Pseudomonas fluorescens SBW25 as a dissipative system:
on-equilibrium thermodynamics offers insight into key evolutionary
processes

LOUDON, Claire Marie

Abstract
Physicists have long been interested in the possibility that the complex living structures which
have developed on planet earth did so in order to dissipate a free energy gradient. However,
whilst convincing theoretical work has been published, a real world test of these ideas has
proved elusive. This thesis tests these ideas using the bacterial model system Pseudomonas
fluorescens SBW25 and finds convincing evidence that this is an ideal model system for
dissipative systems theory.

Reference
LOUDON, Claire Marie. Pseudomonas fluorescens SBW25 as a dissipative system:
non-equilibrium thermodynamics offers insight into key evolutionary processes.

URN: urn:nbn:ch:unige-835600
DOI: 10.13097/archive-ouverte/unige:83560

Available at:
http://archive-ouververte.unige.ch/unige:83560

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*Pseudomonas fluorescens* SBW25 as a dissipative system:

Non-equilibrium thermodynamics offers insight into key evolutionary processes.

THÈSE

présentée à la Faculté des Sciences de l’Université de Genève pour obtenir le grade de Docteur ès Sciences, mention l’environnement

par

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de
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Thèse N°4887
GENÈVE
2015
“The world is a thing of utter inordinate complexity and richness and strangeness that is absolutely awesome. I mean the idea that such complexity can arise not only out of such simplicity, but probably absolutely out of nothing, is the most fabulous extraordinary idea. And once you get some kind of inkling of how that might have happened, it's just wonderful. And ... the opportunity to spend 70 or 80 years of your life in such a universe is time well spent as far as I am concerned.”

Douglas Adams
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Abstract

One of the most fascinating questions in modern science is how and why living organisms evolved on planet earth. Dissipative systems theory (DST) offers a fascinating explanation, it suggests that in order to dissipate free energy gradients such as that imposed on the earth by solar energy, complex structures that can dissipate this energy through irreversible processes (those that produce entropy) will emerge. The theory posits that complex structures such as living organisms would emerge in order to allow for maximum gradient dissipation. Whilst this theory is fascinating, it has proven difficult to test in a real world system as ecosystems are large and complicated, and imposing the necessary free energy gradient manipulations is difficult and the time scales necessary to study the effects are too long. These same problems have been experienced and overcome in experimental evolution through the use of microbes. A microbe that generates complexity and maximally dissipates a single free energy gradient may fulfill the criteria of an ideal real world test for the predictions of DST. This thesis explores the possibility that Pseudomonas fluorescens SBW25 may be an ideal model system for testing this theory. We test the two main predictions of DST using Pseudomonas fluorescens, these being: (1) Maximal dissipation of a free energy gradient is a possible outcome of a system that develops complex structures that dissipate energy and evolve strategies for maintaining energy dissipation over time and (2).In some cases, feedbacks may develop between the system and the external environment. In this thesis I will present compelling evidence to support both these predictions on basis of experimental manipulations of Pseudomonas fluorescens. The general conclusion of the thesis is that Pseudomonas fluorescens SBW25 indeed has the capacity to be used as an effective model system in which to test the predictions of
dissipative systems theory. This may lead to further work on understanding how free energy gradients can contribute to the generation of complex life forms.
Résumé

L’une des questions les plus fascinantes de la science moderne est de comprendre comment et pourquoi les organismes vivants ont évolué la Terre. Selon la théorie des systèmes dissipatifs (DST), les gradients d’énergie libre, comme celui de l’énergie solaire sur la terre, provoquent l’apparition de structures complexes qui permettent la dissipation de cette énergie par le biais de processus entropiques irréversibles. La théorie pose comme principe que des structures complexes, telles que la vie, apparaissent afin d’assurer la dissipation maximale de gradient. Malgré l’intérêt de cette théorie, il s’est avéré difficile de la vérifier dans la réalité. En effet, la taille et la complexité des écosystèmes, la difficulté de manipulation des gradients d’énergie libre, ainsi que l’échelle de temps nécessaire pour étudier les effets rendent son application à la vie réelle difficile. Ces mêmes problèmes ont été surmontés en évolution expérimentale par l’utilisation de microbes. Un microbe, qui génère la complexité afin de dissiper au maximum un dégradé unique d’énergie libre, remplit donc les critères nécessaires pour tester les prédictions de la DST dans le monde réel. Cette thèse étudie la possibilité que Pseudomonas fluorescens SBW25 puisse être un organisme modèle de cette théorie. Pour cela, les deux prédictions principales de la DST ont été testées ainsi à l’aide de Pseudomonas fluorescens : 1. afin de dissiper au maximum l’énergie libre dégradée, un système devrait élaborer des structures complexes pour dissiper l’énergie et des stratégies pour son maintien dans le temps ; 2. Pour assurer la dissipation d’énergie continue, des phénomènes de rétroactions doivent exister entre le système et le milieu extérieur. La similitude entre la théorie et les résultats obtenus en laboratoire permettent de soutenir ces deux prédictions. Cette thèse permet donc de conclure que Pseudomonas fluorescens SBW25 est en effet un
système modèle efficace pour tester les prédictions de la théorie des systèmes dissipatifs. Cela permet donc la poursuite des travaux concernant la contribution des gradients d’énergie libre à la génération de formes de vie complexes.
Glossary of terms

Adaptive radiation: Adaptive radiation is a process by which one ancestral species rapidly diversifies into two or more daughter species. It may occur as a result of the availability of a new niche, a new resource or as the result of extinctions. (i.e. ecological opportunity)

Complexity: Complex systems research is the study of how the small parts in a system contribute to the overall functioning of the system. Complexity therefore refers to the parts of the system, but in principle is measured at the system scale, although there is no unique and agreed upon general quantification. Complexity throughout this thesis is used interchangeably with species diversity.

Dissipative structures: Non-equilibrium structures which have a degree of complication that could grow rapidly in time. They have the potential to become more complicated in response to free energy gradients.

Dissipative processes: In thermodynamics dissipative processes are defined as those that generate heat from work. An example could be the force of friction between two solids, picture the heat generated when rubbing two sticks together quickly, this process generates heat energy and increases entropy. Many biological processes generate heat from work using the same principle but the one that is particularly of interest in this study is respiration in which oxygen uptake is used for biological processes (work in this case) which generates heat energy. Heat energy is more disordered than chemical energy so the entropy is the system increases in accordance with the second law of thermodynamics.
High energy resources: High energy resources are defined as ones that can be taken up by a system and used for work (processes that generate internal order) and can be dissipated as heat energy. Therefore their use produces entropy.

Niche construction: Niche construction is a process by which organisms can affect the selection pressures that are faced by themselves and other organisms by altering the biotic or abiotic environment. Whenever it is used in this thesis, niche construction is defined following the criteria set out by Matthews, (2014). These being: First, a candidate niche construction agent must significantly alter environmental conditions and second the agent mediated environmental conditions must influence selection pressures on a niche construction recipient. Thus, the irreducible element of niche construction is that there is feedback between the organisms that structure an environment and the environment acting back on the organisms themselves.
Chapter 1

General introduction

1.1 Models of early life and the generation of complexity on earth – The physics approach

One of the most interesting questions that has arisen in the study of early life on earth is how and why complex forms of life would evolve from a simple ancestor and how this development may relate to the environmental conditions on the planet. Indeed, why life should evolve at all is an interesting question in itself. Although their work is largely overlooked by the biological community, physicists have been using the tools available to the physical sciences to consider this question for some time. In particular, much of the work in complexity science has tried to reconcile the complexity found in the natural world with the much more basic laws of physics (Goldenfeld and Kadanoff. 1999). This is noted in particular by Sneppen (2014) when he considers Gould's (1989) famous thought experiment on replaying the tape of life. Gould wondered if we could rewind all of the “tape of life” e.g. the evolution from a single common ancestor to the complex biosphere present on earth today and set the process off again, would the processes of evolution, adaptive radiation and extinction repeat exactly as they have before. Sneppen considered this same idea from the physics perspective and remarked that if you “replayed the tape” a hydrogen atom could not possibly be any different than it was but a living cell most certainly could (Sneppen, 2014). At first glance, living systems also appear to defy the second law of thermodynamics which states that all complex forms must break down as the universe tends towards thermodynamic equilibrium (a dead state). However, this realisation that biological systems cannot be explained by the basic rules which can be applied to non living physical systems (in
equilibrium) has not deterred attempts to understand the development of biodiversity using physics approaches. Goldenfeld and Woese (2011) have argued that we need a non-equilibrium statistical mechanics approach in order to fully understand the interaction between evolution and ecology. They also argued that there was a need to understand life through the lens of physics but this would need to be achieved at different levels and using various appropriate physical theories (Goldenfeld and Woese, 2009). One such physical theory that may be applied to early life is that of dissipative structures (Prigogine, 1955; Nicolis and Prigogine, 1977) which are postulated to have a degree of complexity that can grow rapidly in response to free energy gradients (Goldenfeld and Kadanoff, 1999). The application of dissipative systems theory to biology is discussed in detail below. This thesis attempts to explore how this theory may be applied to a well-studied bacterial system, *Pseudomonas fluorescens* SBW25, which is widely regarded as a microbial model system for the study of adaptive radiation (see definitions) (Rainey & Travisano, 1998) and discusses how this might contribute to our understanding of the development of complex life on planet earth.

1.2 *Life and the second law of thermodynamics*

Biological processes have long been of interest to physicists and many have attempted to model biological theories to bring them in accordance with the laws of physics. Physicists have looked at biological phenomenon such as morphogenesis (Turing, 1952), self-replication (England 2013) and adaptation (Perunov et al, 2014). However, the primarily theoretical tools available to physicists are often limiting because they can only work within known constraints that can be applied mathematically and it is difficult to test their predictions in
real life systems. Yet, understanding how the emergence of life on earth fits in with the fundamental laws of physics is a worthwhile endeavor as it is likely to be the predictions of a physical theory that guide the search for life on other planets.

The second law of thermodynamics states that all complex forms cannot persist as the universe tends towards disorder, therefore entropy (a measure of disorder) must always increase. However, at first glance living systems appear to defy this law because throughout the course of evolution they have developed ever more complex forms and interactions (Schrodinger, 1944). Lovelock (1965) observed that in fact most living systems push their physical environments out of thermodynamic equilibrium. Erwin Schrodinger (1944), Nicolis and Prigogine (1977) and most recently England (2013) have attempted to use theoretical thermodynamics to understand if the complexity and diversity of biological systems is increasing without defying the second law of thermodynamics. The work of Nicolis and Prigogine (1977) and England (2013), proposed the theory of dissipative structures in which a system develops greater complexity in order to maximally dissipate a higher energy gradient and generate maximal global entropy by taking up the energy in this gradient and releasing it in a more disordered form. A perfect example of this is metabolism when high energy food is taken up by an organism, this chemical energy is used for biological processes by the organism that help it maintain its far from equilibrium state and is eventually released as heat energy which is a much more disordered form of energy. Therefore metabolism is linked to an increase in entropy in accordance with the second law. Their theories build on work in non-equilibrium thermodynamics which suggests that when a system is pushed out of equilibrium by an energy gradient (such as solar energy or oxygen (Aoki, 2006)) then it will develop strategies to dissipate that gradient and resist being pushed further out of equilibrium. Boltzmann (1872) observed that the biosphere is pushed out of equilibrium by a solar energy gradient and postulated that that biodiversity on earth had evolved along with
strategies to maximally dissipate this solar energy. Since this time, studying dissipative systems theory has been an area of great interest to theoretical physicists who are interested in the living world. Dissipative systems theory has been used to model biological processes (Aoki, 2006) (Schneider and Kay 1994 (a) and (b)) (England, 2013). Of particular relevance are the theoretical and data analysis studies of Aoki (1995, 2001, 2006) who used dissipative systems theory to study how the production of entropy was related to diversity in aquatic ecosystems and communities.) Aoki uses species diversity of the community as a measure of the complexity of the physical “system” and oxygen uptake and respiration as a proxy for the entropy production of the system. His rationale is that oxygen uptake decomposes macromolecules and releases chemical energy which can be used to maintain order in the living organism and is eventually turned into heat energy (a much more disordered form of energy) and dissipated into the environment (Aoki, 2006). Aoki names this heat energy the dissipative function. Aoki also uses oxygen uptake and respiration rate as a proxy measure for the dissipative function. Using data on the diversity, biomass and respiration rates from three lakes, Aoki was able to link the trophic diversity of the system, and by proxy the dissipative function, to the degree of eutrophication in these aquatic ecosystems. He therefore argues that gradient dissipation leads to increased diversity and eutrophication in aquatic communities. He uses this community level data to argue that the degree of eutrophication in lakes represents an “arrow of time” in aquatic ecosystem, (Aoki, 2006). Understandably, this theory has been difficult to test with any tool other than mathematics. However, a microbial system that relied primarily on one resource might provide an excellent model to study the main theoretical predictions.
1.3 The role of adaptive radiation and niche construction in generating complexity

Adaptive radiation is an important mechanism by which new species (diversity) arise to generate biodiversity on earth. It is defined as a process in which new species arise from an ancestral species in order to exploit different resources or occupy a different niche from the ancestor (Schluter, 2000). Perhaps the most famous example of adaptive radiation are the finches on the Galapagos islands known as “Darwin’s finches”. These birds have evolved a range of beak shapes which relate to the foods that they can utilize. These birds evolved from a common ancestor but avoid direct competition with each other by feeding on different food sources and this is made possible by this evolution of different beak shapes (Darwin, 1859; Lack, 1947). Moreover, adaptive radiation has been observed in a variety of other species, the most widely studied being Anolis lizards (Losos et al, 1998; Knox et al, 2001), cichlids in the African lakes (Seehausien et al, 2006) and Hawaiian honey creepers (Lerner et al, 2011; Lovette et al, 2001). The soil bacterium Pseudomonas fluorescens SBW25 (used for this study) is another famous study system for adaptive radiation (Rainey and Travisano, 1998) and its suitability for use as a model of early life is discussed below. Central to the ecological theory of adaptive radiation (Schluter, 2000) is the idea that in response to ecological opportunity (Yoder et al, 2010) a new niche arises and a species will develop in order to exploit this new resource/area.

One process that generates the ecological opportunity that drives adaptive radiation is niche construction. Niche construction has developed over the past 15 years as part of modern evolutionary theory. Niche construction may be defined as any organism–mediated modification to the external environment that alters selection pressures experienced by the
organism doing the modification or by any other organism in the system (Odling-Smee et al., 2003, Odling-Smee et al., 2013). Matthews et al. (2014) establish two criteria for identifying niche construction. First, a candidate niche construction agent must significantly alter environmental conditions and second the agent mediated environmental conditions must influence selection pressures on a niche construction recipient. A common example of niche construction is lactose tolerance in human populations. Tolerance for lactose originally only lasted until weaning; however with the development of agriculture (a human mediated modification) milk increasingly became a highly nutritional food source for children and adults. This nutritional subsidy altered selection pressures on human populations, allowing individuals with a lactose tolerance to gain a fitness advantage. A gene for lactose tolerance is now widespread in human communities, particularly in the descendants of those associated with early agriculture (Gerbault et al., 2011).

