. In their work, Hesse et al. used copper pollution as environmental stressor and some of their results pointed in the same direction of the concepts that we explained 4 [49]. Indeed, they also found that interactions shift towards more positive interactions in higher copper stress, as predicted by the SGH. Another important similarity between their study and ours is that they also found that the net interaction sign shifted back to negative with competitive behaviour in the absence of toxic stress. This second result supports our finding that the presence of toxicity can be crucial in determining whether the net observed interaction is facilitative or competitive.

Another similar approach was adopted by Fetzer et al [29]. They set up a system in which the single compound provided was benzoate. Similarly to our LA system, benzoate was the only carbon source and increasing concentrations were stressful to bacteria [29]. They assembled more than 800 synthetic communities with variable numbers of species starting from a shared pool of 12 species and they exposed the communities to increasing benzoate concentrations. Although their aim was not to specifically test the SGH, their results on the changes in the interaction network and in community composition over a benzoate gradient support its predictions.

If I have to name one thing that we did not manage to explore as much as we wanted in our simple setting, that would be investigating whether there is any evidence for cross-feeding. As already discussed in Chapter 4, it was quite disappointing for us that the very same chemical features that were at the origin of toxicity (i.e.

ROS accumulation) prevented us from testing the presence of crossfeeding. We were expecting to observe crossfeeding on a single nutrient source, as shown in a former study that showed that metabolic facilitation is widespread over a multitude of bacterial communities growing only on glucose as a carbon source [38]. Crossfeeding can also be responsible for controlling competition and promoting coexistence among bacteria within the same community [38, 86]. Goldford et al. used a very simple media-filtering approach to first test the eventuality of coexistence. Although we also considered applying the same approach, the not-very-stable nature of the LA emulsion media out of shaking conditions did not allow a smooth filtering procedure, resulting in visible clumps being stuck on the filter. With these premises, it was hard for us to predict if we could have obtained filtered media with adequate consistency among replicates of the same conditions, so we decided to directly try with the GC-MS approach, as explained in details in Chapter 4.

However, this last mentioned unsuccessful experiment does not invalidate the efficacy of simple, single-compound systems in reproducing and investigating more complex circumstances. If anything, it provided us with further knowledge on which techniques are more or less suitable to investigate a specific question depending on the chemical composition of the environment of interest.

6.5 Bacterial growth in Minimal Medium (MM): the role of assimilable organic carbon (AOC)

In Chapter 3 paragraph 3.2.6 “Bacterial growth in Minimal Medium (MM)”, we discussed the unexpected phenomenon of bacterial growth in the Minimal Medium (MM) in which no carbon source was provided. We tried multiple strategies to first understand the cause of the growth in MM and second, to remove it. After multiple attempts, we could only acknowledge that there was a proportion of basal bacterial growth induced by the presence of unknown contaminants in our media.

The observation of bacterial growth in unexpected nutrient-deprived liquid conditions is not unknown in the field and the most common explanation for this is that bacteria can use the fraction of labile water-dissolved organic carbon to grow [105, 26, 5]. This fraction is called assimilable organic carbon (AOC) [44]. This phenomenon is particularly relevant for the evaluation of drinking water stability, and it is important to quantify the AOC amount for bio-safety reasons: traditional quantification methods are based on the comparison between the growth of an indicator species in the water sample of interest at defined times and temperature conditions and its growth in pure defined solution at the same time and temperature conditions [60, 56, 21, 63].

The presence of AOC can influence the precision of biodegradation assays performed at low substrate concentration. This is because in some systems, AOC concentration can be even higher than target substrate concentration, making it very complicated to assess the real source of the observed growth yield [47]. It is possible to estimate the AOC also from the observed bacterial growth in multiple no-carbon added controls, as done by Duygan et al [24]. Their primary goal was to analyze variation in lake community composition as a function of low chosen substrate concentrations and the AOC indication helped them to better understand which was the minimum substrate concentration necessary to have an effect on bacterial growth that was not due simply to AOC [24].

It is true that having an estimate of AOC quantity can help to predict how much bacterial biomass can

grow on it, but often predictions do not match reality: a study performed on *E.coli* showed that the effective bacterial growth was five time lower than the prediction made on the AOC quantity [108]. This is because AOC quantification are based on the growth of standard indicator species which can have very different features compared to the bacteria of interest: for example, the average cell size of *E.coli* in sterile freshwater is significantly bigger than the cell size of the indicator species and it needs more carbon per unit of cell, hence the origin of the overestimated prediction [108].

Returning back to the results shown in this thesis, it is quite clear at this point that all four species could grow on AOC in the compound-free MM medium, as shown in multiple experiments (see Supplementary Material, growth in MM as control for compound testing in Figs. SI6, SI7, SI8, SI9, SI10, SI11, SI12, SI13, SI14, SI15, SI16, SI17, SI18, SI19). However, the chosen concentration range for most compounds allowed us to obtain compound-dependent and concentration-dependent effects that were significantly different from what was observed in the MM and we always normalized our growth data with the observed growth in MM.

We included the presence of basal growth in our mathematical models too. The detailed explanation can be found in Supplementary Material A, but briefly we modelled an extra equation to predict the variation of the unknown nutrient concentration N over time. Then, we chose an arbitrary N concentration and used this value to estimate growth-related parameters in MM (growth rate, yield and half saturation constant). Lastly, we fixed these parameters and we included them in the estimation of the final parameters of At and Ct growth in monoculture in a range of LA concentrations.

For us, it was crucial to include a parameter that accounted for the basal growth in MM because we wanted to be sure that we were not neglecting something that could have been involved in establishing interaction dynamics and coexistence. Our experimental data showed that, even in a scenario where a single carbon source was provided, unpredicted contaminants can be present. At a first look, having an unknown additional mild nutrient source could sound as a failure in our will to design a system with a single chosen compound acting as a nutrient. However, we included this phenomenon in our mathematical models and still we saw that long-term coexistence between At and Ct was not possible, despite the unplanned extra complexity given by the additional unknown contaminant.

Overall, the growth on AOC taught us that unplanned and unexpected complexity can be present even in a carefully designed and controlled system. However, it is important to take into account this and to incorporate it in the best possible way in the result analysis and progression of work.

6.6 Future perspectives: what's next?

Understanding the ROS-dependent toxicity mechanism and how it affected interactions and coexistence is a good first answer to the questions we asked at the beginning of this Chapter, but it also paves the way to additional paths to explore.

Spatial structure

The role of spatial structure in community dynamics is a topic that has not been addressed in this thesis but it is of crucial relevance. Spatial structure represents a dominant trait in defining assembly and properties of many microbial communities, spanning from the dental plaque biofilm, to the structured communities

growing on river rocks and even affecting how the human gut microbiota interacts with its host [111, 42, 61]. Spatial structure reduces environmental homogeneity, because it impacts the diffusion of both nutrients and waste products, as opposed to liquid and shaking conditions. This results in the creation of several potentially diverse local environments. But how can we translate the exploration of the effects of toxicity to a structured system? Probably the simplest place to start from would be the analysis of mixed-species colony structure and composition when exposed to a gradient of toxicity. An easy way to test that could be to inoculate a drop containing a mixture of two species, one being sensitive and the other resistant to a diffusible stress of interest, on agar plates containing a gradient of that stress. A two-species drop test that followed a similar strategy was applied by Liu et al. when they tested how the presence or absence of a carcinogenic compound found in cigarettes affected interaction among the normal lung microbiota [71].

To link back to the system extensively discussed in this thesis, we could mix liquid cultures of At and Ct and put a drop of the mix on agar plates containing different ROS concentrations. The nutrient-toxicity balance in this situation could be achieved in different ways:

1. we could first test the feasibility of LA-supplemented agar plates;
2. if this proves difficult (for example if LA is not well miscible with agar or agarose), one could try something like H_2O_2 as source of ROS.

It most likely would not be possible to measure ROS in the same way as described in [4]. However, recent techniques were developed to perform real-time ROS measurements in carcinogenic tissues. These techniques are based on the use of amperometric sensors and can be used on both cells and live animals [114, 112, 30]. After some fine-tuning, it is likely that these techniques could be adapted to detect ROS through bacterial colonies.

My hypothesis for the outcome of this type of experiment would be to have more species clustering in low stress concentrations and more intermixed patterns of the two species in the high stress concentration: the sensitive species might need the physical proximity of the resistant partner to be able to cope with the oxidative stress and to establish the same positive interaction as we observed in liquid medium. However, this intermixing would be more likely to happen if bi-directional beneficial interactions occurred [81], since some work showed that competing species tend to create segregated spatial arrangement when exposed to stress [98].

However, the structured solid system might affect also the possibilities of both short- and long-term coexistence, resulting in a reduced coexistence time. My explanation for this scenario would be that in a solid system there is less nutrient circulation compared to a liquid system. This means that the species with higher growth rate would more likely outcompete faster the slower growing species since it would be more efficient in absorbing the surrounding nutrients, unless the two species establish a two-way cross-feeding interaction.