There are a wide range of activities that organisms perform such as trophic interactions and metabolism, which may affect the selection pressures faced by that organism or other organisms in the surrounding environment. Bacteria provide good model systems for exploring how an organism’s metabolism modifies the surrounding environment and affects the selection pressures faced by itself and other organisms in the ecosystem. For example, when Escherichia coli is grown in medium which contains glucose and acetate as carbon sources, it will preferentially use glucose as a primary carbon source (excreting further acetate) before switching to acetate when glucose is depleted (Herron and Doebeli, 2013). As a result, selection pressures faced by subsequent generations of bacteria are strongly determined by how much metabolism has been carried out by previous generations. Prior metabolism not only alters the levels of the preferential nutrient, glucose, but it also excretes
acetate during the metabolism of glucose, and this can affect the fitness landscape of organisms in subsequent generations.

Niche construction is interesting in the context of a dissipative ecosystem as it provides a mechanism by which organisms can alter the selection pressures in the system and give rise to new strategies for energy use, in effect establishing the feedbacks predicted by Nicolis and Prigogine (1977). This fits well with a viewpoint in which niche constructing activities can be seen to create “ecological opportunity” (Yoder et al, 2010) that could influence either coexistence or diversification. This could occur for example if the metabolic byproducts of one species provide a source of nutrition for another species. Ecological opportunity has often been cited as a possible reason for adaptive radiation as species will evolve to exploit empty niches and under exploited resources (Yoder et al, 2010. Losos et al. 2010). In fact the idea that a system will evolve leading to an increase in the dissipation of an external resource, supports the role of ecological opportunity in adaptive radiation. Evolution of new pathways to dissipate the gradient may involve the evolution of new species or innovations within existing species to exploit resources. In the E. coli example above, the ability to metabolize a new carbon source arises as a result of niche construction and this in turns allows for an even more efficient dissipation of the resources available in the media as acetate is now used in addition to glucose. Without the feedback between environmental modification and evolutionary responses (i.e. evolution by niche construction), dissipation of the resources would be limited to only glucose.
1.4 *Pseudomonas fluorescens* SBW25 as model system for Ecology, Evolution and Dissipative systems theory.

The soil bacterium *Pseudomonas fluorescens* SBW25 is a widely used model system to study evolutionary processes and has been studied extensively since the seminal work of Rainey and Travisano in 1998. In this key work they determined that when allowed to grow under static (unshaken) conditions the SBW25 strain would diverge into a variety of phenotypically different morphotypes. This divergence progresses in 30ml glass vials filled with 6ml of liquid KB media. Figure 1 shows the main morphotypes as they first appeared in Rainey and Travisano, 1998. The diversification of the morphs is driven by competition for the limiting resource oxygen, (Rainey and Travisano, 1998; Ibelings et al, included in this thesis). The ancestral smooth type (SM) grows in the broth phase of the liquid microcosm while oxygen is plentiful. As the bacteria grow this oxygen is rapidly depleted and a new niche is created at the air to liquid interface, this is the only area in the microcosm where oxygen is replenished (Ibelings et al, included in this thesis). A new morphotype the wrinkly spreader (WS – of which there is a range, large spreading wrinkly spreader is shown in figure 1) arises and occupies this new niche through the overproduction of cellulose (Spiers et al, 2003). The wrinkly spreader arises on day 2-3 and forms a mat that monopolizes the oxygen supply in the microcosm until day 4-5. Smooth types arise as cheats in the mat through mutation, however as they contribute nothing to mat strength they lead to the ultimate collapse of the mat (Rainey and Rainey, 2003). After the collapse of the mat the fuzzy spreader can be detected, in the original work this morphotype was thought to occupy the bottom of the glass vial below the broth phase and it is shown growing such in figure 1. However, a recent revision by Ferguson (2013) has shown that the FS also competes for oxygen at the air liquid
interface by forming flimsy rafts. These rafts are weak and readily collapse leaving the debris to fall to the bottom of the glass vial.

**Figure 1.1**

![Figure 1.1](image-url)

**Figure 1.1** is adapted from Rainey and Travisano, 1998 and shows the colony morphology of the three dominant morphotypes (SM (smooth), WS (wrinkly spreader), FS (fuzzy spreader)) which arise during the adaptive radiation of *Pseudomonas fluorescens*. This figure also shows the niche of the SM and WS types. The SM grows in main column of the broth while oxygen is plentiful and the WS type forms a biofilm at the AL interface. The FS is shown growing at the bottom of the vial but this niche preference has recently been revised based on work by Ferguson *et al.*, (2013) and Ibelings *et al.*, included in this report.

Although originally used as a model system for the study of adaptive radiation the system has been exploited to study a variety of other processes in evolutionary biology. For example Fukami and colleagues (2007) used the system to study whether or not the immigration history in a population could affect the diversity that could be achieved in the community.
They found that when wrinkly spreader types were added before or soon after the ancestral type then its presence vastly reduces the diversity that can be achieved in the radiation. The system has also been used to study bet hedging by Beaumont et al (2009). In this study the authors discovered that when faced with a fluctuating environment the bacteria could develop a phenotypic switch. This work was important as it demonstrated that the system could adapt in order to maximize fitness in a variable environment. This is corroborated by work on the adaption of the bacteria to high or low quality environments by Buckling et al (2007). Kassen et al, (2004) also reported that the environment could create ecological constraints that would influence the size of the adaptive radiation. McDonald and colleagues (2009) found that genetic constraints determined which would be the most likely mutations leading to the wrinkly spreader phenotype. This exploration found that in the majority of cases the wrinkly spreader phenotype arose from one of three genetic pathways, and it was these genetic constraints that allow a focus on the phenotypic differences in the system. The research presented in this thesis is also not the only case in which the system has been used to explore concepts of early life, Hammerschmidt and colleagues (2014) among others (Rainey and Kerr, 2010; Libby and Rainey, 2013), have used this system to study the transition from unicellular to multicellular life.

Like all bacteria, *Pseudomonas fluorescens* SBW25 is a thermodynamically open non-equilibrium system that is capable of self-organization, self-reproduction and can pass its genetic material on to subsequent generations (Trevors, 2010). This organism lends itself particularly well to studying the concept of a dissipative ecosystem within modern evolutionary theory as it has already proven to be an effective model system to study adaptive radiation (Rainey and Travisano, 1998) and niche construction (Callahan *et al*, 2014, Ibelings *et al*, included in this thesis).
In this thesis the *Pseudomonas fluorescens* system is proposed as a model system to test the predictions of dissipative systems theory which are summarized in table 1. It appears the common evolutionary trajectory is towards a more effective dissipation of the gradient. In the case of *Pseudomonas fluorescens* it is likely that the ability to maximally dissipate the gradient is not present in a single genotype as the growth of the bacteria themselves alter the optimal strategy for utilizing oxygen on a temporal scale. In this way the system provides a compelling example of the role of eco-evolutionary feedbacks in the acceleration of the gradient dissipation. In this system niche construction theory can help us to understand the evolution of the dissipative ecosystem. Metabolism tends to evolve towards the highest level of global entropy production (Unrean and Srienc, 2011) and maximum energy dissipation, and so metabolism is the most effective method by which *Pseudomonas fluorescens* can dissipate the oxygen gradient in the microcosm. However, little is known about how metabolic variation of *Pseudomonas* genotypes both affects environmental conditions and responds to environmental conditions. The smooth types have the highest rate of metabolism as they expend less energy as they do not produce an extra cellular polymer such as cellulose, (as in the case of the wrinkly spreader). Therefore, in the initial stages of the reaction (i.e. ecosystem inoculation), the smooth types alone achieve high levels of dissipation of the oxygen gradient through metabolism. However, this metabolism depletes the oxygen within the broth phase and changes the selective environment of the bacteria. This metabolism creates ecological opportunity by creating a new niche at the air to liquid interface. As this soon becomes the only place where oxygen is replenished, and being able to colonize it provides a selective advantage (analogous to a key innovation). The activities of the SM create a selective environment that favours the WS, which by colonizing fully the AL interface leads to a progressive increase in the dissipation of the oxygen gradient over time. However, as the
metabolism of the smooth types more efficiently dissipate the gradient than the metabolism
of the wrinkly spreader, cheats can colonize the mat further enhancing the rate of oxygen
consumption in the mat. After the mat collapses under impact of the cheaters, the fuzzy
spreader evolves and this leads to greater dissipation at the AL interface until the mat can
form again. In this light, both niche construction and adaptive radiation in *Pseudomonas
fluorescens* make perfect sense within the context of a dissipative ecosystem as diversification
is driven by ecological opportunities that develop over time that eventually lead to a greater
dissipation of the oxygen.

**Table 1:** Is an excerpt from Loudon et al, 2015 and gives the main predictions of dissipative
systems theory in the first column, the requirements of a model system to test these in the
second column and the features of *Pseudomonas fluorescens* SBW25 that meet these
requirements in the third column.

<table>
<thead>
<tr>
<th>Prediction of dissipative systems theory</th>
<th>Requirements of a model system</th>
<th><em>Pseudomonas fluorescens</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximal dissipation of a free energy gradient by the system can be achieved by the development of complex structures that dissipate energy and strategies/processes for maintaining energy dissipation over time.</td>
<td>• The system must have a method of generating complex structure or strategies • Association between diversity change and change in resource utilization</td>
<td>• <em>Pseudomonas fluorescens</em> undergoes an adaptive radiation (<a href="#">Rainey and Travisano, 1998</a>), driven by the limiting resource oxygen (free energy source). • Each of the species that arise in the system has a different strategy for oxygen use, (<a href="#">Rainey and Travisano, 1998</a>; <a href="#">Rainey and Rainey, 2003</a>; <a href="#">Ferguson et al, 2013</a>; <a href="#">Ibelings et al, in prep</a>)</td>
</tr>
<tr>
<td>To ensure continued energy</td>
<td>• Organisms alter their</td>
<td>• In the <em>Pseudomonas</em></td>
</tr>
</tbody>
</table>
dissipation, feedbacks should exist between the system and the external environment.

- Organisms mediated environmental modification influences the development and maintenance of diversity (i.e. selection feedbacks are present and can be manipulated)

- The growth of the smooth morphotype creates the niche at the air to liquid interface which creates the environmental conditions which make the growth of the wrinkly spreader beneficial.

- System we can estimate the entropy production of the bacteria based on the respiration rate. We can accurately measure the oxygen use of the bacteria using microsensors.

- We can estimate the entropy production of the bacteria based on the respiration rate. We can accurately measure the oxygen use of the bacteria using microsensors.

It is worthwhile to note that the adaptive radiation of *Pseudomonas fluorescens* follows a pattern similar to that proposed by Nicolis and Prigogine (1977) for a single species in a resource limited medium. In a simple model they found that when new mutants arose from a single population, a successful mutant would either be a more efficient colonizer of the same niche, in which case competitive replacement would occur or it would occupy a different niche. Although different, both of these conditions lead to greater exploitation of the resources available in the media and greater dissipation of the gradient. Models of dissipative systems and resource competition (such as have commonly be applied to *Pseudomonas fluorescens* SBW25) have a common structure, in that mutants can either be successful by colonizing a new niche (i.e. a novel innovation) or by competitively displacing an existing species utilizing the same resource. Models of dissipative systems predict that the strength of the feedback between environmental modification and evolutionary dynamics will change over time. Microbes are an excellent model system to explore such feedbacks, particularly when one can control the environmental distribution of resources and track
evolutionary changes over the same time scale. Microbes are particularly useful for the study of dissipative systems theory as applied to the biological sciences as it is possible to exert external gradients on them in laboratory manipulations. The use of microbes such as Pseudomonas fluorescens to explore ideas in ecology and evolution is reviewed in Buckling et al (2009). Although this thesis tests specifically whether or not Pseudomonas fluorescens could be used as a model system to test the predictions of dissipative systems theory, there were two previous works in the system that seemed particularly compatible with DST. Bailey and colleagues, (2013), examined the effect of competition on the adaptive radiation of Pseudomonas fluorescens. One of the key results of this study was that strong competitors for the same resources as the focal lineage could prevent diversification due to niche pre-emption and competitive exclusion, this is concurrent with the prediction from Nicolis and Prigogine’s work that new mutants arising in a resource limited environment will competitively replace existing species if they occupy the same niche. Interestingly, this work also found that the presence of weak competitors (such as the rare morph explored in chapter 5) of this thesis which occupies the very edge of AL interface can actually promote diversification as they will have sufficient numbers in the first days of the adaptive radiation to increase resource competition without diversifying themselves. The rare morph in question is found to be incapable of diversifying into any of the other Pseudomonas niche specialists. The theory of a dissipative system may also shed some light on the “overshooting” phenomenon studied by Meyer and colleagues, (2011), which refers to the rapid increase in diversity found in the initial stages of an adaptive radiation, some of which is lost over time as ecological opportunity decreases. Meyer et al. (2011) found that the loss of diversity observed in the later stages of the Pseudomonas fluorescens adaptive radiation was due to a loss of wrinkly spreader genotypes, although the wrinkly spreader phenotype itself remained and became more adapted to the conditions in the microcosm. This was expected when oxygen levels
were increased in order to test prediction 1 of DST. It is expected that greater complexity will be found when the bacteria are incubated under oxygen, because increased oxygen functions as an abundant free energy gradient but also creates ecological opportunity by increasing levels of a resource which is usually limiting. If this greater complexity represents the system’s strategy to most efficiently dissipate the oxygen gradient then it makes sense that the greatest complexity would be found in the initial stages of the experiment carried out by Meyer and colleagues before ecological opportunity decreases and only the most efficient wrinkly spreader types remain. The sustained complexity found under pure oxygen compared to air may be due to the continued opportunity created in the search for more pathways to dissipate the oxygen.

1.5 Aims of the thesis

The main goal of this thesis is to test if the bacterium *Pseudomonas fluorescens* SBW25 can be used as model system to test the predictions of dissipative systems theory from statistical mechanics. We aim to test theory by testing several sub predictions.

- To determine if *Pseudomonas fluorescens* does indeed undergo its adaptive radiation in order to maximally use oxygen (the free energy gradient) it would need to be established that oxygen played an important role in the system. It has been postulated since Rainey and Travisano, (1998) that oxygen is the limiting resource but a further understanding of how the oxygen gradient relates to complexity (diversity) in the system is required. Chapters 2 and 4 test this prediction.
• One of the main predictions of DST is that feedbacks will develop between a dissipative structure and the abiotic environment in order to allow continued free energy dissipation. This is tested experimentally in chapter 3.

• Another prediction of DST is that complexity in the system is related to an increase in the free energy to be dissipated through irreversible entropy generating processes. This prediction is tested in chapter 2 and 4.

• Nicolis and Prigogine (1977) predicted that poor dissipaters of free energy gradients would be competitively replaced by more efficient ones, this is explored in chapter 5.

1.6.1 Thesis structure

The study presented in this thesis provides several tests to assess the suitability of the bacterium *Pseudomonas fluorescens SBW25* to be used as a model system to test dissipative systems theory. The thesis chapters are presented in the format of articles that have been submitted or are being prepared for submission. Each chapter contains an introduction and a methodology section specific to its study. The structure of the thesis allows for a more concise understanding of the results found in each study without having to refer to other areas of the thesis. A general discussion of the results found across the studies is presented as Chapter 6.

1.6.2 Institutional framework

The results presented in this thesis were obtained through a collaborative work between three institutions:

• University of Geneva (UNIGE), Faculty of Sciences, Earth and Environmental sciences, Institute FA Forel and Institute of Environmental sciences, Bd Carl Vogt 66, CH- 1211
Geneva, (PhD carried out under the Supervision of Professor. Bastiaan Ibelings and in collaboration with Dr. Patrick Venail)

- Max Planck Institute for Marine Microbiology, Celsiusstraße 1, 28359 Bremen, Germany (Work carried out in the Microsensor group led by Dr. Dirk De Beer in collaboration with Dr. Duygu Sevilgen)

- New Zealand Institute for Advanced Study, Massey University, 102904, North Shore Mail Centre, Auckland 0745, New Zealand (Work carried out under the supervision of Professor Paul Rainey in collaboration with Dr. Katrin Hammerschmidt)

A substantial collaboration was also carried out with Dr. Blake Matthews at EAWAG, Switzerland.

1.6.3 Scholarships and funding

- PhD assistant funding from the University of Geneva (November 2012 – January 2016)

- Academic society of Geneva award (September 2014 – December 2014)

1.6.4 Publication from this research


The following are a list of publications in preparation

- **Loudon CM, Matthews B, Venail P, Sevilgen DS, Ibelings BW**, “Historical energy conditions can have an effect on the generation of complexity and energy dissipation, even in a completely new environment”
• **Loudon CM**, Hammerschmidt K, Matthews B, Sevilgen DS, Farr AD, Lind P, Rainey PB, Ibelings BW, “The evolution and later extinction of a low fitness morphotype within a dissipative ecosystem”

• Ibelings BW, Stief P, **Loudon CM**, Gieseke A, Scwhermer C, Seehausen O, Rainey PB, “Fallible strategies in the acquisition of limiting oxygen maintain coexistence at the air-liquid interface of *Pseudomonas fluorescens* microcosms”
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Fallible strategies in the acquisition of limiting oxygen maintain coexistence at the air-liquid interface of *Pseudomonas fluorescens* microcosms

**Manuscript status:** This manuscript is included in this thesis as it was prepared for submission in December 2014. The manuscript is currently being improved with the addition of a mathematical model. It is likely to be submitted some time in 2015 but was not ready in its final form before the submission of this thesis.

**Candidate contribution:** I am a co-author on this paper and a substantial portion of the lab time in my PhD was dedicated to this work so it is included in my thesis. I carried out experiments to confirm the oxygen profiles, the diversity results and the invasion from rare results.

**Context:** These initial experiments inspired the perspective which is explored in the remainder of the experimental chapters of this thesis. Importantly this work established the critical role of oxygen gradients in generating diversity (complexity) in the *Pseudomonas fluorescens* SBW25 system. One of the key results of this paper that increasing the oxygen concentration leads to increased diversity is one of the key features of the system which makes it an amiable model to study the key features of dissipative systems theory.
Fallible strategies in the acquisition of limiting oxygen maintain coexistence at the air-liquid interface of *Pseudomonas fluorescens* microcosms

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BWI designed and performed experiments, analysed data and wrote major parts of the manuscript; PS, AG, CS and CL designed and performed experiments, provided methods for microelectrode measurements, analysed data and wrote parts of the manuscript; OS interpreted data and wrote parts of the manuscript; PBR designed experiments, interpreted data and wrote large parts of the manuscript.
Abstract

Adaptive radiation in experimental populations of *Pseudomonas fluorescens* is seen as an exemplar of ecological diversification driven by divergent selection, resulting in the emergence of spatially distinct niche specialists (ecotypes). Coexistence and diversity is promoted through competitive non-transitivity of specialist strategies (as in a game of rock-paper-scissors) which evolve in response to decreasing availability of the key limiting resource, oxygen. We propose a partial revision of current understanding of the *Pseudomonas* radiation and demonstrate that, whilst retaining frequency-dependent selection, coexistence of the main three, heritably stable ecotypes is explained by the dynamic fallibility of each resource-utilization strategy. Fallibility, which is a consequence of disturbance events that are themselves a consequence of the various ecoytypes growth dynamics. The key effector of evolutionary change is dynamics in formation and collapse of wrinkly-spreader fabricated mats at the air-to-liquid interface, which provides a striking example of niche-construction. Since renewable oxygen is only available at the interface, all ecotypes compete for the same spatial niche. Diversity is maintained dynamically since no singular resource acquisition strategy is superior at all times. Concluding, we provide evidence that non-transitivity maintains diversity of *Pseudomonas* ecotypes through temporal rather than spatial coexistence, as has been the prevailing theory in literature.
2.1 Introduction

Biological diversity arises from a complex interplay of factors. From an ecological stance, vacant niche space alone can be sufficient to promote rapid lineage splitting. Provided niches are sufficiently discrete to cause trade-offs between niche-specialists, a process of diversification, known as adaptive radiation, ensues. Where niches overlap – as in sympatry – competition is a necessary additional factor (Rundle & Nosil 2005). Adaptive radiation results in the formation of new species (Schluter 2000) and adaptation of constituent species to divergent niches (Gavrilets & Losos 2009). In classical evolutionary theory it was assumed that speciation could only occur through allopatry, allopatric speciation is facilitated by divergent natural selection between isolated environments, unimpeded by gene flow (Mayr 1963). However, in the last decade, significant progress has led to the conclusion that parapatric and sympatric speciation are also possible (Coyne & Orr 2004; Seehausen et al. 2008). This work suggests that diversification may be an adaptive response to frequency-dependent (FD) interactions (Bridle & Jiggins 2000; Tautz 2003).

As a model for studying adaptive radiation, experimental populations of *Pseudomonas fluorescens* SBW25 have proven useful (Rainey & Travisano 1998; Buckling et al. 2000; Kassen et al. 2000; MacLean 2005; Fukami et al. 2007; Jasmin & Kassen 2007; Bailey et al., 2013). When propagated in a spatially structured environment populations founded by a single ancestral type rapidly and predictably diversify into a range of niche-adapted ecotypes. These studies confirmed experimentally that diversification is dependent upon ecological opportunity, and that intraspecific competition drives lineage-splitting. Explanations for diversification point logically to sympatric processes fuelled by divergent selection. While evidence of trade-offs and FD-interactions support this view (Rainey & Travisano 1998), much rested on observational evidence that the derived types are adapted to spatially distinct niches. For example, smooth (SM) types were described to inhabit the broth phase, while the wrinkly spreader (WS) and fuzzy spreader (FS) morphotypes, inhabit the air-to-liquid (AL)
interface, and microcosm bottom, respectively. Wrinkly spreaders as a group are able to colonize the AL interface, in contrast to the ancestral smooth morphotype, by producing a cell to cell glue (Spiers 2014). Eventually WS mats collapse through invasion of SM cheats (Rainey & Rainey, 2003), which are at the same time fundamental for the maintenance of collective mat reproduction (Hammerschmidt et al., 2014).

Recent work on the system, however, has required a revision of the niche of the fuzzy spreader (Ferguson et al., 2013). As all the main morphs of *Pseudomonas fluorescens* SBW25 are motile and like all *Pseudomonas* species tax towards oxygen, (Smith & Doetsch, 1969), it was not clear why the FS would show an apparent preference for the vial bottom—an environment that for the most part is completely devoid of oxygen (Koza et al., 2010). In an attempt to understand the seemingly paradoxical niche preference of FS, Ferguson et al. (2013) used time-lapse photography to monitor growth in static microcosms over the course of three days. Based on these movies they concluded that FS does not grow at the bottom of the microcosm, but instead at the AL interface, just like wrinkly spreaders. FS, however, forms thin rafts (flocs) of cells which, once of a certain size (~2-3 mm in diameter), cannot be sustained at the surface, and sink rapidly to the bottom. The biomass that accumulates at the base of the vial is cellular detritus. FS, like WS, has thus evolved the capacity to compete for limiting oxygen at AL interface, and this begs the question how the ecotypes manage to stably coexist competing for a single limiting resource in the same spatial niche.

While ecological diversification indeed is a likely scenario the details and underlying mechanisms are not sufficiently clear. For example, oxygen is often cited as the primary limiting resource that drives the radiation (Rainey & Travisano 1998; Rainey & Rainey 2003). The notion that the activities of bacteria themselves deplete the available oxygen is both intuitive and supported by steep oxygen profiles in static cultures (Koza et al. 2010), but how the various ecotypes which evolve can coexist while competing for a single limiting resource in the same spatial niche remains uncertain. Here, recognizing the need for deeper insight into
ecological processes underpinning adaptive radiation in *Pseudomonas* cultures, we focus on the role of oxygen and show that the three dominant ecotypes are all “oxygen specialists” in their own right, each with a different strategy for acquiring this limiting resource at AL interface. Importantly, none of these strategies is superior at all times. Diversity is maintained in a dynamic manner and through disturbance events which are in part the outcome of the activities of the main ecotypes, which modify the environmental conditions in the microcosms. Our experimental results necessitate a partially revised understanding of the type of the mechanisms maintaining coexistence and the type of selection leading to adaptive radiation. Our study provides novel insight into the nature of the frequency dependent ecological interactions, showing new ways of species co-existence on a single resource.

2.2 Materials and methods

*Pseudomonas fluorescens* SBW25 wild type was grown in standard microcosms as described earlier (Rainey & Travisano 1998). Briefly, 5 mL of Kings-B-liquid-broth was dispensed into glass vials left under static conditions at 28° C. Vials were inoculated with ca. 1000 *Pseudomonas* cells (either SM, WS or FS). In addition, controls were included comprising uninoculated vials. Experiments were performed under different oxygen atmospheres. For this, vials were placed inside gas-tight Perspex incubators, and continuously flushed with nitrogen, air or pure oxygen at 0.5 L per min. At inoculation and every 24 h for 8 days, oxygen microprofiles were recorded in three replicate vials of each of the three morphotypes incubated in each of the three oxygen atmospheres. Oxygen microsensors were constructed at MPI-Bremen, calibrated before and after profiling, and used in a measuring set-up as described previously (Polerecky et al. 2007). An oxygen microsensor was inserted into the incubator through a narrow hole in the top lid of the incubator, exactly above one of the inoculated vials. The sensor tip was positioned precisely at the AL-interface aided by a computer-controlled micromanipulator and a dissection microscope. Custom-made programs
were used for measurement automation and data acquisition. Vertical profiles were started at the AL-interface with increments of 50 µm down to 500 µm depth, and with increments of 250 µm between 500 and 4000 µm depth. The slope of the oxygen gradient was calculated by regression analysis for the linear stretch of the concentration profile just below AL-interface.

The diversity of SM, WS and FS morphotypes was checked every other day by destructive harvesting and plating using methods described earlier (Rainey & Travisano 1998). An additional experiment was specifically set up to study how collapse of the WS mat alters oxygen penetration: triplicate vials were inoculated with WS, and mats were allowed to form and mature over 96h. At that time the mats were displaced by carefully disconnecting them from the glass wall of the vial using a sterile glass rod, resulting in their collapse. Oxygen profiles were measured at inoculation, just prior to disturbance, directly after mats had collapsed, and at 2, 4, 10 and 24 h after disturbance. In a following experiment, we tested how the capability of a rare morphotype to invade a resident population of an abundant morphotype depends on oxygen concentration and steepness of resource gradients. At the start, rare (invasive) morphotypes were mixed with common (resident) morphotypes in a ratio of 2:100 with a total cell number of ca. 1250 cells per vial. All possible six pair wise combinations of SM, WS and FS were included in triplicate competition experiments, performed under nitrogen, air or oxygen. Abundance of the two competing morphotypes was checked after seven days (Rainey & Travisano 1998). The Malthusian growth parameter (r) for a particular morphotype was calculated as \( r = \ln \left( \frac{N_i(t)}{N_i(0)} \right) / t \), where \( N_i(t) \) and \( N_i(0) \) are the final and initial densities, respectively. The relative fitness of genotype i to genotype j was calculated as the selection rate constant \( s_{ij} = r_i - r_j \).

Statistical tests were performed with PASW Statistics-18. Results from the temporal dynamics experiments were tested with repeated measures ANOVA. Results from the competition experiments were analysed with a two-way ANOVA for each of the pairs of competitors. Bonferroni was used as post hoc test. ANOVA was performed after testing for
compliance of normality of data using a Kolmogorov-Smirnov test (p > 0.05), where necessary after log transformation of the data, and homogeneity of variance using Levene’s-test (p > 0.05). Repeated measures ANOVA were tested for sphericity using Mauchly’s-test (p > 0.05).

2.3 Results

Growth of ancestral *Pseudomonas fluorescens*-SBW25 in three day old spent, but filter-sterilised, King’s-Medium-B is indistinguishable from growth in fresh medium (P.B.Rainey, unpublished data) and demonstrates that resources available in the growth medium are not limiting – at least during early stages of the radiation. In contrast, as we show, oxygen becomes severely limiting within 24 h. The middle panel of Fig. 1 shows the oxygen profile from the moment just prior to inoculation and through successive 24 h periods for eight days. Prior to inoculation oxygen is evenly distributed through the microcosm, with the concentration being unaffected by depth into the broth phase. In controls, without *Pseudomonas*, this homogenous oxygen distribution was maintained throughout (not shown). Within 24 h of growth a steep oxygen gradient develops. This is a direct result of the metabolic activities of the growing population: oxygen is depleted from all but the top ca. 150 μm of the broth phase. The gradient steepens through subsequent days, but after day five the slope gradually lessens.

In microcosms founded by the ancestral SM genotype, depletion of oxygen from all but the uppermost zone creates an environment that favours mat-forming WS types. In turn, WS-mats provide opportunity for various SM genotypes to prosper by hitchhiking within the fabric of the mat while contributing nothing toward mat strength. However, the presence of these hitchhiking cheats ultimately causes the mat to collapse (Rainey & Rainey 2003; Rainey & Kerr, 2010). To determine the impact of mat collapse on availability of oxygen, fresh microcosms were founded with a single WS genotype and the mat was allowed to develop over a three day period. At day three, oxygen penetration was monitored by carefully
lowering the oxygen probe through the mat and down into the broth phase. Measurements show that oxygen is absent from all but the top few micrometers (Fig. 2). After gently dislodging the mat from the glass wall to the point at which the mat collapses (and sinks) oxygen once again penetrates into the microcosm. However subsequent growth ensures rapid re-establishment of the steep gradient (Fig. 2).