Changing the type of stress and comparing different stress-coping mechanisms

In Chapter 1 we introduced the definition of stress as any condition that negatively impacts the fitness of an organism. We found that the type of stress impacting At's survival in high LA concentration was oxidative stress caused by accumulation of ROS over time, but would we get the same results if we had a different

source of stress? On this note, more work could be done in exploring community robustness when exposed to different types of stress. The focus could be directed to how different stresses affect interaction balances, coexistence and, additionally, spatial arrangement. For example, let us consider a mixed community of At and Ct. We could imagine that, if this co-culture is exposed to a physical stress such as higher than optimal temperature, bacteria could react by producing a more pronounced biofilm as a protection mechanism. It has been documented both in marine environments and on river rock surfaces that there is a positive correlation between increased temperature and increased biofilm production [107, 57], so it would be intriguing to test which and how many stresses a community can endure and how it affects the internal dynamics: would there be a switch in which is the stress-reducing species? If two species have the ability to cope with two different types of stresses, can the simultaneous presence of both stresses influence the establishment of a longer-term coexistence even if nutrient conditions are limiting?

Increasing the number of compounds: steps towards a synthetic MWF medium

The concept of increasing the type of stress bring us straight to other variables that we could increase, such as the number of available compounds. We established this very simple one compound-two species system, so it would be interesting to build up from there and see how the increase of available compounds, whether they are nutrients or toxic, can affect the established dynamics. Moreover, it is still unclear what the source of toxicity is in MWF: as mentioned before, I performed the same ROS-detection assay that I did on LA media on MWF as well, but no ROS were detected over time (no significant variation between MWF samples and MM controls, data not shown). This result was not shocking because we know that in MWF there are stabilizers that prevent fatty acid oxidation [18]. However, maybe the incremental addition of more MWF-derived toxic compounds could elucidate more on this still open question.

The idea of combining multiple compounds deriving from MWF was something that I had already considered during the first half of my PhD. However, eventually we realized that this exploratory assembly of a synthetic MWF required more time and dedication that we expected, so we decided to take it aside from my PhD project and it became part of the PhD project of a fellow lab member, Andrea Dos Santos.

Introducing more species

In line with the scenario outlined in the last paragraph, one additional thing to test could be the impact of increasing number of species. The presence of one or more additional species could result in different outcomes: it could be an advantage if they contribute to ROS neutralization, but it could also simply result in stronger competition for the single nutrient source. It is hard to speculate what the outcome of this higher order interaction would be. We know from the literature that cross-feeding can spontaneously occur even in communities growing on a limiting nutrient resource leading to coexistence (tested both experimentally and with a mathematical model [38]). However, the situation drastically changes in the presence of toxicity. A theoretical prediction showed that, when increasing the number of species in a stressful environment, there is a first phase of facilitation due to the increased degradation capacity; however, competition could arise if the species number increases up to a point that toxicity is sufficiently reduced and stress is removed [87]. Given the simplicity of our toxicity-tracking system, it would be interesting to explore both with mathematical models and with wet lab experiments the outcome of adding more species to our LA At-Ct system.

6.7 Final remarks

To summarize this PhD project, I aimed to develop a simple system to study a challenging question. I am of course not the first scientist to wonder how the exposure to differently challenging habitats can influence the establishment of different types of interactions and coexistence among members of a community, but I think that with this PhD work we have reached some important realizations:

1. We advanced our understanding of the role of toxicity and specifically of ROS in shaping interspecies interactions, with results that are in line with SGH;
2. We developed a controlled and easily manipulable system with a nutrient-toxic gradient to investigate how the environment affects community dynamics. This first exploratory part took a lot of effort and time, but eventually led to a final system that leaves room for multiple future explorations;
3. We highlighted how crucial it can be to couple experimental procedures with mathematical modelling. The constant interplay between model predictions and experimental data allowed us to better understand our results and to predict further beyond what we were able to test in the lab.

After these past years, I surely do not yet have all the answers to the big open question of how the environment affects microbial community interactions, but our findings contribute to bringing us one step closer to these answers.

Appendix A

Supplementary Material

A.1 Model

A.1.1 Equations for the first model with implicit toxicity

We used a mathematical modelling approach to fit the data from the experiments and give predictions regarding whether species are expecting to engage in competition, facilitation, and to coexist over long term serial transfers.

The first model that we used is a modified Monod model with maximum growth rate r, half-saturation constant K, and yield Y, in which we incorporate a mortality term to take into account concentration-dependent toxicity of linoleic acid. We assume that toxicity of the environment $T(t)$ increases linearly over time and is proportional to the linoleic acid concentration $T(t) = (\beta + \gamma t)$.

The simplest equations that we start from are expressed for a single species B in a batch culture with linoleic acid (C) are:

$$\frac{\mathrm{d}B}{\mathrm{d}t} = (\frac{r}{C(t)+K} - (\beta+\gamma t))C(t)B(t) \quad \text{(SI0)}$$

$$\frac{\mathrm{d}C}{\mathrm{d}t} = -\frac{1}{Y}\frac{r}{C(t)+K}C(t)B(t) \quad \text{(SI0)}$$

The equations for two species B_1, corresponding to Ct and B_2, corresponding to At, in coculture in linoleic acid are:

$$\frac{\mathrm{d}B_1}{\mathrm{d}t} = (\frac{r_1}{C(t)+K_1} - (\beta_1+\gamma_1 t))C(t)B_1(t) \quad \text{(SI0)}$$

$$\frac{\mathrm{d}B_2}{\mathrm{d}t} = (\frac{r_2}{C(t)+K_2} - (\beta_2+\gamma_2 t))C(t)B_2(t) \quad \text{(SI0)}$$

$$\frac{\mathrm{d}C}{\mathrm{d}t} = -\frac{1}{Y_1}\frac{r_1}{C(t)+K_1}C(t)B_1(t) - \frac{1}{Y_2}\frac{r_2}{C(t)+K_2}C(t)B_2(t) \quad \text{(SI0)}$$

Because the bacteria showed some growth in the Minimal Medium, we assume an additional unknown nutrient to be present in the minimal medium, which concentration N(t) is modelled in an extra equation of the system. The updated model becomes:

$$\frac{dB_1}{dt} = (\frac{r_{C1}}{C(t)+K_{C1}} - (\beta_1+\gamma_1 t))C(t)B_1(t) + \frac{r_{N1}}{N(t)+K_{N1}}N(t)B_1(t) \quad \text{(SI0)}$$

$$\frac{dB_2}{dt} = (\frac{r_{C2}}{C(t)+K_{C2}} - (\beta_2+\gamma_2 t))C(t)B_2(t) + \frac{r_{N2}}{N(t)+K_{N2}}N(t)B_2(t) \quad \text{(SI0)}$$

$$\frac{dC}{dt} = -\frac{1}{Y_{C1}}\frac{r_{C1}}{C(t)+K_{C1}}C(t)B_1(t) - \frac{1}{Y_{C2}}\frac{r_{C2}}{C(t)+K_{C2}}C(t)B_2(t) \quad \text{(SI0)}$$

$$\frac{dN}{dt} = -\frac{1}{Y_{N1}}\frac{r_N 1}{C(t)+K_{N1}}C(t)B_1(t) - \frac{1}{Y_{N2}}\frac{r_{N2}}{C(t)+K_{N2}}C(t)B_2(t) \quad \text{(SI0)}$$

We then first estimate the parameters of the growth in the minimal medium using an arbitrary concentration for this unknown nutrient (0.01), by using the data from both monocultures and cocultures of Ct (species B_1) and At (species B_2) in the minimal medium. This allows us to obtain estimates for the parameters r_{N1}, r_{N2}, Y_{N1}, Y_{N2}, K_{N1} and K_{N1}. Then, we fix these parameters and estimate the parameters for the growth of At and Ct in monoculture using a range of concentrations of linoleic acid (0.05%, 0.1%, 0.5% and 0.075%). This leads to estimating r_{C1}, r_{C2}, Y_{C1}, Y_{C2}, K_{C1}, K_{C1} and the toxicity parameters for At β_2 and γ_2, and we set β_1 and γ_1 to zero for Ct (assuming no toxicity of the linoleic acid for Ct).

A.1.2 Equations for the second model with explicit toxicity (ROS)

In this second model, we add a new state variable corresponding to the concentration of ROS. Linoleic acid is now only a nutrient, and the toxicity is proportional to ROS concentration (which can increase), so we do not need a specific parameter for the toxicity accumulation. The parameters β_1 and β_2 are the sensitivity of Ct and At to ROS (high value meaning low tolerance). The uptake of linoleic acid does not change from the previous model. The ROS intrinsic dynamics depend on their production by the oxidation of LA (spontaneous oxidation from the air, d, and positive feedback by ROS presence in the media, e, their half-life l and the yield of ROS production m. To this intrinsic part, we add the detoxification by the cells, through parameters α_1 for Ct and α_2 for At.