Oxygen availability is restricted to a narrow layer at the AL-interface for much of the incubation period (Fig. 1), hence likely to severely limit population development. Competition for this limiting resource, during the initial stages of the radiation is the engine behind the *Pseudomonas* radiation. This means that the evolving populations experience strong directional selection for oxygen. If correct, then the notion that SM, FS and WS are adapted to spatially distinct niches (Rainey & Travisano 1998; Kassen 2009) is called into question. If oxygen is the primary limiting resource, then different morphotypes should each have a different strategy to ensure access to oxygen, for which the only renewable source is at the AL-interface. SM grows in the broth when oxygen is plentiful, WS by virtue of over produced adhesive polymers constructs a mat at the AL interface ensuring that cells have access to oxygen, (Spiers *et al*, 2002), and FS grows in rafts at the AL interface whilst this niche is temporarily empty after the collapse of the mat, (Ferguson *et al*, 2013).

The patterns of diversification under different oxygen availability are likely to arise from the ecological interactions among the different types and these are likely to be affected by resource availability. To test this – and thus understand the ecological mechanism underpinning the observed changes in diversity – we examined the invasion capability of each type when rare under nitrogen, air and oxygen. Fig. 3 shows the results from these pair wise competition experiments. This figure shows that the nature of the interactions changes with oxygen content in the atmosphere (also see results of post-hoc analysis in Appendix 3, Supporting Information). Under nitrogen, SM and FS cannot invade against WS (as indicated by the zero selection rate constants in the boxed arrows of Fig. 3). The fitness of WS relative
to SM and FS is significantly higher under nitrogen than under air or oxygen. The superior strategy of WS ensures access to the little oxygen (ca. 5–10 μM) that is available at the very surface of the broth under nitrogen, which leaks into the sealed incubators during profiling. Under air, SM can invade against WS, but FS cannot invade against WS, in accordance with results in earlier studies (Rainey & Travisano 1998). Under oxygen both SM and FS can invade from rare against WS, and the fitness advantage of WS decreases even more than under air. Logically, if it were possible to continue to increase oxygen, the fitness of WS would continue to decline. Indeed, it is perfectly reasonable to suppose that if oxygen were abundant and available evenly throughout the broth that there would be no advantage to mat building at the AL-interface by WS (or FS) and the cost - slow growth relative to SM - of being either of these two types would not be offset by any fitness advantage. We note that Koza and colleagues did not find an advantage for WS under the “low oxygen” conditions in their fitness experiments and this appears to contrast with our results. We can offer two potential explanations for this discrepancy: the first being that the competition experiments in their study were carried out at a ratio of 1:1 whereas ours were carried out as an invasion from rare with a ratio of 2:100, given the importance of frequency dependent interactions in the Pseudomonas system it is likely that the results from the experiments of Koza et al, (2010) and ourselves are not comparable. The second possible explanation may be that the competition experiments carried out by these authors were carried out for a shorter (3-5 days) period than the 7 days in our experiments, this is relevant as we found that the adaptive radiation was delayed by at least a day under the pure nitrogen conditions. Therefore, it may take longer for the fitness advantage that we observed for WS in the nitrogen experimental condition to become established.
2.4 Discussion

2.4.1 Dynamic coexistence

Existing models of diversification in static *Pseudomonas fluorescens* microcosms closely follow the ecological theory of adaptive radiation (Schluter, 2000) in which competition for limiting resources in a heterogeneous environment drives the diversification through frequency dependent interactions. Whereas our results are still in line with this model, a model of divergent selection leading to the evolution of spatially distinct niche-specialists that trade-off fitness across environments (MacLean 2005; Jasmin & Kassen 2007; Kassen 2009) seems no longer tenable. It would indeed seem that we can no longer rely on spatial niche segregation as a basis for stable coexistence, given the rapid development of anoxia in the broth phase, forcing all ecotypes to compete for limited space at the AL interface. Nevertheless competitive exclusion clearly is prevented and the diversity which evolves in the radiation is maintained. At first sight it would appear that wrinkly spreaders evolve a winning strategy, ensuring access to oxygen by building a strong mat at the AL interface (Spiers et al., 2013). Mat formation which in itself is the key event leading to formation of the steepest oxygen gradients (Figs. 1 and 2). This apparent superiority could lead to competitive exclusion, but as demonstrated in our experiments - which link patterns of diversification to dynamics in oxygen availability - disturbance events prevent this from happening. Once again the key event is in the evolution and growth dynamics of the main ecotypes themselves, in this case appearance and expansion of evolutionary cheats. Recent work by Hammerschmidt et al (2014) also points out how the production of SM cheats in the WS mats is essential for the long term dynamics of the system. Under conditions where cheats are maintained selection at the level of the collective (the mat) becomes decoupled from selection at the individual level and this results in perpetuation of mat formation and breakdown, which is not the case when cheats are purged from the radiation and mat formation is not sustained.
Hence we put forward that disturbance events, which are the outcome of evolutionary and ecological interactions between the ecotypes themselves are key to the maintenance of diversity in the single spatial niche occupied by the various Pseudomonas ecotypes. Often the relationship between the frequency - or intensity - of disturbance and diversity is assumed to be unimodal (Connel, 1978, Huston, 1979, MacKay & Currie, 2001). Recently this intermediate disturbance hypothesis (IDH) has come under severe scrutiny (Fox, 2013), who claims that a hump shaped relationship is rarely found in empirical studies and the three main mechanisms invoked to explain the IDH are logically invalid. Huston (2014) in a reply alleges that peak diversity at intermediate disturbance levels is only expected at intermediate productivity, and the two hypotheses – diversity peaking at intermediate levels of diversity and intermediate levels of productivity - cannot be seen separate. A hump shaped relationship between disturbance and diversity in Pseudomonas radiation has been reported by Buckling et al. (2000), Brockhurst et al. (2007) and Hall et al (2011), whilst Kassen et al. (2000) also provide an account of diversity peaking at intermediate productivity. Fox (2013) asserts that disturbance only results in the long term maintenance of diversity when the fluctuations act as stabilising mechanisms causing negative frequency dependent selection so that there is a clear advantage to being rare. Clearly this is the case for Pseudomonas (see Fig. 3), which would explain why IDH is supported in Pseudomonas experiments.

2.4.2 Fallibility of strategies

We see three main solutions to overcome the challenge of acquiring oxygen at the AL-interface: WS-mats, FS-rafts and SM-hitchhikers. What we observe is a dynamic interplay between the evolving population and a landscape which continuously changes as a consequence of the changing phenotypic composition of the population through FD-interactions (Weissing et al. 2011). In the case of Pseudomonas, co-existence through FD-interactions is a direct consequence of the fallibility of each of the strategies leading to time-
lagged frequency dependence and thus, in principle, perpetual cycling of types. The pathway leading to the evolution of WS from the ancestral SM phenotype, resulting in mat formation has been described earlier. In brief, the ability to colonize the AL-interface is dependent upon spontaneous mutations (McDonald et al. 2009) that lead to the production and excretion of an acetylated form of cellulose, combined with a pili-like attachment factor (Spiers et al. 2002; Spiers & Rainey 2005). Through colonization of the AL-interface WS monopolises almost all of the oxygen, creating severe oxygen limitation for cells within the broth phase. However, as previously shown, presence of the WS mat creates opportunity for ‘cheating’ types to hitchhike within the mat (Rainey & Rainey 2003). These ancestral-like SM-types, while obtaining access to the limiting resource to an extent not possible in the absence of the mat (Rainey 2005), bring about the demise of the mat. Along with collapse of the mat comes a further alteration of the environment: oxygen now penetrates into the broth phase (Fig. 2). Thus, while mat-forming WS triumphs over the broth colonizing ancestral type, the ancestral type eventually outcompetes the mat-forming type. In successfully competing with the mat-forming WS types, the ancestral SM type not only destroys WS's advantage, but also its own, giving a window of opportunity for FS to expand and form rafts at the momentarily ‘empty’ AL-interface. FS rafts being too weak to be invaded by SM cheats, help to restore WS dominance, thereby also reducing its own opportunity to remain at the AL interface.

Mats, once collapsed, cannot immediately reform, the previously built mat cannot be reinvigorated (Meintjes 2009). This delay in occupation of the AL-interface provides opportunity for FS to gain an advantage. Its capacity to form single-cell flocs at the meniscus means that it gains maximal access to limiting oxygen, but at the same time, the feeble and transient nature of the rafts means that FS is exempt from exploitation by SM (Ferguson et al., 2013). In fact, the growth of FS in a single layer at the meniscus, combined with the fact that SM cannot hitchhike with FS flocs, explains the previously reported observation that FS, when grown in direct competition with SM, results in significant inhibition of the latter (Rainey
2005). The next phase of the dynamic involves return of the WS, which readily out-competes FS at the AL-interface, and so the cycle continues. The three types all compete for the same resource and are maintained by FD-interactions that stem from the temporal dynamics of the different types, in true RPS fashion.

2.4.3 Niche construction

Hence the various mutants which evolve engage in a dynamic interaction, showing non-transitivity and this interaction prevents competitive exclusion. The disturbance events which maintain diversity are a direct consequence of the growth and metabolic activity of the organisms themselves. Organisms not only adapt to their environment, but actively modify and construct it as well (Laland et al. 2003; Odling-Smee et al. 2003). Niche construction results in an eco-evolutionary feedback in which the evolutionary trajectories of the organisms are modulated by their own impact on the environment. Feedback also occurs across generations with individuals adapting to the environment being shaped by the activity of their ancestors. Evolutionary feedbacks may be adaptive provided that they enhance the fitness of the niche constructor (Kylafis & Loreau 2008). Others argue that for eco-evolutionary feedbacks to occur populations must not only shape their environment, but must also possess the ability to evolve in response to the changes in the environment, i.e., construction of the environment must cause directional or disruptive selection on the population in question (Post & Palkovacs 2009, Matthews et al, 2014). Comprehensive empirical evidence requires documenting (1) strength of niche construction, (2) strength of selection and direction of evolution, and (3) strength of niche construction after evolution. These steps would indicate evolution by niche construction. The occurrence of these sequential steps during the process of *Pseudomonas* diversification is explained below.

In the *Pseudomonas* radiation niches do no pre-exist, but are generated in the process of evolution towards an initially singular adaptive peak. Ancestral SM was inoculated into an
environment with weak oxygen gradients resulting from diffusion limitation. Metabolic activity of the founding genotype rapidly generates a gradient in the supply of the limiting resource. The generation of this gradient provides opportunity for the evolution of diversity as it modifies the environment in a way that creates a new niche at the AL interface (step 1). We will argue in the next section that diversification in *Pseudomonas* may support a model in which the evolving populations experience a phase of directional selection, which turns disruptive and leads to evolutionary branching (step 2). One of the main morphotypes that evolves in response to opportunities provided by the niche-constructed environment, the WS type, has a disproportionately strong effect on the environment (step 3) through creation of oxygen gradients of extremely steep slope. With on-going evolution (in particular the evolution of SM hitchhikers from WS) the WS mat eventually collapses and the system reverts to a gentler environmental gradient (this constituting a further round of niche construction; step 4). Reduction of the oxygen gradient permits further diversification which would be denied if the gradient remained steep. Despite the temporary setback for WS, the niche constructing ability of WS remains advantageous in the long run, since WS types eventually rebuild a mat (Fig. 2), and with this, this morphotype regains preferential access to oxygen. FS rafts, filling the void at AL interface in the absence of a fully developed WS mat, do not sustain growth of the cheats, and this will help to support a return of WS (step 5). In formation of a physical structure that affects supply of the primary limiting resource, WS demonstrates key activities of an ecosystem engineer (Jones et al. 2010). But where ecosystem engineering is often focused on feed-forward ecological effects (Kylafis & Loreau 2008), WS activity includes clear evolutionary feedbacks from the constructed environment on the engineering organisms – an essential part of niche construction.
2.4.4 *Adaptive dynamics*

The notion that the activities of bacteria themselves deplete the available oxygen is both intuitive and supported by steep oxygen profiles in static cultures (Fig 1). But if true, then this fact carries with it the suggestion that the *Pseudomonas* radiation is fuelled, at least initially, by directional selection. This raises the possibility that diversification may result from a shift from a phase of directional selection where the population evolves towards a fitness optimum dictated by the abiotic environment, to a phase of disruptive selection dynamically emerging from the overexploitation of the original resource optimum. While well studied in theory, this dynamic change of an adaptive landscape and subsequent lineage splitting has to the best of our knowledge never been shown experimentally and we will now explore to what extent this scenario of evolutionary branching may explain the radiation of *Pseudomonas* in microcosms.

The establishment of ‘extreme’ oxygen gradients logically points at an initial phase of directional selection, during which variant types are generated by mutation and intense competition for oxygen favours diversification through the evolution of frequency dependent trade-offs (Bridle & Jiggins 2000; Doebeli & Dieckmann 2003; Rueffler et al. 2006). Directional selection is expected to drive the evolution of the mean phenotype towards the nearest adaptive peak, with selection turning negative frequency dependent as the population reaches carrying capacity at the adaptive optimum, such that resources for the most common phenotype are depleted, leaving this type at a fitness minimum. This then facilitates colonization of dynamically emerging adaptive peaks, driven by disruptive selection (Abrams et al. 2008). We envisage that evolution of WS can be seen as a key evolutionary innovation, allowing colonization of a novel adaptive peak, which was not available in the adaptive landscape of the ancestor.
This view of diversification in *Pseudomonas* has resemblance to speciation models based upon adaptive dynamics theory (Geritz et al. 2004) although there are clear deviations from standard theory too. Adaptive dynamics are a framework to model trait evolution - trait diversification - driven by FD-selection and are particularly suitable for incorporating ecological complexity (Brannstrom et al., 2013). Although adaptive dynamics has made important contribution to speciation theory (Gavriletz, 2014), opinions differ to whether the branching scenario is biologically plausible (Gavriletz, 2005). In the adaptive dynamics framework evolution proceeds in small steps, by successive invasion events where a resident population is challenged by mutants slightly differing from the residents. Adaptive dynamics reveal the evolutionary trajectory in a given ecological scenario and in particular it shows if a population will evolve towards disruptive selection, promoting diversification (Friesen et al. 2004; Weissing et al. 2011). Typically branching occurs when the slopes of the competition function are steeper than those of the resource distribution so that the under-utilized tails of the resource distribution can be monopolized by newly evolved phenotypes (Weissing et al., 2011). The model assumes a continuous spectrum of resources, for instance in case of the Darwin finches a distribution in seed size (Weissing et al., 2011).

For *Pseudomonas* the key limiting resource, oxygen, has no distribution. The oxygen gradient is initially created by the activity of ancestral SM-type. Steepness of the gradient (Fig. 1) creates a mismatch between the niche of the broth-inhabiting ancestor and availability of the primary limiting resource, possibly resulting in a phase of directional selection, restoring a match between availability of the resource – which is restricted to AL interface – and the newly evolved phenotype, mat forming WS. Density dependence of fitness among *Pseudomonas* types competing for shared resources in the same niche, guides the population towards a dynamically emerging fitness valley, the eventual escape from which happens through evolutionary branching (Geritz et al. 2004). Disruptive selection results in the promotion of polymorphism (Doebeli et al. 2004), and newly evolved ecotypes can –
temporarily – escape the most stringent competition (Dieckmann et al. 2004). We note that in case of *Pseudomonas* the opportunity – underutilized resources – may not be in underutilization of a non-existing resource distribution but in underutilized periods in time, moments when availability of oxygen temporarily increases as an outcome of the dynamic interactions between the ecotypes. In particular when SM hitchhiking cheats bring about the temporally demise of the WS mat, oxygen becomes available – as shown in Figs. 1 and 2 - for the raft building FS. Thus as proposed by adaptive dynamics theory evolutionary branching in the *Pseudomonas* radiation may be seen to create new opportunities to exploit gaps in utilization of the key resources. In this perception the sequence of phenotypic innovation would follow WS, which are the first to appear during an initial phase of directional selection, followed by evolution of cheats and FS when selection turns disruptive in response to the extreme competition for limiting oxygen when WS mats are fully developed.

Our experimental data may not fit the 'classical' criteria of disruptive selection in sexually reproducing organisms, which includes selection against intermediate phenotypes. The essential outcome of disruptive selection, however, is the creation of polymorphism, and in clonal *Pseudomonas* this polymorphism can be maintained without the need for assortative mating or the need to remove intermediate phenotypes. Evolutionary branching, a process driven by disruptive selection under conditions of FD-competition, can explain polymorphism, and ultimately speciation in strictly asexual populations.

**2.5 Conclusions**

Diversification of *Pseudomonas fluorescens* in spatially structured microcosms has become a "paradigmatic experimental model for adaptive radiation" (Kassen 2009). Although the development of steep oxygen gradients during the radiation has been shown before (Koza et al., 2014), our study adds significance by directly linking dynamics in the `oxygen landscape`
to patterns of diversification. The change in perception which arises from our observations is a shift from FD selection in a spatial context to frequency dependence maintaining diversity in a single spatial niche through perpetual cycling of the ecotypes. Revision of the existing model entails a transformation of the type of selection driving the diversification, a model of diversifying selection which has prevailed since the original study by Rainey & Travisano (1998). Our data are not inconsistent with a process of evolutionary branching, during which directional selection drives the population to a phenotype where ecological interactions cause disruptive selection at a moment where the evolving populations slide into a dynamically deepening fitness valley, because of intense competition for oxygen. Importantly the emergence of forces responsible for speciation is intrinsic to the system, emerging dynamically from adaptation in conjunction with spatially localised ecological interactions.

**Acknowledgments**

BWI is grateful to Dirk de Beer for hospitality received at Max Planck Institute for Marine Microbiology in Bremen, Germany. The authors are grateful to Michael Doebeli (University of British Columbia, Canada) for fruitful discussions.
References


Fig. 2.1. Oxygen microprofiles. Oxygen microprofiles recorded at daily intervals in vials inoculated with Pseudomonas, either SM (white symbols), WS (grey symbols) or FS (black symbols). The figure has three panels, the top panels shows results for nitrogen incubation, the middle panel for air and the bottom for oxygen. For the bottom panel a separate scale of the abscissa is included, showing oxygen concentrations for this treatment. Each profile represents the mean of 3 independent replicates (3 vials).
Fig. 2.2 Collapse WS mat. Oxygen micro-profiles of vials inoculated with WS. Profiles shown in the graph represent means of 3 independent vials. At \( t = 0 \) h the WS mat was carefully dislodged from the glass vial. Further profiles show reformation of the mat and steepening of the oxygen profile over first 24 h following disturbance. Colour coding: time at inoculation: black; time at pre-collapse with fully intact WS mat in position: grey; collapse at \( t = 0 \) h: pink; \( t = 2 \) h since collapse: cyan; \( t = 4 \) h: blue; \( t = 10 \) h: red; \( t = 24 \) h:
Fig. 2.3 Invasion from rare. Numbers in the arrows represent the selection rate constant $s_{ij}$, the difference in the Malthusian growth parameter $r_i$ of a rare morph invading an abundant resident morph $r_j$ ($s_{ij} = r_i - s_j$). Six pair wise competitions are shown between SM, WS and FS, each morph acting both as resident and as invading morph in each combination. Competition experiments were performed under 3 different levels of oxygen in sealed containers (nitrogen, air and oxygen). Zero values indicate negative values of $s_{ij}$, i.e. rare morph unable to invade.
Chapter 3

Experimental evidence that evolution by niche construction affects dissipative ecosystem dynamics.

Manuscript status: This manuscript was published in a special edition of the journal Evolutionary Ecology on the 21st September 2015. The research appears here as it was submitted for final review.

Candidate contribution: I carried out all of the experimental work for this manuscript and wrote the manuscript in collaboration with my co-authors.

Context: One of the major aims of this thesis was to explore the possibility of using Pseudomonas fluorescens SBW25 as a model system to test the predictions of dissipative systems theory. In this chapter we test one prediction of theory, this being that feedbacks between life and its environment will be essential to ensure efficient dissipation of free energy gradients and also the generation of complexity in a natural community. We test this in the context of the ecological theory of niche construction and find interesting results for both.
Experimental evidence that evolution by niche construction affects dissipative ecosystem dynamics

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Running head: Evolution by niche construction in a dissipative ecosystem

Keywords: Non-equilibrium thermodynamics, dissipative ecosystems, evolution by niche construction, entropy, Pseudomonas fluorescens SBW25
Abstract

Evolution by niche construction occurs when organism-mediated modification of the environment causes an evolutionary response. Physicists have postulated that evolution in general, and evolution mediated via feedbacks between organisms and their environment in particular (i.e. evolution by niche construction), could increase the capacity of biological systems to dissipate free energy in an open thermodynamic system, and help them maintain a state far from thermodynamic equilibrium. Here, we propose using the bacterium *Pseudomonas fluorescens* (strain SBW25) as a model system to experimentally test theories in both evolutionary biology (e.g. niche construction) and physics (e.g. dissipative systems theory). *P. fluorescens* rapidly and predictably evolves multiple strategies for exploiting oxygen in unmixed culture flasks. This evolutionary dynamic is mediated by feedbacks between the modification of the oxygen gradient by *P. fluorescens* and the ecological and evolutionary responses of *Pseudomonas* to modified environmental conditions. To confirm this, we experimentally manipulated two aspects of the system that influence the strength of the feedback between *P. fluorescens* and oxygen gradients in the system. First, we inhibited the metabolism of the strain used to inoculate the cultures, and, second, we disturbed the formation of mats at the air-liquid interface. Overall, we found convincing experimental evidence of evolution by niche construction, and conclude that this study system is amenable to experimental investigations of both niche construction and dissipative systems theory.
3.1 Introduction

For decades, physicists and biologists have been fascinated with how both the origin of life and the evolution of complex organisms seemingly defy the second law of thermodynamics, which states that the entropy of a closed system must always increase, and, as a result, all complex forms should break down as the universe tends towards disorder. In his seminal work “What is life?” (1944), Erwin Schrodinger suggested that living organisms could maintain their low entropy (highly ordered) state by operating as open thermodynamic systems, essentially taking in free energy from their environments and transforming it (via metabolism) into a more disordered and higher entropy form. Building on this idea, Prigogine (1955) used principles of non-equilibrium thermodynamics to develop dissipative systems theory (DST), and although it has been studied in the context of ecological, evolutionary, and ecosystem dynamics (Aoki, 2006; Schneider and Kay 1994(a) and (b); England, 2013), there have been no experimental tests of DST with a biological model system.

Dissipative system theory (DST) has two key predictions (Table 1). First, DST posits that when free energy is supplied to a system and pushes it out of thermodynamic equilibrium, the system can develop (or evolve) complex structures (or strategies) that will dissipate the source of free energy through irreversible processes (i.e. those that generate entropy). In biological systems, metabolism (e.g. respiration) is a fundamental irreversible process that generates entropy (Figure 1) and can be quantified by measuring gas fluxes in ecosystems. Second, DST predicts that when there is an increase in free energy applied to the system, feedbacks will develop between the system and the external environment leading to an increase in overall energy dissipation (or entropy production). In biological systems, such feedbacks can arise either with or without evolution. In the simplest ecological case, feedbacks can occur between population growth and resource utilization by organisms (e.g. growth feedbacks in Figure 1). Feedbacks can also be involved in evolutionary dynamics (e.g.
selection feedbacks in Figure 1), when selection pressures are altered by organism-mediated modification of the environment and this influences fitness variation within and among populations (Lenton et al. 1998, Matthews et al. 2014). Such evolutionary feedbacks are a hallmark of niche construction theory (Odling-Smee et al., 2013, Matthews et al. 2014), and their presence in a biological model system is useful for testing DST.

We propose that *Pseudomonas fluorescens* (SBW25) growing in a unshaken liquid broth culture with a continuous supply of oxygen at the surface may provide a useful model system for experimentally testing both dissipative systems theory (Nicolis and Prigogine 1977) and niche construction theory (Hamblin et al, Callahan et al, 2014) (Figure 1). The *Pseudomonas* system provides a rare opportunity to track both ecological and evolutionary dynamics along with modifications of resource gradients (i.e. oxygen concentrations in the culture) over short time periods. Respiration by *Pseudomonas* converts oxygen into carbon dioxide, and in doing so, increases entropy production (lowering the internal entropy) and maintains the system far from an equilibrium state (Figure 1). In such cultures, *P. fluorescens* undergoes an adaptive radiation (Rainey and Travisano, 1998), characterized by the rapid evolution of strains with observable phenotypic traits that reflect differences in their oxygen utilization strategies (Figure 1, 2). These phenotypes evolve repeatedly in these systems via mutation (Rainey and Travisano, 1998; McDonald et al, 2009), and have different effects on the oxygen gradient in the microcosms (Figure 1). Over the course of the radiation, oxygen dynamics can be quantified by using microelectrode measurements (Figure 2).

In the *P. fluorescens* model system, the outcome of the adaptive radiation is highly predictable (Figure 1, 2), making it a tractable system to test the relationship between the evolution of diversity and the modification of oxygen gradients in the system. In microcosms without *Pseudomonas*, oxygen is evenly distributed and the system is thermodynamic equilibrium.
because there is no flow of energy within the system (Figure 1, Scenario 1). At this point, both the slope of the oxygen gradient and the dissipation index is zero (Figure 2). The microcosms are initially inoculated with a smooth morphotype (SBW25), and respiration then generates a steep oxygen gradient over the depth of the flask (Figure 1, scenario 2). If no new strains were to evolve, then the system would equilibrate at an oxygen gradient (and dissipation index) that reflects feedbacks between the strain’s ability to grow in the prevailing oxygen conditions. However, as the gradient steepens, conditions become more amenable for wrinkly spreader morphotypes to increase in frequency and they begin to colonize the air-liquid interface (AL). Wrinkly spreaders repeatedly arise by mutation in these experiments (Spiers et al, 2002; Bantinaki et al, 2007; McDonald et al, 2009), and by overproducing cellulose they are able to form a biofilm that monopolizes oxygen availability at the surface (Figure 2). Two wrinkly spreader morphotypes are readily observable: a small wrinkly spreader (SWS) that colonizes the sides of the flasks, and a large wrinkly spreader (LWS) that establishes a floating mat in the flask. The mat, which becomes established in 2-3 days, is unstable because smooth types begin to appear as “cheats” in the mat (i.e. they produce no cellulose), and by contributing nothing to mat strength they ultimately cause it to collapse (Rainey and Rainey, 2003).

During this process of mat formation and collapse (Figure 1, Scenario 3), there is a selective feedback between the oxygen modification by the bacterial community and evolutionary responses. Selection pressures change from being stabilizing, where only a single strain is favored and wrinkly spreader mutants are selected against (i.e. Figure 1, scenario 2), to being disruptive and favoring the coexistence of multiple stains with different oxygen utilization strategies (Figure 1, scenario 3). After the mat collapses, a new fuzzy spreader (FS) mutant is able to colonize the vacant AL interface by forming fragile rafts of cells (e.g. typically around day 4-6), which then eventually sink to the bottom of the flask (Ferguson et al, 2013). The
process of mat formation, collapse, and re-colonization is then repeated over time, and there are presumably reciprocal interactions between environmental modification by the strains and relative fitness of different strains. The prevailing hypothesis is that coexistence of the multiple strains is maintained by non-transitive interactions (e.g. rock-paper-scissors competitive interactions), because none of the oxygen utilization strategies are infallible. We would predict that this stable coexistence maintains the steepest oxygen gradients in the system (Figure 1, Scenario 4).

A key feature of this model system that makes it useful for testing both evolutionary and physics theory, is that the evolutionary dynamics are likely mediated by feedbacks between how organisms modify the environment, and by how these modifications alter selection pressures and drive evolutionary responses (Figure 1). In Table 1, we summarize two key predictions of dissipative system theory and how the *Pseudomonas* system is well suited to test them (Table 1). The first prediction, and the focus of this paper, is that the presence of feedbacks between organisms and their environment is one way that systems can evolve more complex strategies (e.g. oxygen utilization) and dynamics (e.g. adaptive radiation) that increase the dissipation of external sources of free energy (i.e. oxygen in the system) and increase entropy production (Nicolis and Prigogine, 1977; Aoki, 2006). In order to test for the influence of feedbacks on the system dynamics (Table 1, Prediction 1), we manipulated two aspects of the *Pseudomonas* system that could affect the occurrence and strength of these feedbacks, and the progression of the adaptive radiation. Using a full factorial experimental design, we (i) manipulated the metabolism of *Pseudomonas* so as to reduce the ability of *Pseudomonas* to modify the oxygen environment, and we (ii) mechanically disturbed mat formation so as to limit the ability of wrinkly spreaders from monopolizing oxygen at the air-liquid interface (Figure 2). We used a metabolic inhibitor (Sodium Nitrite) at a concentration that would limit respiration but not dramatically alter growth rates and cell densities in the
system (Davidson et al, 2005; Quastel and Woolridge, 1927). One limitation of this approach is that inhibiting metabolism might also alter cell density dynamics, and in this model system it is extremely difficult to simultaneously track cell densities, diversity, and oxygen profiles in microcosms because sampling can be destructive. In order to compensate for this, we used duplicate sets of microcosms to track diversity and density in parallel with the oxygen profiles.