The coculture equations become:

$$\frac{dB_1}{dt} = \frac{r_{C1}}{C(t)+K_{C1}}C(t)B_1(t) - \beta_1 B_1(t)R(t) + \frac{r_{N1}}{N(t)+K_{N1}}N(t)B_1(t) \quad \text{(SI0)}$$

$$\frac{dB_2}{dt} = \frac{r_{C2}}{C(t)+K_{C2}}C(t)B_2(t) - \beta_2 B_2(t)R(t) + \frac{r_{N2}}{N(t)+K_{N2}}N(t)B_2(t) \quad \text{(SI0)}$$

$$\frac{dC}{dt} = -\frac{1}{Y_{C1}}\frac{r_{C1}}{C(t)+K_{C1}}C(t)B_1(t) - \frac{1}{Y_{C2}}\frac{r_{C2}}{C(t)+K_{C2}}C(t)B_2(t) - \frac{1}{m}(d+eR(t))C(t) \quad \text{(SI0)}$$

$$\frac{dN}{dt} = -\frac{1}{Y_{N1}}\frac{r_N 1}{C(t)+K_{N1}}C(t)B_1(t) - \frac{1}{Y_{N2}}\frac{r_{N2}}{C(t)+K_{N2}}C(t)B_2(t) \quad \text{(SI0)}$$

$$\frac{dR}{dt} = (d+eR(t))C(t) - lR(t) - \alpha_1 B_1(t)R(t) - \alpha_2 B_2(t)R(t) \quad \text{(SI0)}$$

The monoculture equations can be derived by putting one of the two bacteria densities to zero. Because we now have data on the spontaneous oxidation of LA in cell-free media, we can first estimate the parameters d, e, m, and l using ROS proxy in different linoleic acid concentrations. We then use these fixed parameters in the monoculture estimation to estimate the parameters of growth, toxicity, and detoxification

for single species. Then, predictions can be made, as in the previous model, for the dynamics of coculture, short- and long-term dynamics (serial transfers), and mimicking the addition of a ROS quencher to the media (putting initial ROS concentration to zero, as well as parameters d, e, and l).

A.2 Supplementary figures

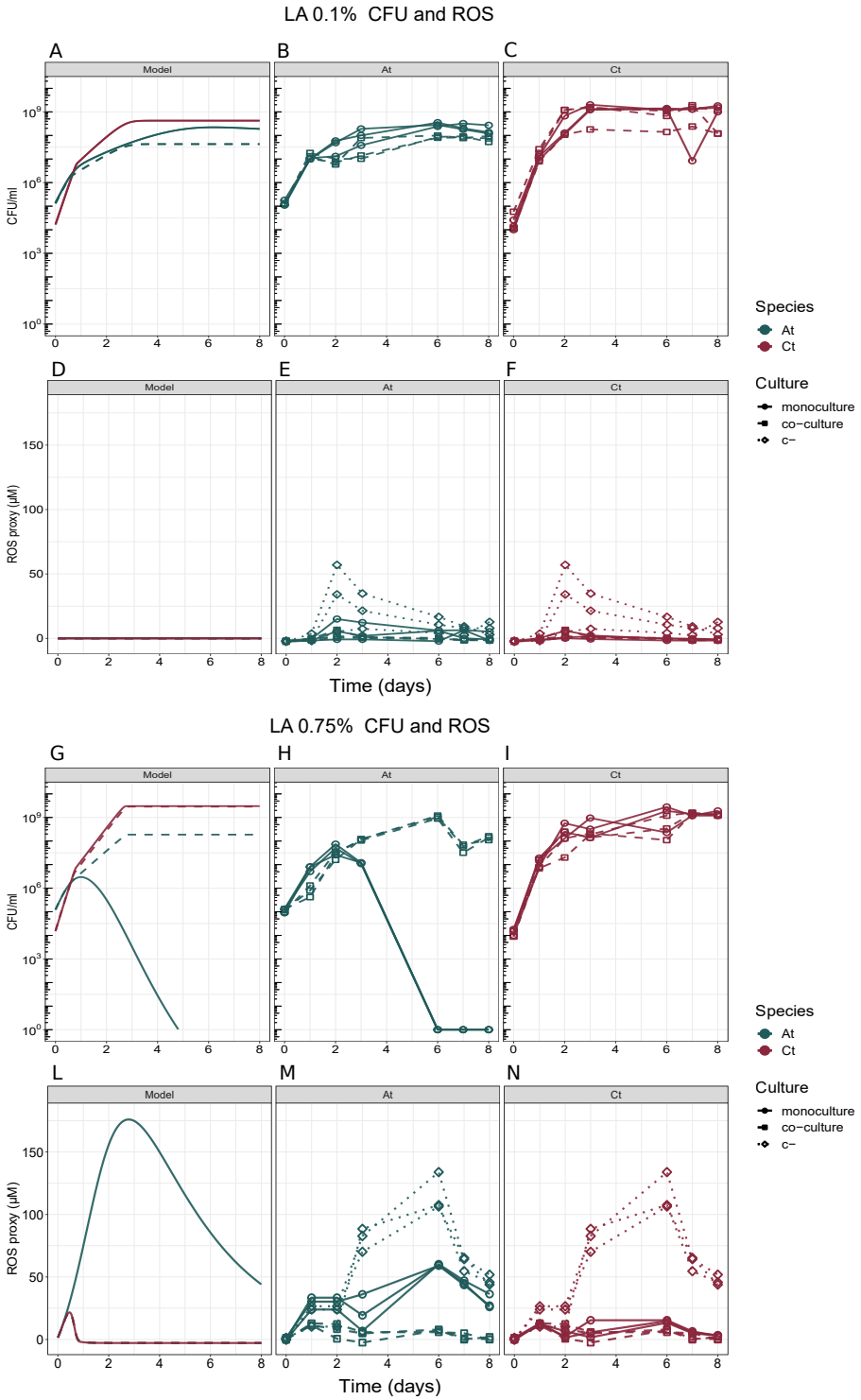


Figure SI1 – Comparison CFU and ROS LA 0.1% and LA 0.75%. Upper row shows CFU/ml data (y-axis), bottom row shows the variation of a ROS-proxy abundance (y-axis) over time (x-axis). At is in green, Ct is in red (three technical replicates per condition, graphs show the results of one biologically independent experiment). Squares-solid lines are monoculture data, round-dashed lines are co-culture data, diamond-dotted lines are bacteria-free data (c-, data available only for bottom row on ROS, since the CFU data of c-samples was always 0.). The yellow-haloed curves are extrapolated from Model 2 predictions and cover both mono- and co-cultures at both LA concentrations. ROS abundance in LA 0.1% is lower in all monocultures, co-culture and bacteria free conditions compared to the same culture conditions in LA 0.75% (comparison panels C-G and D-H. Growth curves in panels A, B, E and F are described in Chapter 4, Fig. 4.2). Panels E-H are described in Chapter 4, Fig. 4.4

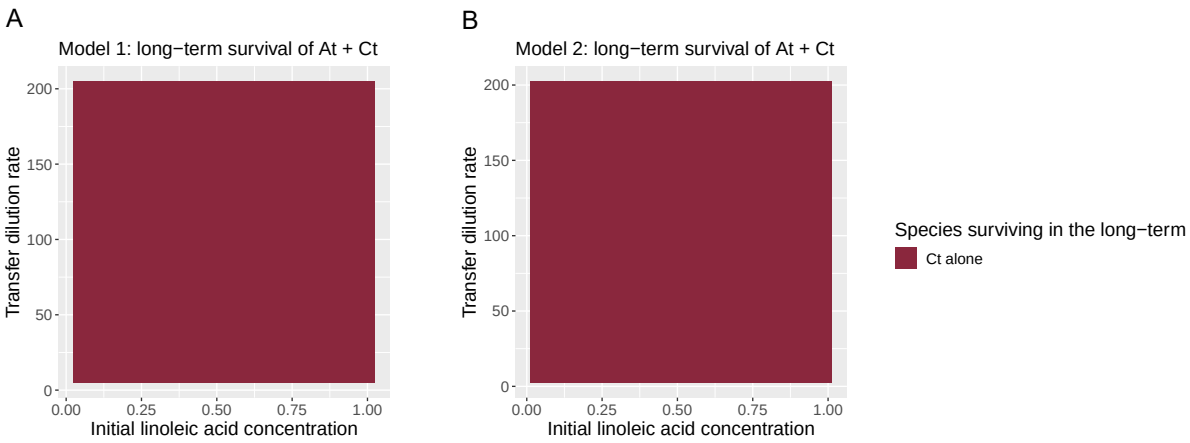


Figure SI2 – Placeholder for: Testing for long-term coexistence in both Model 1 and Model 2. Both models predict that there is no long term coexistence: Ct outcompetes At over longer time scale and it is the only species surviving.