3.2 Materials and Methods

3.2.1 Design of the microcosm experiment

Static glass vial microcosms were prepared by adding 6ml of King’s B (King et al, 1954) growth media to 30 ml glass universal vials. Vials were inoculated with 6µl from an overnight culture of the ancestral strain SBW25 and then subjected to the following manipulations: (i) Sodium nitrite was added at a concentration of 0.3%, and (ii) the area at the AL interface was cleared using a sterile needle 3 times daily for the duration of the experiment. The design was as complete randomized block design with 4 treatment combinations (Table 2) replicated in 6 temporal blocks, each of which each ran for 6 days. Vials were sealed in an airtight Perspex chamber and compressed air was pumped into the chamber at a flow rate of 0.5L/per min using an electronic flowmeter. Triplicate vials were profiled using an oxygen microsensor over 6 days, and an additional 3 vials were prepared in order to destructively sample and plate for diversity on days 2 and 4.

3.2.2 Quantifying diversity in the system

The ancestral Pseudomonas fluorescens SBW25 strain was isolated from the leaf of a sugar beet plant from Oxford University farm, Wytham in 1989, (Rainey and Bailey, 1996). This was the only strain used in these experiments for the inoculation of the vials. The phenotypes that evolve in such experiments (Figure 2) can easily be distinguished by eye on an agar plate
These phenotypes are known to be genetically different (Spiers et al., 2003; Fukami et al., 2007, McDonald et al., 2009; Ferguson et al., 2013), and reflect different oxygen utilization strategies of the strains (Rainey and Travisano, 1998). Based on this previous work, we chose to quantify diversity by counting the number of different phenotypes on each plate (Rainey and Travisano, 1998; Rainey et al., 2011; Fukami et al., 2007; Hammerschmidt et al., 2014). This certainly underestimates the genetic diversity of the system, but it is a good approximation of the phenotypic diversity and is the most tractable approach for testing our hypotheses. We also quantified the density of the bacteria by counting the number of colony forming units (cfu) on a plate (Rainey and Travisano, 1998).

3.2.3 Quantifying the oxygen gradient using microsensors

The oxygen distribution within the microcosms (vials) was measured using oxygen microelectrodes with a guard cathode. These were constructed at MPI Bremen and as described previously (Revsbech, 1989). Measurements were conducted in triplicate per profile, and duplicate profiles were obtained for each vial. For each profile, the oxygen sensor was placed directly at the AL interface with the assistance of custom programs and a dissection microscope. Automatic profiles were generated using custom software and were recorded from 250 microns above the AL interface and at increments of 50 microns until a depth of 500 microns into the medium. Below this depth, readings were recorded at increments of 250 microns until a depth of 4000 microns (following Ibelings et al. in preparation). Linear calibration of the sensors was derived from measurements in air bubbled King’s B (King et al., 1954) growth media and MilliQ water (100% air saturation) and in N2 bubbled medium or (later in the experiment) in the anoxic part of the medium (0% air saturation). Oxygen concentrations were calculated using Unisense gas tables (Li and Gregory, 1974; Garcia and Gordon, 1992).
Data analysis of the oxygen profiles was carried out using the statistical analysis software R. A negative exponential function \( y = ae^{-bx} \) was fit to each oxygen profile in order to estimate the slope \( b \) of the oxygen gradient. We also calculated a “dissipation index” (Figure 2), to indicate differences in the thermodynamic state of the system (Figure 1) by comparing total oxygen consumption of the bacterial community.

3.3 Results

3.3.1 Diversity and abundance the bacterial community

As expected from previous studies (Rainey et al. 1998, Buckling et al. 2007; Kassen, 2002; Ferguson et al, 2013, Ibelings et al, in prep), we observed the rapid evolution of a diversity of oxygen utilization strategies (i.e. an adaptive radiation). More importantly, our manipulations of metabolism and mat formation had profound effects on the dynamics of the adaptive radiation (Figure 2). In all treatments, small wrinkly spreaders evolved within 2 days (Figure 3A), and diversity did not differ among treatments (ANOVA, \( p > 0.05 \)). After 4 days, diversity was higher in the treatments without sodium nitrite (i.e. MCTC and MDTC) (ANOVA: Metabolism effect: \( p < 0.001 \)), because of the evolution of both wrinkly spreaders (in both MCTC and MDTC treatments) and fuzzy spreaders (only in MCTC treatment). Diversity was also higher in the treatments with an undisturbed mat (MCTC and MCTD) (ANOVA: Mat Disturbance effect: \( p = 0.001 \)), because fuzzy spreaders evolved in the MCTC but not the MDTC treatment, and small wrinkly spreaders persisted in the MCTD but not the MDTD treatment. Bacterial densities varied widely among microcosms, but after 2 days the inhibition of metabolism by sodium nitrite did not lead to significantly lower bacterial densities (Figure 3B: ANOVA all \( p \)-values>0.05). After 4 days, we found a significant interaction effect on the bacterial density between the mat (M) and the metabolism (T) modification manipulations (ANOVA interaction \( p = 0.03 \)). However, this was only because cell densities in the MDTD were
significantly higher than those in the MCTD treatment (Tukeys post hoc test: p=0.03), but no other differences were found between treatments (Tukeys post hoc test: all other pairwise comparisons p>0.05). Importantly, cell densities were similar between treatments with (MCTD and MDTD) and without (MCTC and MDTC) sodium nitrite (Figure 3B, ANOVA: p=0.37). Overall, this suggests that the concentration of sodium nitrite that we used successfully manipulated metabolism without significantly affecting population growth. Nevertheless, we also acknowledge that there is substantial unexplained variation in bacterial densities among our experimental microcosms.

3.3.2 Dynamics of the oxygen profiles

The inhibition of respiration using sodium nitrite had an immediate effect on overall oxygen utilization of the bacterial community (Figure 4, figure 5). By the end of the experiment, treatments with sodium nitrite (MCTD, MDTD) had shallower oxygen slopes (Figure 5A: Day 5, p=0.0001; Day 6 p=0.0001) and a lower dissipation index (Figure 5B: Day 5, p < 0.0001; Day 6, p <0.0001) than those without sodium nitrite (MC_TC, MD_TC). The effect of mat disturbance on the distribution of oxygen in the system depended on whether sodium nitrite was present in the system and on the sampling time (Figure 5). On Day 5 (Figure 5, closed circles), we found a significant interaction effect, both for the slope of the oxygen gradient (Figure 5A: p<0.01) and the dissipation index (Figure 5B: p <0.01). However, these interaction effects were no longer evident on day 6, (Figure 5, open circles: Figure 5A, p=0.25; Figure 5B, p=0.17). On day 6, there was only a significant effect of the metabolism manipulation on both the oxygen gradient and the dissipation index (Figure 5 closed circles: Figure 5A, p<0.001; Figure 5B, p<0.001).
3.4 Discussion

We tested how manipulating the two aspects of environmental modification by *Pseudomonas* affected the development of diversity of the bacterial community and the overall utilization of oxygen in a liquid broth culture (Figure 2). Our experiments clearly show that the metabolism of the smooth morph influenced the dynamics of the oxygen gradients through time (Figure 4), and that variation in how the oxygen gradients are modified by different bacterial communities can strongly influence the progression of the adaptive radiation in the system (Figure 3A).

Vials in which we modified the metabolism of *Pseudomonas* (i.e. using sodium nitrite) had shallow oxygen gradients and low dissipation indices (Figure 5). This might be explained if sodium nitrite lowered cell density and limited population in the culture, however mid-way through the experiment (i.e. Day 4) treatments with and without sodium nitrite did not differ in cell densities (Figure 3B). The effects on oxygen caused by physical disturbance of the mat at the air-liquid interface were also significant, but more nuanced than those of sodium nitrite. On day 5 of the experiment, the effect of mat disturbance depended on whether or not metabolism had been altered by sodium nitrite, but this effect was not evident the following day (Figure 5). One significant drawback of this model system is that it sampling the abundance and diversity of bacteria can destroy the measurements of the oxygen gradients in the same vial, and so it is difficult to simultaneously quantify environmental modification (e.g. oxygen gradients) along with the ecological (e.g. abundance of bacteria) and evolutionary dynamics (e.g. number of strains).

We found clear evidence that variation in the progression of the adaptive radiation was associated with differences in the oxygen dynamics in the system, suggesting feedbacks between oxygen utilization strategies and evolutionary dynamics. The phenotypic diversity of the strains was clearly affected by our manipulations of both mat formation and metabolism.
As predicted in Figure 2, disrupting both the mat formation and metabolism (i.e. MDTD treatment) prevented the adaptive radiation from proceeding (Figure 3). Furthermore, wrinkly spreaders only evolved in treatments where only one factor was manipulated, either the mat formation (MDTC – SWS and LWS evolved) or metabolism (MCTD – only SWS evolved). In systems without the evolution of WS and FS, the oxygen gradients were shallower and dissipation indices were lower (Figure 5). This might suggest that the persistence of steep oxygen gradients over time is only possible with a sustained diversity of oxygen utilization strategies in the community.

Overall, our results suggest that altering the specific mechanisms underlying evolution by niche construction can affect the overall dissipation of oxygen. In the unmanipulated treatment (i.e. MCTC), the adaptive radiation proceeded in a manner consistent with strong feedbacks between the *Pseudomonas* community and the oxygen environment. For example, by day 6 the mat in the MCTC vials likely collapsed due to cheating smooth types weighing down the mat, as has been observed in previous experiments (Rainey and Rainey, 2003). However, repeatedly disturbing the mat formation prevented the evolution of the fuzzy spreaders, which typically colonizes the air-liquid interface after the collapse of the mat formed by the wrinkly spreaders. The overall effect of this on the oxygen gradients is difficult to discern, because in the case of the oxygen slopes, the effect of removing the physical structure (i.e. the mat) is substantially different depending on whether metabolism has been disrupted or not. When we disrupted metabolism and disturbed the mat then this led to low levels of oxygen dissipation. However, if metabolism was undisturbed then removing the mat may lead to an increase in oxygen utilization, because smooth types are able to exploit oxygen that penetrates deeper into the vials. Future work on the spatial structure of oxygen use of the different bacterial strains would be necessary to more clearly identify the mechanisms causing the variation in the oxygen profiles among our treatments.
P. fluorescens is a particularly interesting system in which to study the effects of evolution by niche construction because the fitness of different oxygen utilization strategies are highly dependent on the temporal dynamics of the system, the composition of the bacterial community, and the ability of previous generations to modify the environment. For example, the formation of a mat at the AL interface is only beneficial if the bacteria cannot grow in the oxygen rich broth, because of previous exploitation during the early stages of growth by the ancestral SM types. However, if the mat is continually disturbed then it does not offer the same monopoly of the oxygen supply. Overall, our results suggest a dynamic interplay between the ability of organisms to modify their environments and the resulting evolutionary dynamics, consistent with niche construction theory. However, the dynamics of these feedbacks remain poorly understood, and more work is need to understand the temporal dynamics of organismal traits associated with ecosystem modification, and of the heritable traits that respond to altered environmental conditions (Matthews et al. 2014).

In this Pseudomonas system, both the presence of feedbacks and the ability to manipulate their strength are useful features of a biological model system that can be exploited to further test dissipative systems theory (Table 1). Our experiments provide some initial support for the first prediction of dissipative systems theory (Table 1), namely that systems can develop processes (e.g. evolution of oxygen utilization strategies) that will maintain the efficient dissipation of an imposed free energy gradient (Nicolis and Prigogine, 1977; Schneider and Kay 1994 (a) and (b)). We show that manipulating the strength of feedbacks between Pseudomonas and the oxygen environment can influence overall oxygen utilization, and the dynamics of the adaptive radiation. Future experiments could focus on more explicitly testing relationships between trajectories of diversity and energy dissipation over time (Figure 1).
Our experiments also suggest that this model system could be used to test a second key prediction of dissipative system theory, namely that when systems are confronted with increasing free energy they will evolve increasingly complex strategies that lead toward higher levels of entropy production (Table 1). This could be tested by directly manipulating the magnitude of the free energy gradient (e.g. oxygen concentration in the air) imposed on the system, and testing for a relationship between diversity and maximum oxygen utilization by the system over time (Table 1).

This model system can also provide novel insights into ecological research on niche partitioning and coexistence (Carroll et al., 2011; Behmer and Joern, 2008; Johnson et al., 2006; Chesson, 2000) and the evolutionary theory of character displacement (Schluter 2000), both of which have fundamental connections to our understanding of niche construction (Matthews et al. 2014). Coexistence in competitive communities can be facilitated when niche partitioning among species leads to greater intraspecific versus interspecific competition (Chesson 2000). In character displacement theory, phenotypic differentiation among organisms evolves at a rate by which interspecific competition declines per unit of phenotypic divergence (Schluter 2000). Organisms evolve greater resource use efficiency on rarer resources (e.g. those on the tails of a symmetric resource distribution), leading to an expansion of the utilization of available resources. One could predict that the processes involved both in coexistence of competitive communities and in character displacement can lead to biological systems that more efficiently dissipate external energy gradients. There are a growing number of experimental tests of coexistence (Adler et al., 2013; Kraft et al., 2015) and character displacement (Rundle et al., 2003; Brockhurst et al., 2006; Tyerman et al., 2008; Ellis et al., 2015), but there are few model systems where one could observe such ecological and evolutionary dynamics in a simple system and simultaneously quantify modification of resource gradients in real time. Experiments with the *Pseudomonas* model system that
explicitly quantify oxygen gradients over time and space, might lead to new insights into the ecological and evolutionary dynamics that underlie both niche construction and dissipative system theory.