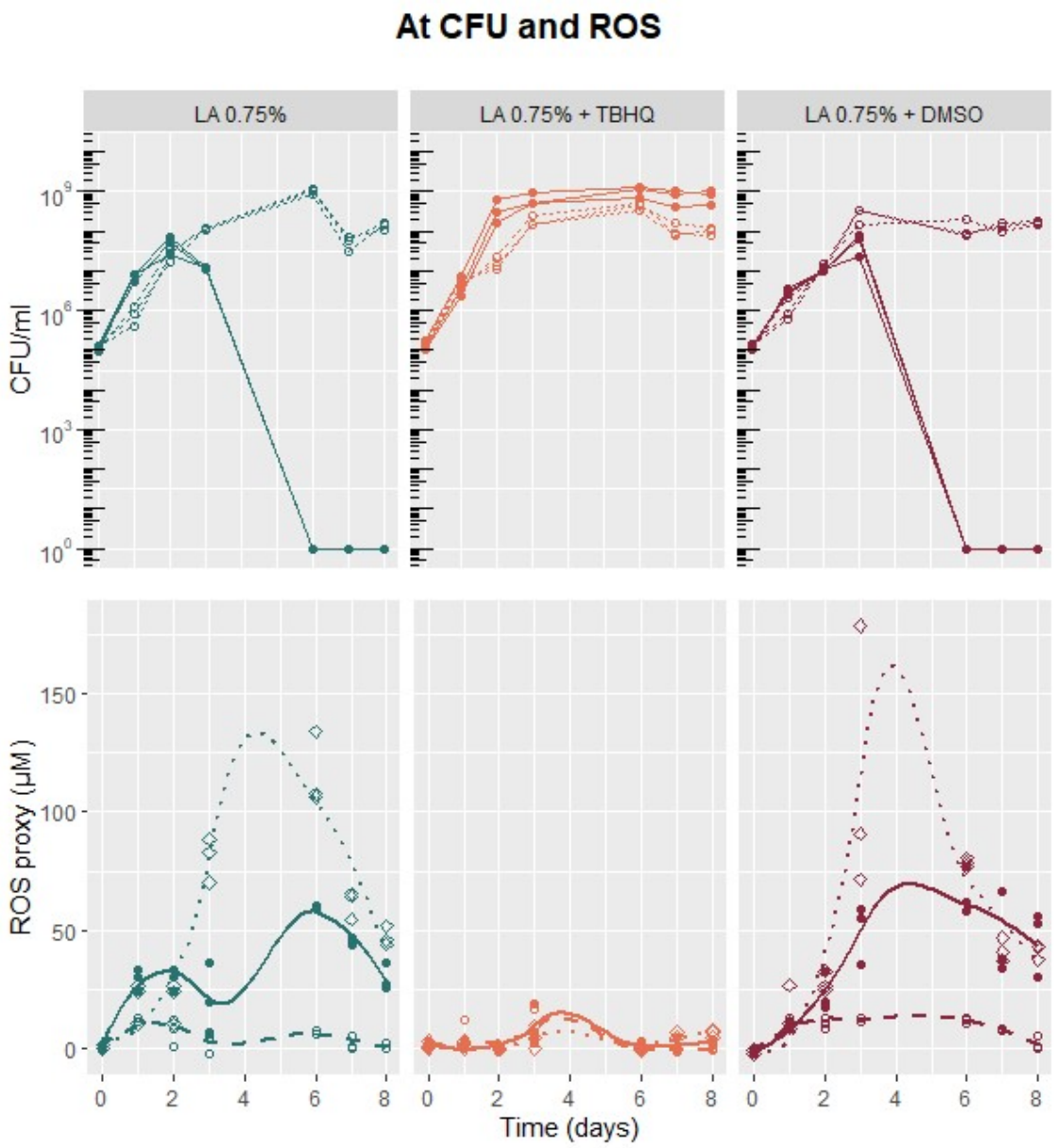


Figure SI3 – ROS abundance over time and comparison with correspondent bacterial growth in At mono and co-culture. Upper row shows CFU/ml data, bottom row shows the variation of a ROS-proxy abundance over time. Solid lines are monoculture data, dashed lines are co-culture data, dotted lines are bacteria-free data (c-, data available only for bottom row on ROS, since the CFU data of c- samples was always 0. Please note that c- data are the same for both C and D plots and G and H plots, since they were controls for the same respective experiment). Three different media condition are showed: LA 0.75% (green), LA 0.75% + TBHQ (orange), LA 0.75% + DMSO (red). Comparison between CFU data and ROS data across the three media conditions shows that DMSO does not affect neither bacterial growth nor ROS abundance: both CFU and ROS data of LA 0.75% + DMSO (red) are very similar to what happens in simple LA 0.75% medium (green).

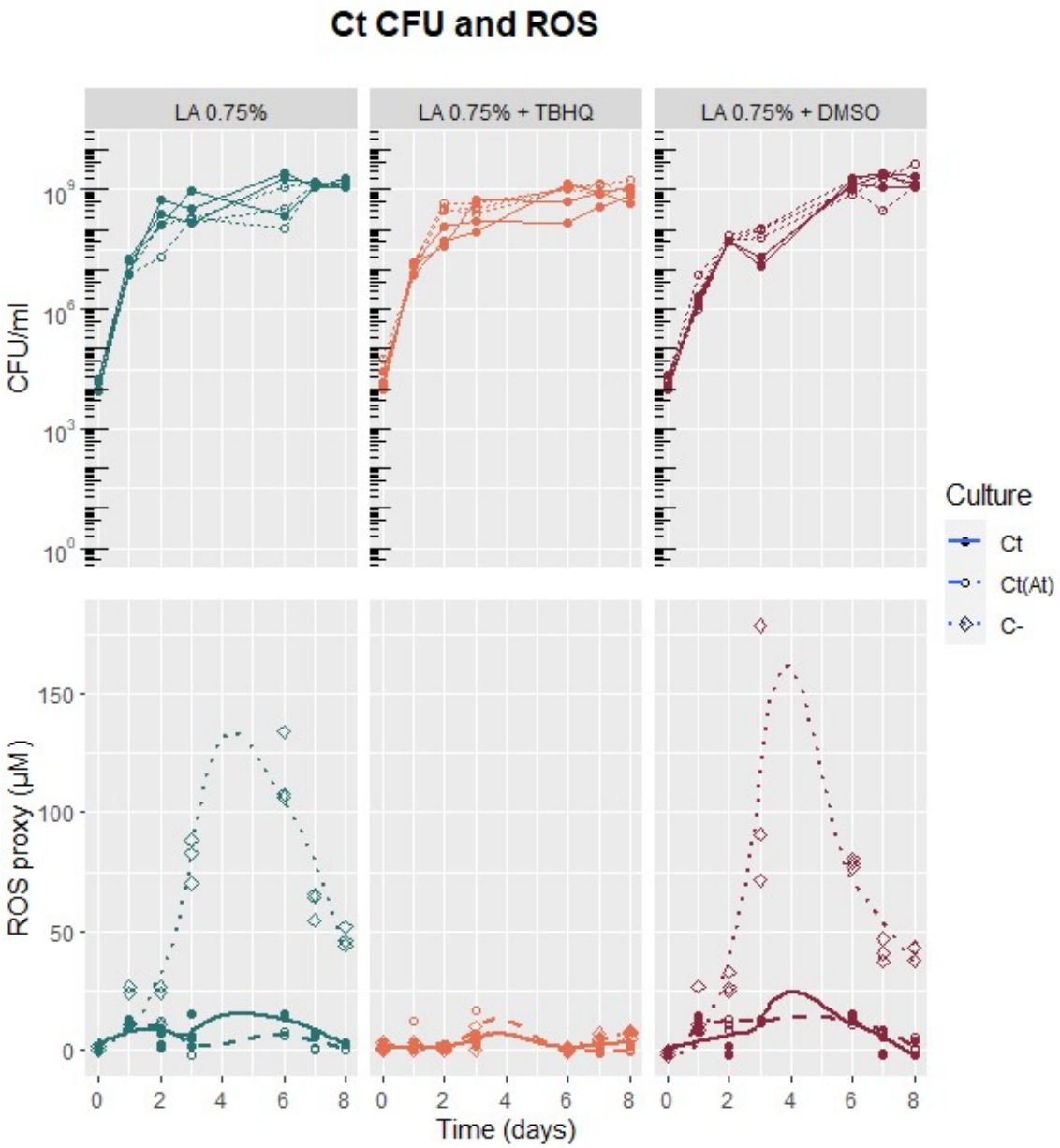


Figure SI4 – ROS abundance over time and comparison with correspondent bacterial growth in Ct mono and co-culture. Upper row shows CFU/ml data, bottom row shows the variation of a ROS-proxy abundance over time. Solid lines are monoculture data, dashed lines are co-culture data, dotted lines are bacteria-free data (c-, data available only for bottom row on ROS, since the CFU data of c- samples was always 0. Please note that c- data are the same for both C and D plots and G and H plots, since they were controls for the same respective experiment). Three different media condition are showed: LA 0.75% (green), LA 0.75% + TBHQ (orange), LA 0.75% + DMSO (red). Comparison between CFU data and ROS data across the three media conditions shows that DMSO does not affect neither bacterial growth nor ROS abundance: both CFU and ROS data of LA 0.75% + DMSO (red) are very similar to what happens in simple LA 0.75% medium (green).