Acknowledgements
CML is grateful to Dirk De Beer for hospitality received at the Max Planck institute for Marine Microbiology in Bremen and to the Academic Society of Geneva for an additional support grant which made this work possible.
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Table 3.1

<table>
<thead>
<tr>
<th>Prediction of dissipative systems theory</th>
<th>Requirements of a model system</th>
<th>Pseudomonas fluorescens</th>
</tr>
</thead>
<tbody>
<tr>
<td>In order to maximally dissipate a free energy gradient a system should develop complex structures that dissipate energy and strategies/processes for maintaining energy dissipation over time.</td>
<td>• The system must have a method of generating complex structure or strategies • Association between diversity change and change in resource utilization</td>
<td>• <em>Pseudomonas fluorescens</em> undergoes an adaptive radiation (Rainey and Travisano, 1998), driven by the limiting resource oxygen (free energy source). • Each of the morphs that arise in the system has a different strategy for oxygen use, (Rainey and Travisano, 1998; Rainey and Rainey, 2003; Ferguson et al, 2013; Ibelings et al, in prep)</td>
</tr>
<tr>
<td>To ensure continued energy dissipation, feedbacks should exist between the system and the external environment.</td>
<td>• Organisms alter their environment by taking in free energy and excreting more disordered, higher entropy products. • Organisms mediated environmental modification influences the development and maintenance of diversity (i.e. selection feedbacks are present and can be manipulated)</td>
<td>• In the <em>Pseudomonas</em> system we can estimate the entropy production of the bacteria based on the respiration rate. We can accurately measure the oxygen use of the bacteria using microsensors. • The growth of the smooth morphotype creates the niche at the air to liquid interface which creates the environmental conditions which make the growth of the wrinkly spreader beneficial.</td>
</tr>
</tbody>
</table>

Table 3.1: Gives the main predictions of DST in the first column, the requirements of a model system in the second and the features of *Pseudomonas fluorescens* that satisfy this criteria in the third column.
### Table 3.2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Brief description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCTC (Mat Control Metabolism Control)</td>
<td>The ancestral strain was allowed to develop under standard static conditions (see Rainey and Travisano, 1998)</td>
</tr>
<tr>
<td>MCTD (Mat Control Metabolism Disturbed)</td>
<td>Sodium nitrite (NaNO2) was added.</td>
</tr>
<tr>
<td>MDTC (Mat disturbed Metabolism Control)</td>
<td>The AL interface was disturbed 3 times daily.</td>
</tr>
<tr>
<td>MDTD (Mat disturbed Metabolism Disturbed)</td>
<td>NaNO2 was added and the AL interface was cleared 3 times daily.</td>
</tr>
</tbody>
</table>

**Table 3.2**: Gives a detailed description of the experimental treatments.
Figure 3.1: A conceptual figure which describes the niche construction experiment within dissipative ecosystems theory. For each of 4 scenarios which are explained in more detail in the next, the figure gives a predicted oxygen gradient, the thermodynamic state, the predicted production of entropy, the expected state of internal entropy, a predicted dissipation index, the predicted diversity and predicted species composition, the type of feedback and the form of selection acting.
Figure 3.2: A conceptual schematic for the niche construction experiment. The top figure shows the 4 morphotypes identified during the experiment and the coloured lines link the number and range of species to the treatment under which they were found. The treatment colours are (MCTC: Black; MDTD: Red; MDTC: Green; MDTD: Blue). The bottom figure shows the oxygen gradients expected for each treatment at the time points at which the diversity
measures were taken. The bottom figure also identifies the slope which was used for data analysis and shows the area of the profile grid which was used to calculate the dissipation index.
Figure 3.3

(A) Number of morphotypes

(B) Log cell density

Day 2

Day 4

MC_TC  MD_TC  MC_TD  MD_TD

Treatment
**Figure 3.3:** Picture A details the bacterial abundance found under each treatment on day 2 (filled circles) and day 4 (unfilled circles). Picture B details the species richness observed on day 2 (filled circles) and day 4 (unfilled circles). The treatments are colour coded as follows: MCTC, black; MDTC, green; MCTD, red; MDTD; blue.

**Figure 3.4**
**Figure 3.4:** Shows the oxygen microprofiles obtained for each treatment at three different time points in the experiment. The treatments are colour coded as follows: MCTC, black;
MDTC, green; MCTD, red; MDTD; blue. These profiles were recorded at a) the beginning of the experiment, b) after the loss of diversity and c) at the end of the experiment respectively.

Figure 3.5
Figure 3.5: shows the slope of the oxygen profile (Picture A) and the dissipation index (Picture B) obtained for each of the treatments on day 5 (filled circles) and day 6 (unfilled circles). The treatments are colour coded as follows: MCTC, black; MDTC, green; MCTD, red; MDTD; blue.
Chapter 4

Historical energy conditions can have a significant effect on the generation of complexity and energy dissipation, even in a completely new environment.

Manuscript status: This manuscript is included in its most recent edition. It will likely be revised further based on suggestions from my co-authors. It is being prepared for submission..

Candidate contribution: I carried out all the experimental work for this chapter. I also wrote the manuscript with the aid of my co-authors.

Context: This paper tests the prediction from dissipative systems theory that the system should develop complexity in such a way that it maximizes the use of a resource such as oxygen. We also test the ability of the bacteria to develop complexity and dissipate a resource when they have been pre exposed to low, natural or high energy conditions.
Historical energy conditions can have a significant effect on the generation of complexity and energy dissipation, even in a completely new environment.

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Abstract

Understanding the origin of complexity in the biosphere depends on understanding how the environmental conditions that interact with living systems contribute to the generation of this complexity. One theory which attempts to explain this phenomenon is dissipative systems theory (DST) which suggests that a system can develop more complex strategies which will allow it to maximally dissipate a free energy gradient. This theory offers compelling predictions which have proved difficult to test in real biological systems. Here we suggest that the bacterium *Pseudomonas fluorescens* SBW25 can be used as a model system to test the predictions of DST. We test the effect of pre-exposure to various levels of oxygen (the limiting resource) on the ability of the bacteria to generate complexity and dissipate the available oxygen in a completely new environment. We find that pre-exposure to high levels of oxygen leads to an increased ability to use oxygen but only in other high oxygen environments. Most interestingly, we find that the historical energy (resource) conditions faced by the bacteria have a significant effect on their ability to generate complexity, regardless of the new environmental conditions.
4.1 Introduction

For decades there has existed a theory in physics known as DST (dissipative systems theory) that postulates that complexity in systems can evolve toward maximal dissipation of a free energy gradient. This theory, first developed for biological systems by Nicolis and Prigogine (1977) suggests that when living systems are pushed further from thermodynamic equilibrium by such a free energy gradient, which in the biological context may be a food resource or oxygen, they can develop ever more complex strategies that dissipate this gradient and therefore avoid being pushed further from equilibrium. In the context of the biosphere this may be the evolution of new species, enhancing biodiversity. New species that play different roles in the ecosystem and it is this definition of complexity that we adopt for this study. However, while data analysis has been carried out on ecological systems and seems to offer support for the theory, (Aoki, 2006; Schneider and Kay 1994 (a) and (b)), a suitable experimental system to test the predictions of dissipative systems theory for biology has been lacking.

In the current study we present the bacterium *Pseudomonas fluorescens* SBW25 as a model system to test the predictions of dissipative systems theory in a biological context. We consider that *P. fluorescens* may undergo its well studied adaptive radiation (e.g. Rainey and Travisano, 1998) in order to maximally exploit oxygen as an energy source. The oxygen in the microcosm is analogous to a free energy source, and as all the morphotypes that arise have different strategies for obtaining oxygen at the air to liquid (AL) interface – the only place where re-renewable oxygen remains available - we can postulate that these different morphotypes arise in response to the ecological opportunity generated by oxygen gradients and possess strategies to ensure the most efficient use of oxygen. The ancestral smooth (SM) morphotype has the fastest rate of metabolism and growth rate and occupies the broth phase.
of the microcosm until oxygen gets depleted there by its own metabolism. Once the broth is
anoxic, wrinkly spreader (WS) mutants evolve (around day 2-3) and overproduce cellulose in
order to form a biofilm and monopolize the little oxygen that remains at the AL interface,
(Rainey and Travisano, 1998). However, as the smooth types have a better metabolism and
use the oxygen more efficiently, they quickly arise as cheats in the biofilm. As they do not
produce cellulose and contribute nothing to mat strength they lead to its collapse, (Rainey and
Rainey, 2003). This leaves the air liquid interface temporarily vacant and this space is then
occupied by the fuzzy spreader (FS) morphotype, which forms rafts of cells, (Ferguson et al,
2013). These fragile rafts act as placeholders until the return of the WS. This sequence fits
well to the prediction of dissipative systems theory that living systems will increase in
complexity in order to maximally utilize a free energy gradient. In this case new morphotypes
arise within the bacterial community (the system) allowing them to most efficiently use the
oxygen available.

One of the main predictions of the dissipative systems theory is that in response to an
increase in the availability of free energy, the system will develop a larger degree of
complexity containing strategies that allow it to maximally use this energy. In the context of
the Pseudomonas fluorescens system this means that we would expect a greater amount of
complexity (i.e., diversity of oxygen exploiting strategies or morphotypes) to develop in
response to an increase in the oxygen concentration in the static glass vials. From previous
experiments, (Ibelings et al, included in this thesis) we know that the diversity of the bacterial
community does increase when pure oxygen is pumped into the microcosms as compared to
air or nitrogen. We revalidated this finding in the current study. Here we attempted to
understand the effects of a higher energy (i.e., oxygen) availability on the subsequent
radiation of the bacterial morphotypes. To this effect we repeated the experiment of Ibelings
et al, detailed in chapter 2, by exposing the ancestral SBW25 strain to either pure nitrogen,
compressed air or pure oxygen and allowing it to undergo an adaptive radiation. On day 4 of these different adaptive radiation scenarios we picked a smooth type to determine what effects the historical energy environment would have on the generation of complexity and the subsequent ability to dissipate the energy gradient. We hypothesize that bacteria pre-exposed to high oxygen conditions will become more efficient oxygen utilizers, which we assess as the capacity to rapidly and effectively use oxygen when faced with a high oxygen environment.

4.2 Materials and methods

4.2.1 Bacterial strains

The ancestral SBW25 strain was isolated from the leaf of a sugar beet plant from Oxford University farm, Wytham in 1989, (Rainey and Bailey, 1996.) This was the only stock strain used in the experiment to inoculate the microcosms in stage 1 (described below).

4.2.2 Microsensor measurements

Oxygen distribution within the microcosms (vials) was measured using oxygen microelectrodes with a guard cathode. These were constructed at MPI for Marine Mirobiology in Bremen, as described previously by (Revsbech, 1989). Triplicate vials were used for each experimental treatment at each time point. Per individual vial measurements were conducted in triplicate. For each profile, the oxygen sensor was placed directly at the AL interface with the assistance of a dissection microscope, Automatic profiles were generated using custom software and were recorded from 250 microns above the AL interface and at increments of 50 microns until a depth of 500 microns into the medium. Below this depth, readings were recorded at increments of 250 microns until a depth of 4000 microns in order to make the profiles comparable to those presented in Ibelings et al, (in preparation). Linear
calibration of the sensors was derived from measurements in air bubbled King’s B (King et al, 1954) growth media and MilliQ water (100% air saturation) and in N2 bubbled medium or (later in the experiment) in the anoxic part of the medium (0% air saturation). Oxygen concentrations were calculated using Unisense gas tables (Li and Gregory, 1974; Garcia and Gordon, 1992).

4.2.3 Experimental stage 1 – The origin stage

Static (unshaken) microcosms were prepared by adding 6ml of King’s B (King et al, 1954) growth media to 30 ml glass universal vials. The ancestral strain SBW25 was streaked from a glycerol stock (frozen at -80°C) on to agar plates to obtain single colonies of smooth (SM) morphotypes only. Single SM colonies were picked and used to inoculate the microcosms which were then sealed in air tight Perspex chambers and allowed to undergo an adaptive radiation under one of three oxygen conditions; Pure nitrogen (0% oxygen), compressed air (20% oxygen) and pure oxygen (100% oxygen). (See the first line of figure 1) The gases for each of these origin treatments were pumped directly into the chamber at a rate of 0.5L/min. The colonies were allowed to diversify for 7 days and each day microelectode oxygen profiles were obtained for each of 3 replicate vials to determine the ability of the colonies to use the oxygen available in gradients along the liquid media. 3 vials were also destructively sampled daily and plated to examine the diversity of morphotypes present in each microbial community. On day 4 single colonies of smooth bacteria from the different origin conditions were picked and used to inoculate each of the microcosms in experimental stage 2, (See the second line of figure 1).
4.2.4 Experimental stage 2

New fresh static microcosms were prepared. Single SM colonies from day 4 of the origin treatments described above were used to inoculate each of the fresh microcosms and these were sealed in airtight Perspex containers and subjected to one of three mixed gas conditions; Low oxygen (20% Oxygen, 80% nitrogen), Mid Oxygen (50% oxygen, 50% nitrogen) or high oxygen (80% oxygen, 20% nitrogen). A smooth colony obtained by streaking directly from a glycerol stock was also added for each condition as a control. Electronic flow meters (brand) were used to create the gas mixes. Gases were pumped into the chambers at a flow rate of 0.5L/min. The colonies were allowed to evolve and undergo a second round of adaptive radiation. In addition, the ability of the cultures to use the available oxygen was measured daily using microsensors, while samples of the bacterial community were destructively sampled and plated on days 3 and 5 to monitor the morphotype diversity present in this second adaptive radiation. Triplicate vials were measured using microsensors for each time point and the entire experiment was repeated to ensure that the pattern observed was replicable. The therefore 6 replicate experimental bacterial communities are therefore treated in this experiment as 6 “blocks”.

4.2.5 Measures of bacterial density and diversity (complexity)

The density of the bacteria was calculated by counting the number of colony forming units (cfu) on a plate and multiplying this by the dilution factor in order to obtain the number of cfu per ml of liquid culture, this is in line with other studies using this model system, (Rainey and Travisano, 1998).
The morphotypes that arise in the *Pseudomonas fluorescens* radiation are phenotypically different and can easily be distinguished by eye on an agar plate. To establish the complexity of the bacterial community the number of different morphotype that arose under each experimental condition was monitored. The differences in colony morphology are heritable and the genetic mutations that lead to the main types examined in this study are known, (Rainey and Travisano, 1998; Spiers et al, 2003; Fukami et al, 2007; Ferguson et al, 2013; Hammerschmidt et al, 2014).

4.2.6 Data analysis

Data analysis of the oxygen profiles was carried out using the statistical analysis software R. A negative exponential function \( y = a e^{-bx} \) was fit to each oxygen profile in order to estimate the slope \( b \) of the oxygen gradient. We also calculated an “Index of dissipation” by dividing the sum of the O2 values for each profile by the max level of O2 available. This quantifies the ability of the bacterial community in each treatment to dissipate the oxygen gradient. Additional data analysis of the profiles was carried out using JMP statistical analysis software. Analysis of the species richness and bacterial abundance data was carried out using JMP statistical analysis software.

4.3 Results

4.3.1 Stage 1 results

We examined the effect of the different origin oxygen concentrations on the diversity and abundance of the bacterial communities on day four of the first adaptive radiation. We ran the statistical analysis on the day 4 data as this was the day that the smooth morphotype was picked in order to inoculate the second adaptive radiation. We found that when origin was
considered as a categorical variable the effect of origin was slightly non-significant (ANOVA p= 0.064) for morphotype richness and not significant for bacterial abundance (ANOVA p= 0.154). This would suggest at first glance that the adaptive radiation produced similarly abundant and diverse communities regardless of the oxygen availability during stage one. However, when origin is instead considered as a continuous variable progressing from 0%, 20% and up to 100% then we find the effect of oxygen becomes highly significant on morphotype richness (ANOVA p= 0.025) and bacterial abundance (ANOVA p= 0.05). Both morphotype richness and bacterial abundance were found to increase linearly with oxygen concentrations. We believe that considering oxygen as a continuous variable gives a more accurate picture of the relationship between oxygen and abundance and richness and bacterial communities. This is based on the results of Ibelings et al, (included in this thesis) which found that bacterial diversity was highest under pure oxygen as compared to under air and pure nitrogen. Bacterial abundance was also found to be higher under oxygen and air but lower under nitrogen. This is also in accordance with our own observations during the stage 1 part of the experiments. From the results of Ibelings et al (included in this thesis) it is clear that the concentration of oxygen can have an effect on the diversity and because we find an increase in diversity which is linear with the increase in oxygen concentration we feel that it is appropriate to consider oxygen as a continuous variable.