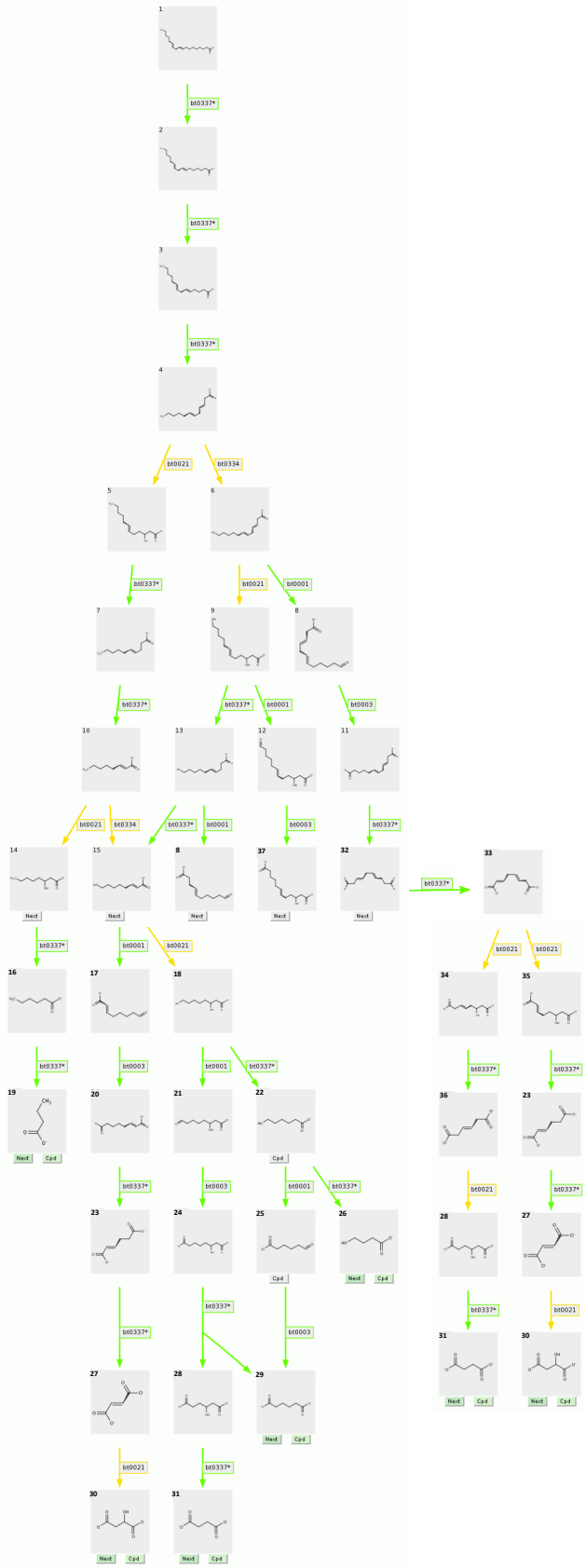


Figure SI5 – One of the possible linoleic acid microbial degradation routes as predicted by Biocatalysis/Biodegradation Database developed by eawag. More branches were predicted, but they are not shown here for visual simplicity.

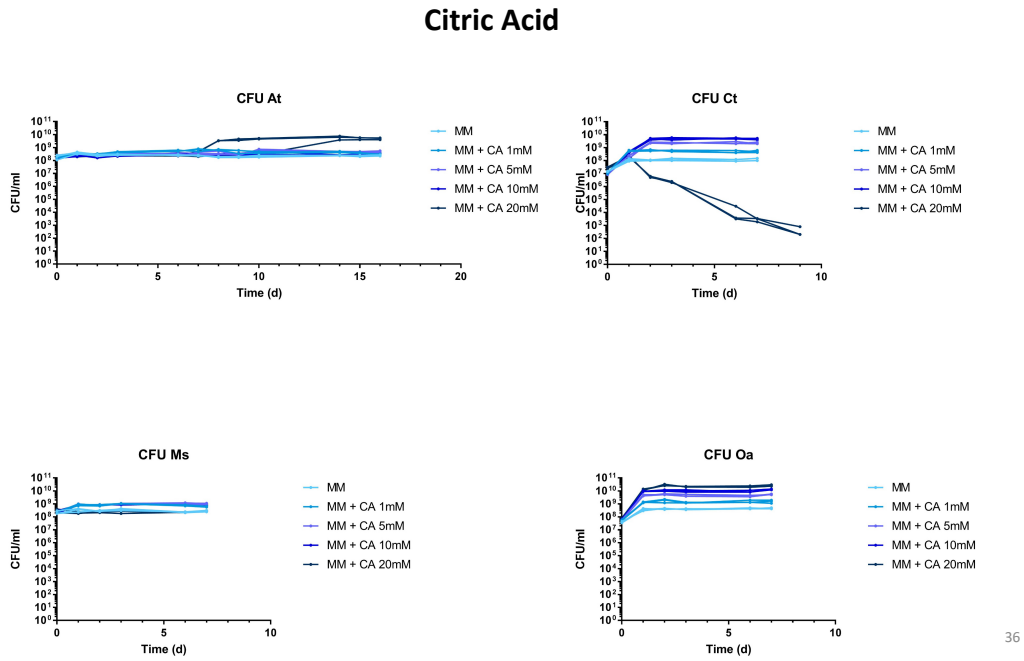


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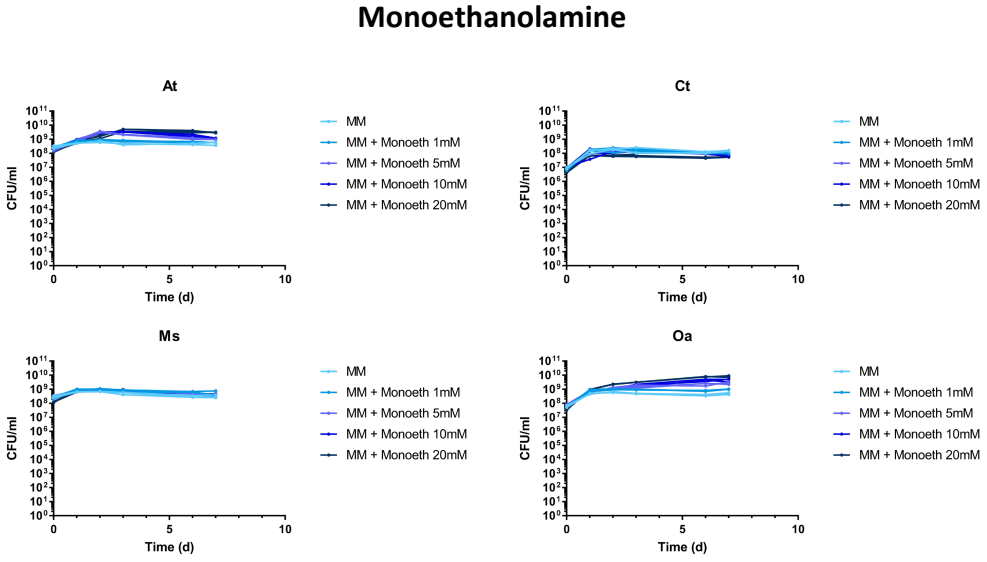
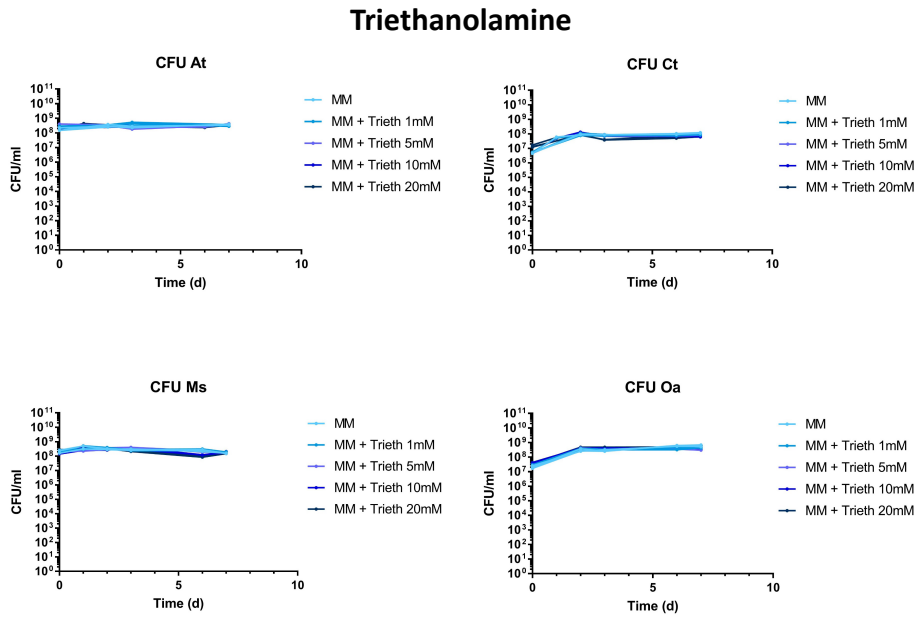
Figure SI6 – Growth curves of At, Ct, Ms and Oa in citric acid at high initial population size. Darker gradient of blue curves represents increasing compound concentration.



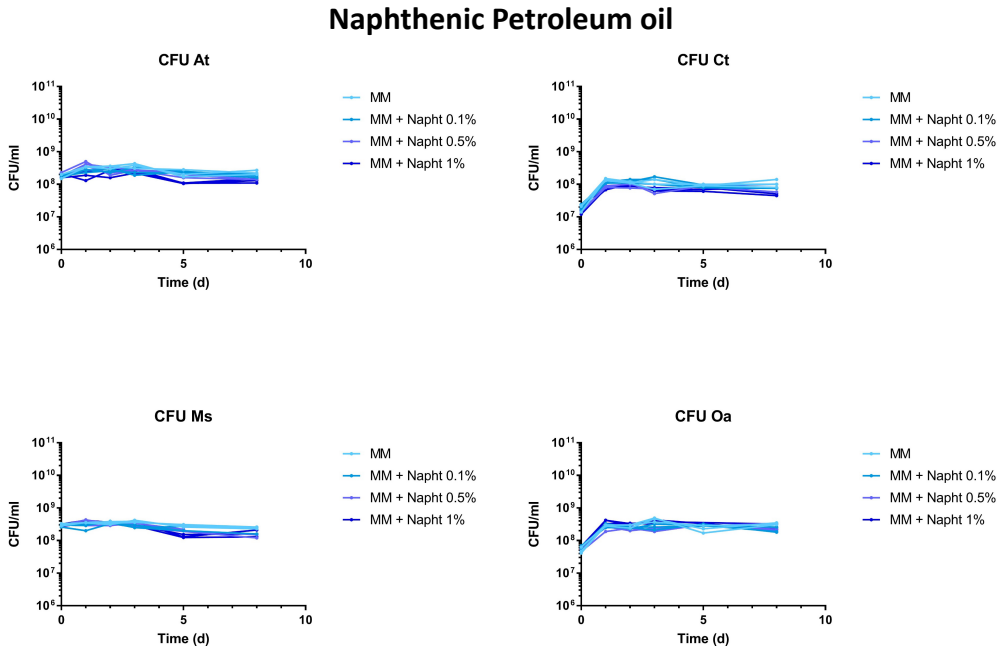
37

Figure SI7 – Growth curves of At, Ct, Ms and Oa in monoethanolamine at high initial population size. Darker gradient of blue curves represents increasing compound concentration.



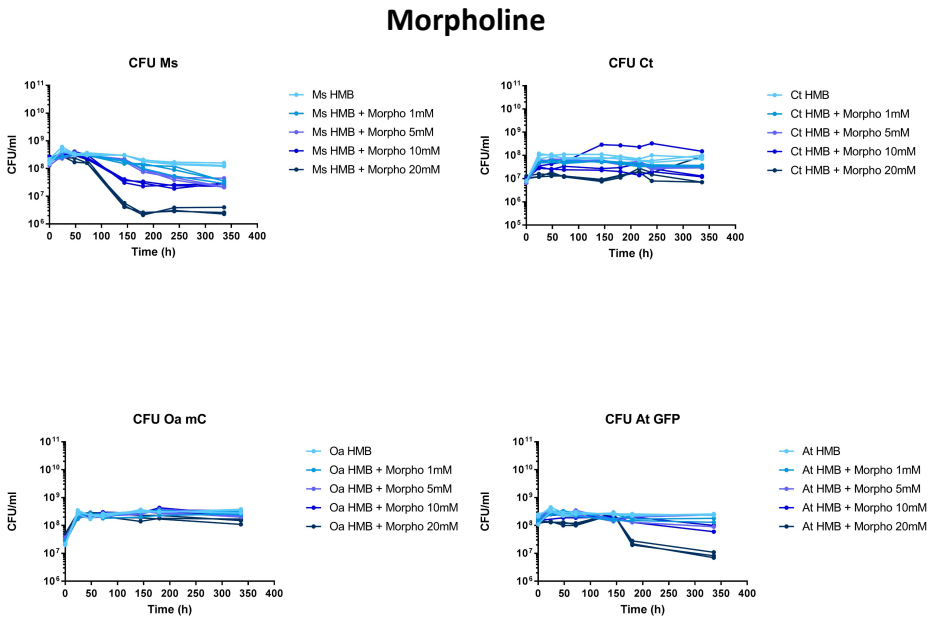
38

Figure SI8 – Growth curves of At, Ct, Ms and Oa in triethanolamine at high initial population size. Darker gradient of blue curves represents increasing compound concentration.



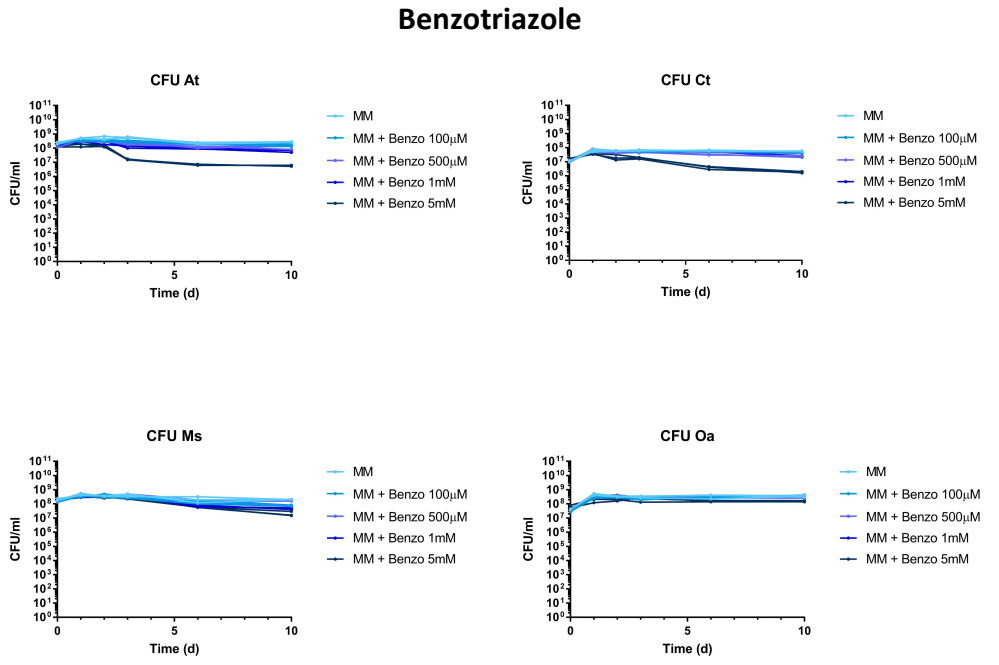
9

Figure SI9 – Growth curves of At, Ct, Ms and Oa in naphthenic at high initial population size. Darker gradient of blue curves represents increasing compound concentration.



40

Figure SI10 – Growth curves of At, Ct, Ms and Oa in morpholine at high initial population size. Darker gradient of blue curves represents increasing compound concentration.



41

Figure SI11 – Growth curves of At, Ct, Ms and Oa in benzotriazole at high initial population size. Darker gradient of blue curves represents increasing compound concentration.

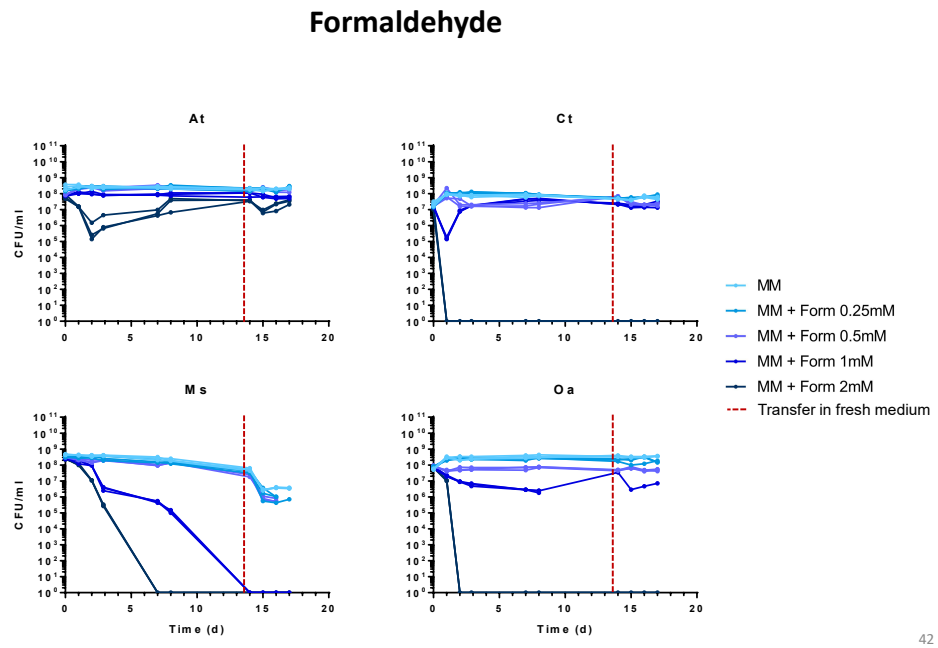


Figure SI12 – Growth curves of At, Ct, Ms and Oa in formaldehyde at high initial population size. Darker gradient of blue curves represents increasing compound concentration. Red dashed line marks the transfer of the entire population in fresh formaldehyde media.

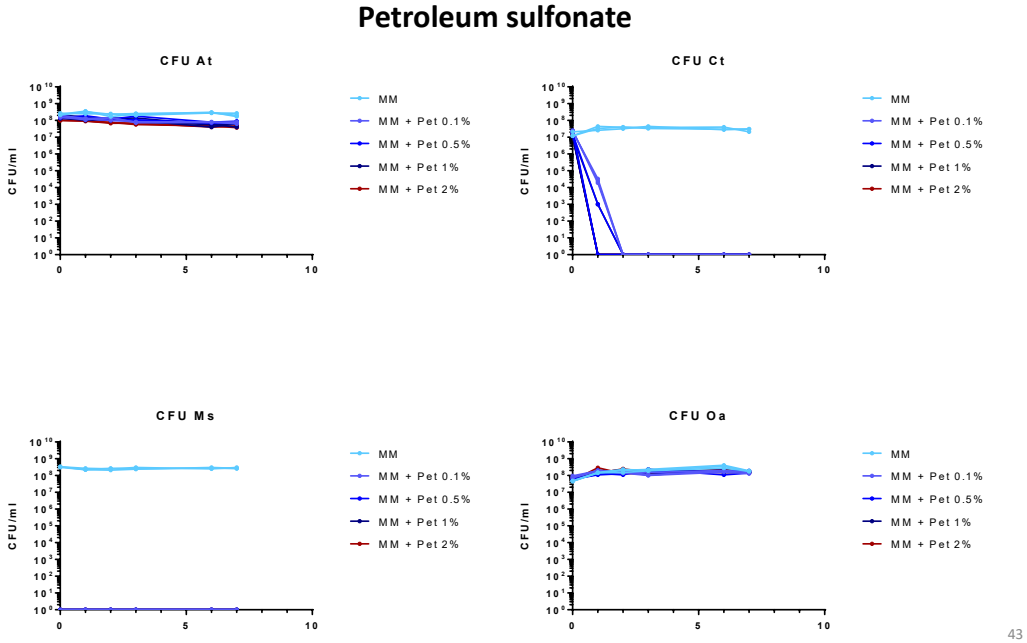


Figure SI13 – Growth curves of At, Ct, Ms and Oa in petroleum sulfonate at high initial population size. Darker gradient of blue curves represents increasing compound concentration.

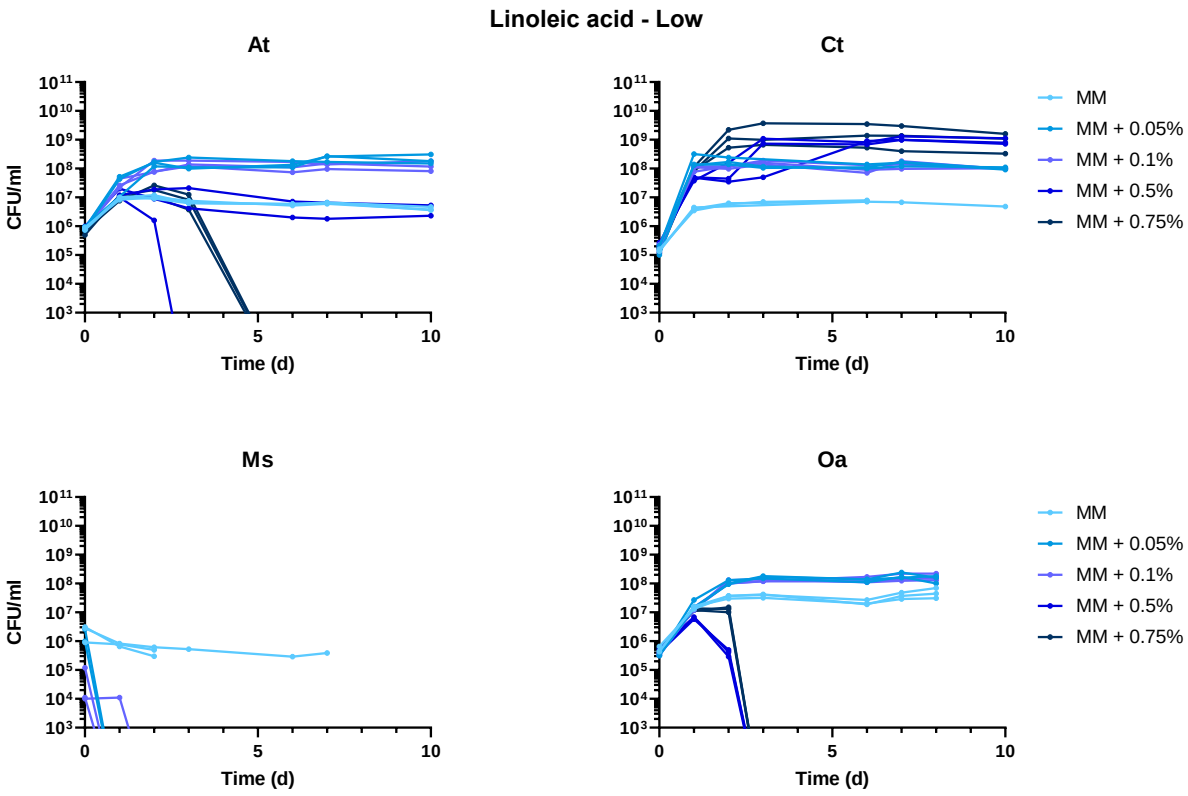


Figure SI14 – Growth curves of At, Ct, Ms and Oa in linoleic acid at low initial population size. Darker gradient of blue curves represents increasing compound concentration.

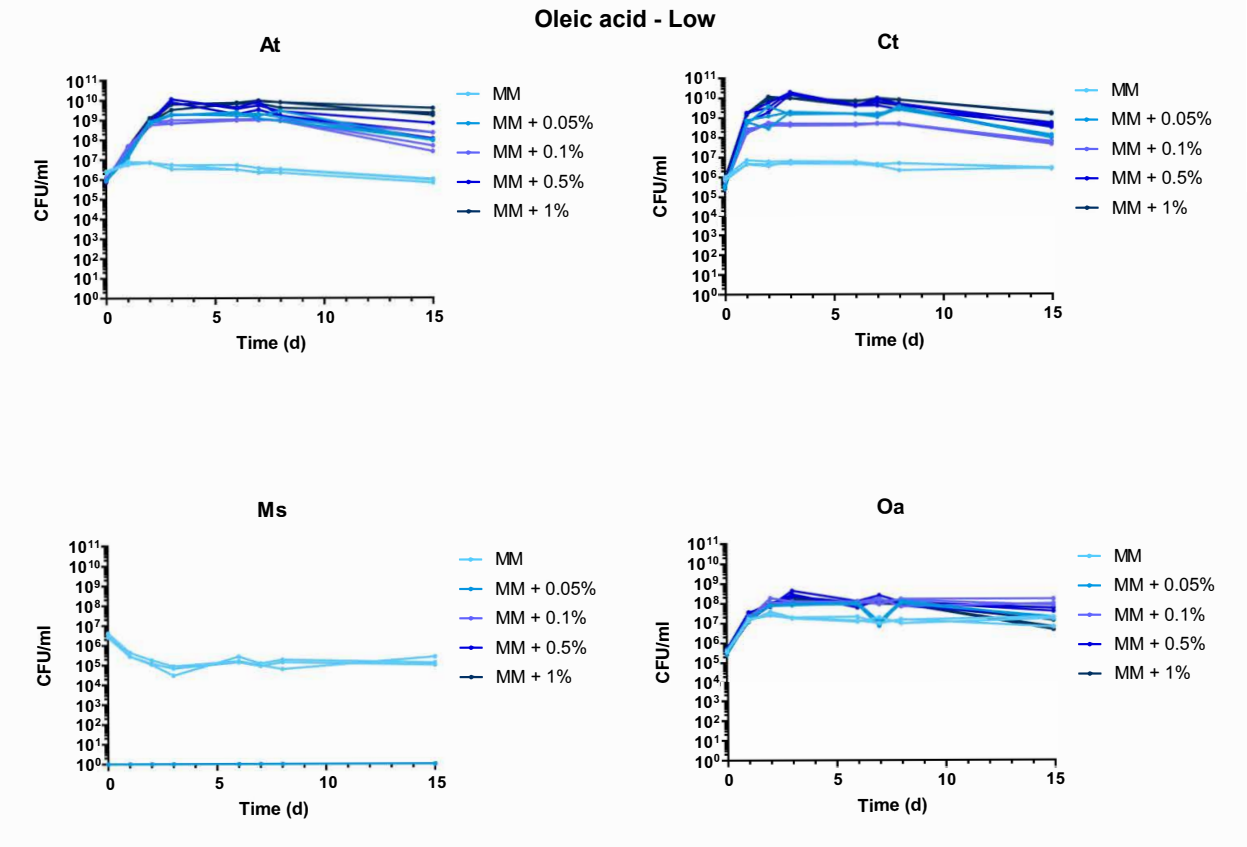


Figure SI15 – Growth curves of At, Ct, Ms and Oa in oleic acid at low initial population size. Darker gradient of blue curves represents increasing compound concentration.

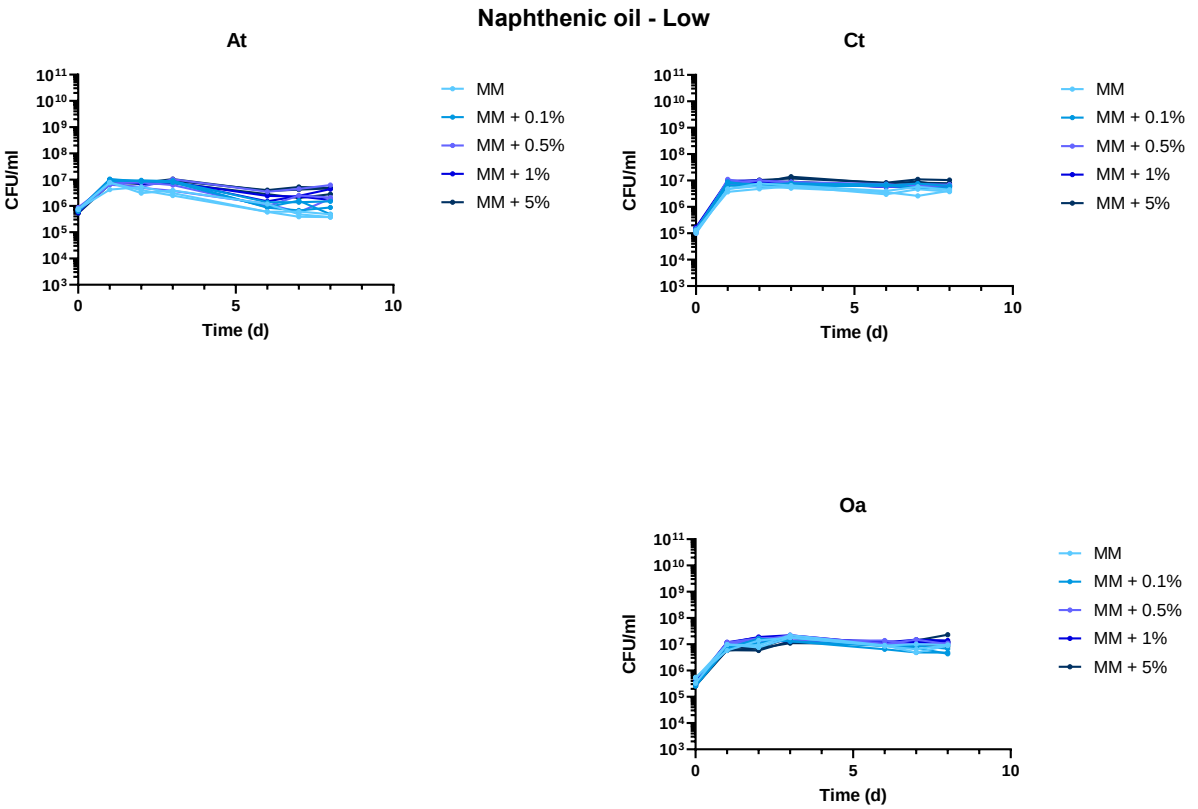


Figure SI16 – Growth curves of At, Ct, Ms and Oa in napthtenic oil at low initial population size. Darker gradient of blue curves represents increasing compound concentration.

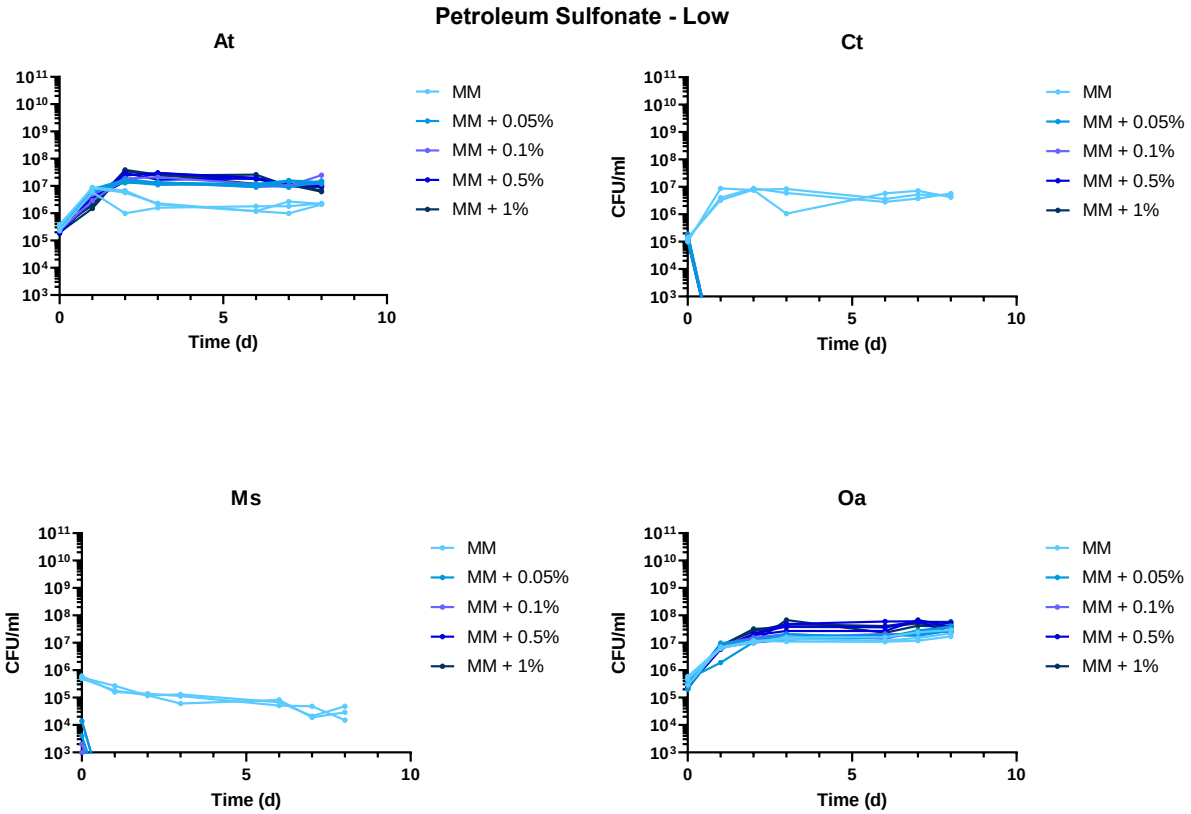


Figure SI17 – Growth curves of At, Ct, Ms and Oa in petroleum sulfonate at low initial population size. Darker gradient of blue curves represents increasing compound concentration.

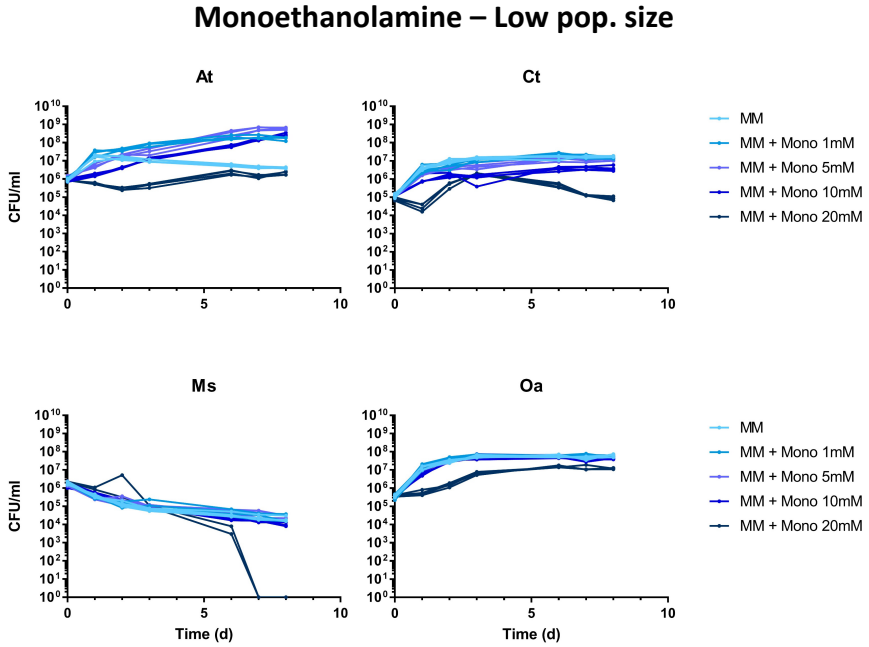


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Figure SI18 – Growth curves of At, Ct, Ms and Oa in monoethanolamine at low initial population size. Darker gradient of blue curves represents increasing compound concentration.

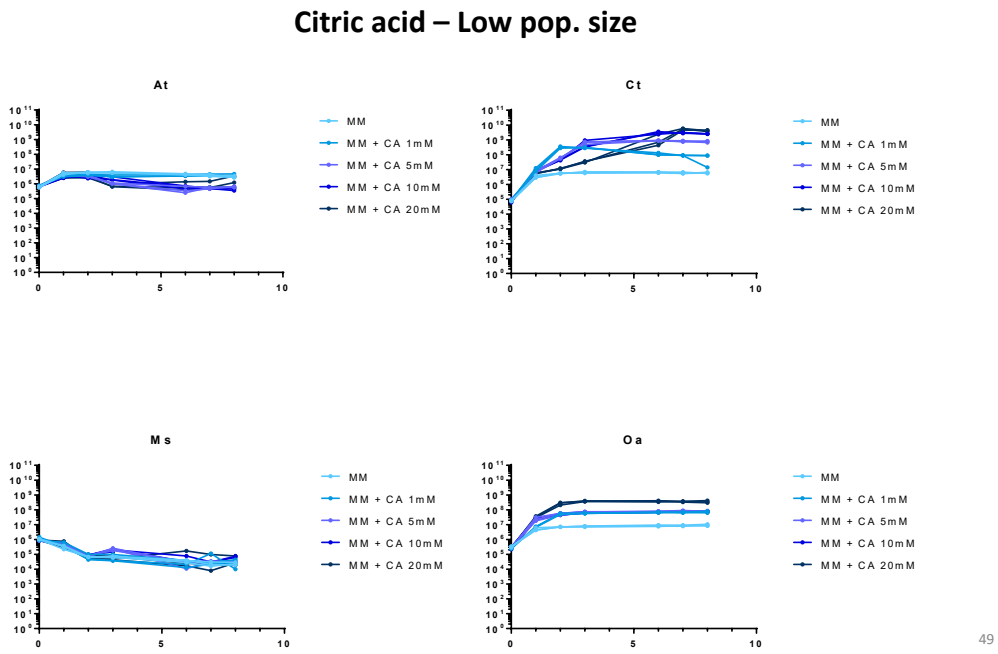

Figure SI19 – Growth curves of At, Ct, Ms and Oa in citric acid at low initial population size. Darker gradient of blue curves represents increasing compound concentration.

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Approved: _____

Tanja Schwander, Prof.
Committee President

Approved: _____

Sara Mitri, Prof.
Thesis Director

Approved: _____

Jan-Willem Veening, Prof.
Thesis Co-Director

Approved: _____

Melanie Blokesch, Prof.
Committee Member

Approved: _____

Orkun Soyer, Prof.
Committee Member