4.3.2 The oxygen slope results

We found that the overall ability to efficiently deplete the oxygen in the liquid media (i.e the slope of the oxygen gradient/ dissipation gradient) did not vary significantly among stage 1 origins (ANOVA p=0.671) or between stage 2 oxygen treatments (ANOVA p=0.074). However, there was a significant interaction effect between stage 1: origin and stage 2: oxygen concentration (ANOVA p=0.0042) on oxygen use efficiency. This meant that in some new
(stage 2) environments, the environment that the bacteria faced in the origin (stage 1) could have a significant effect on the dissipation index / slope and therefore the ability to use oxygen.

This interaction effect is displayed in figure 4.2. We found that bacteria that were pre-conditioned to high oxygen levels (100% oxygen) during the origin-phase depleted the oxygen in the liquid media during the treatment phase more efficiently as the oxygen concentration in the air increased (Figure 4.2). With an increase in oxygen during the treatment phase the slope of the vials inoculated with cells pre-adapted to high oxygen got more negative, indicating a steeper slope, indicating that cells became more efficient in dissipating the oxygen gradient. The opposite was true for the bacteria that were pre-conditioned to lower oxygen levels (0%, 20%), they depleted the oxygen in the liquid media less efficiently as the concentration of oxygen increased (Figure 4.2): with an increase in oxygen concentration along the x-axis the slope of the oxygen gradients in the vials became less negative (less steep), and gentler slopes indicate that oxygen gets dissipated less efficiently. This trend was also observed for the control bacteria but it was not statistically significant, (P=0.06). The full oxygen profiles obtained under each treatment for the five days of the stage 2 treatment are given as supporting information for this chapter.

4.3.3 Morphotype richness results

We found that the oxygen concentration during the first adaptive radiation (stage 1, called “origin”) had a significant effect on the species richness generated in the second adaptive radiation (stage 2), (ANOVA, p=0.001), (Figure 4.3). We also found that the oxygen concentration during the second adaptive radiation (called “treatment”) did not have a significant effect on the morphotype diversity generated in the second adaptive radiation,
(ANOVA, p=0.268) and there was no significant interaction effect between origin (oxygen concentration during stage 1 radiation) and treatment (oxygen concentration during stage 2 radiation) on morphotype diversity during the second adaptive radiation, (ANOVA, p=0.119). Morphotype richness generated during the second adaptive radiation (stage 2) from the bacteria that originated at 20% oxygen (stage 1) had a significantly lower richness than that found in either of the other origin conditions (0% or 100%) or the control.

4.3.4 Bacterial abundance results

We found that “origin” (i.e., oxygen percentage during stage 1 radiation) had a significant effect on the bacterial abundance of the bacteria in the second stage radiation (ANOVA p=0.016). The colonies that originated from the 20% oxygen conditions (stage 1) had a significantly higher abundance than those originated under the other oxygen conditions or the control (Figure 3). We found no significant effect of the oxygen concentration present in the second adaptive radiation, (ANOVA, p=0.809) on the bacterial abundance. There was also no significant interaction effect between origin and treatment on bacterial abundance during the second adaptive radiation, (ANOVA, p=0.681).

4.4 Discussion

Our results confirmed the findings of Ibelings et al, included in this report, showing that the diversity of the bacterial community increases as oxygen concentrations increase. We also confirmed the finding that when the bacteria were incubated under nitrogen they had a significantly lower abundance than when they were incubated under air or pure oxygen. We found that both diversity increased linearly with oxygen concentration. These stage 1 results (
see figure 1) would support the prediction from dissipative systems theory that an increase in energy will lead to an increase in diversity. We consider this increase in diversity to be analogous to the increase in complexity and postulate that this complexity allows for the more efficient use the available oxygen. This suggestion that the bacterial communities pre exposed to a high oxygen environment are better able to cope with a new high oxygen environment seems to be corroborated by our results which can be seen in figure 2 which show that the dissipation of the oxygen by colonies pre exposed to pure oxygen increases as the oxygen level in the new environment increases. The opposite trend is observed for the bacteria pre exposed to any other condition than high oxygen and the control. This suggests that the oxygen pre exposed bacteria have indeed developed in a way that leads to more efficient oxygen dissipation as predicted by dissipative systems theory. However the fact that the oxygen pre exposed colonies perform poorer in the low and medium oxygen concentrations suggest that high energy exposed bacteria can only thrive in conditions with similar concentrations of free energy.

Perhaps the most interesting result of this study was the finding that the historical energy conditions(i.e adaption) which drove the first adaptive radiation detailed in stage one had a highly significant effect on the diversity, and therefore complexity, that could arise in the second adaptive radiation regardless of the new energy conditions that the bacteria faced. This is important for theories of early life as it suggests that the resources and energy gradients present on early earth could constrain the potential for diversity and complexity in species that now faced entirely different environmental conditions. This result may also offer some compelling insight into the ecology of adaptive radiation as it has often been wondered why when species are similar and face similar ecological conditions, some will undergo an adaptive radiation and diversify and other may not, (Fukami et al, 2007; Seehausen, 2006; Pinto et al, 2008; Lovette et al, 2001). This work offers a suggestion that historical ecological
opportunity and resource availability may influence the potential for phenotypically similar species to radiate even when they face identical resource availability in a secondary environment.

We found that the highest complexity (diversity) was found for bacteria that had been pre exposed to pure oxygen or pure nitrogen, the former result makes sense with the DST interpretation of the *Pseudomonas* system as it is expected that the community will develop a potential to generate new types in order to find novel routes to dissipate the available oxygen. The latter result fits well with the ecological theory of adaptive radiation as the bacteria previously exposed to nitrogen experience a burst of a novel resource in all of the oxygen environments in the stage 2 experiments, this provides ecological opportunity (Yoder et al, 2010) and may lead to a burst of speciation. This result may also be an example of the overshooting effect (Meyer et al, 2011) in which many new forms arise in response to an abundance of a newly available resource, however in this case usually diversity would reduce rapidly after the initial burst as the best competitors drive others to extinction, however the diversity did not appear to reduce in this case. This ecological theory is not incompatible with DST as it again suggests that the system will develop complexity to allow for the maximal use of the resource that is available, which in the case of *Pseudomonas* will result in the maximal use of oxygen.

In general, we find that the conditions that the bacterium were pre exposed to can have significant effects on the functioning of the bacterial community, we expected to see this effect in the first days of the stage 2 experiments during the four days that the bacteria were grown under the stage 1 “origin” conditions we would have expected the bacteria to acclimate to their origin environment. However, as the doubling time of *Pseudomonas fluorescens* is roughly once per 20 minutes, we would not usually expect 4 days to be a suitably long time for significant “adaptation” (including genetic changes) to occur. For this reason, the fact that we see a sustained effect of origin across the 5 days of the stage 2 adaptive radiation is
surprising. The mechanisms that lead to this extended effect of acclimation are not clear from the results but we can offer some potential explanations. One such explanation could be that maintained acclimation is the result of epigenetic effects, these are heritable and therefore could be sustained across the 5 days of the secondary adaptive radiation. Recent work by Klironomos and colleagues (2013) estimates that the epigenetics play a much greater role in adaptation than has previously been appreciated. A second possible explanation is that the acclimated cells have different effects on the early environment in the stage 2 experiments, in this case we might seem something similar to a priority effect, where the starting “origin” of the cells can constrain or expand the adaptive radiation. Another possible explanation may be genetic mutation, although all of the colonies used in the stage 2 adaptive radiations were phenotypically smooth like the ancestral SBW25, it is possible that mutations occurred which were unrelated to colony morphology and oxygen strategy but nonetheless had an effect on the potential of the bacteria to undergo a second adaptive radiation.

It is not immediately clear why the lowest complexity (diversity) is observed in bacteria that were pre exposed to air. It is possible that pure oxygen and pure nitrogen lead to diversification as a response to stressful conditions but air does not put the same pressure on the system. It is also possible that when the system cannot generate complexity and new strategies that aid in the maximal use of the oxygen then the system increases the number of cells in order to ensure maximal metabolism and maximal resource use. When diversity is low the population is dominated by the metabolically efficient smooth type which offers some support for this theory. This scenario is also supported by another experiment from our laboratory, which found that in a treatment where complexity was completely lost from the system, the highest abundance of bacterial cells was recorded, (Loudon et al, 2015). Another explanation may also be found in recent work by Leopold et al, (2015) , in which priority effects were found to be stronger in less stressful environments. If air is indeed the least
stressful of the origin environments then this may lead to an increased level of competition during the initial origin adaptive radiation that translates into stronger priority effects in the stage 2 adaptive radiation. However, further work is required to fully understand the mechanisms that lead to a reduced diversity in the bacteria that had been pre exposed to air.

4.5 Conclusions

We found that the bacteria *Pseudomonas fluorescens* SBW25 is a useful model system to test the theoretical predictions of dissipative systems theory. We confirmed previous results that a higher concentration of oxygen would lead to a higher diversity. We found that pre exposure to high oxygen conditions led to a better ability to use the oxygen supply in a new high oxygen environment but this led to reduced performance in lower oxygen environments. Importantly our results show that the historical resource conditions faced by a bacteria can have an effect on the complexity that will develop when the bacteria is moved into a new environment regardless of the environmental conditions in the new location. This offers insight into how dissipative systems theory can explain the generation of complexity on earth and may also be important for the ecological theory of adaptive radiation which has struggled to understand why seemingly identical species vary in their ability to radiate under similar ecological conditions, we suggest that historical resource conditions may play a role.
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Figure 4.1

STAGE 1 - ORIGIN

NITROGEN

AIR

OXYGEN

STAGE 2 - TREATMENT

LOW O2  MID O2  HIGH O2

LOW O2  MID O2  HIGH O2

LOW O2  MID O2  HIGH O2
**Figure 4.1:** Conceptual figure showing the design of the precondition experiment. A single ancestral smooth colony was picked to inoculate each vial, these were then incubated under either pure nitrogen (0% oxygen), air (20% oxygen) or pure oxygen (100% oxygen). The adaptive radiation that had occurred by day 4 of this incubation is shown in figure 1. At this point a single smooth morphotype was picked from the day 4 agar plates to inoculate the microcosms for the stage 2 experiments, this process is shown on the bottom level of figure 1.
Figure 4.2: Origin by treatment effects on oxygen depletion efficiency. This figure shows the slope reflecting the oxygen utilization of the bacterial community in relation to the oxygen conditions faced by the bacteria, a more negative oxygen slope by depth (y-axis) value indicates a more efficient use of the oxygen present in the vial. Bacteria are color labeled according to their stage 1 origin: 0% oxygen in red; 20% oxygen in green and 100% oxygen in blue respectively. A control streaked directly from the glycerol stock is included in black.
**Figure 4.3:** Average morphotype richness during the second (stage 2) adaptive radiation from each of the origin (stage 1) oxygen concentrations. The average species richness generated from the control is indicated with a black reference line. Dotted line represents one standard deviation around the mean.

**Figure 4.4:** Average bacterial abundance generated during the second adaptive radiation for colonies that originated at different oxygen concentrations (stage 1). The average abundance generated from the control is indicated by the black reference line. Dotted line represents one standard deviation around the mean.
Supporting information for Chapter 4

The following are the oxygen profiles obtained for each of the treatments in the stage 2 experiments carried out in chapter 4 for all 5 days of the experiment. An analysed and summarized assessment of this data is presented in figure 1.

Supporting figure 1 – Low oxygen chamber
Supporting figure 1 - Shows the oxygen profiles obtained in the low oxygen chamber on a) Day 1 b) Day 2 c) Day 3 d) Day 4 e) Day 5.
Supporting information figure 2 – Mid oxygen chamber

a

C50/50 Day 1
N50/50 Day 1
A 50/50 Day 1
O 50/50 Day 1

CONTROL
NITROGEN
AIR
OXYGEN

b

C 50/50 Day 2
N 50/50 Day 2
A 50/50 Day 2
O 50/50 Day 2

CONTROL
NITROGEN
AIR
OXYGEN
Supporting information figure 2 - Shows the oxygen profiles obtained in the mid oxygen chamber on a) Day 1 b) Day 2 c) Day 3 d) Day 4 e) Day 5.
Supporting information figure 3 – High oxygen chamber

a

CONTROL  NITROGEN  AIR  OXYGEN

b

CONTROL  NITROGEN  AIR  OXYGEN
Supporting figure 3- Shows the oxygen profiles obtained in the high oxygen chamber on a) Day 1 b) Day 2 c) Day 3 d) Day 4 e) Day 5.
Chapter 5:

The evolution and later extinction of a low fitness morphotype within a dissipative ecosystem

**Manuscript status:** This manuscript is still in preparation, the experimental work and interpretation is complete but it will be rewritten based on comments and suggestions from my co-authors.

**Candidate contribution:** I designed and performed experiments, analyzed the data and wrote the manuscript.

**Context:** This manuscript studies in detail the ecology of a particularly interesting morph that arises early within the *Pseudomonas fluorescens* adaptive radiation. The morph was found to evolve and rapidly reach appreciable numbers before being competitively replaced by a superior colonizer of the same niche. This paper explores the evolution and extinction of this morph within the context of a dissipative ecosystem. This is particularly relevant because Nicolis and Prigogine (1977) the seminal work that much of this thesis is inspired by predicted that poor gradient dissipaters would be competitively replaced by more efficient dissipaters.
The evolution and later extinction of a low fitness morphotype within a dissipative ecosystem

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Abstract

Adaptive radiation is a process by which diversity can arise rapidly within a system in response to ecological opportunity. The bacterium Pseudomonas fluorescens SBW25 undergoes an adaptive radiation that leads to maximal dissipation of the oxygen gradient in a static glass vial as predicted in theory by Nicolis and Prigogine, (1977), (Loudon et al, 2015). In line with this theory we present here a morphotype, defined by a mutation in nlpD, which evolves early in the adaptive radiation in order to take advantage of a novel niche, before being rapidly competitively replaced by a superior colonizer of the same niche. We demonstrate that this morph is a poor dissipator of the oxygen gradient and discuss this morph within the context of a dissipative ecosystem.
5.1 Introduction

Adaptive radiation is an important mechanism by which new species arise to maintain biodiversity on earth. It is defined as a process in which new species arise from an ancestral species and exploit different resources or occupy a different niche from the ancestral strain, (Schluter, 2000). Perhaps the most famous example of adaptive radiation are the finches on the Galapagos islands known as “Darwin’s finches”. These birds have evolved a range of beak shapes which relate to the foods that they can utilize, they evolved from a common ancestor but avoid direct competition with each other by depending on different food sources, this is made possible by the evolution of different beak shapes (Darwin, 1859 ; Lack, 1947)

The soil bacterium *Pseudomonas fluorescens* SBW25 is a model system for the study of adaptive radiation (Rainey and Travisano, 1998; Kassen et al, 2000). When the ancestral smooth morphotype is inoculated into static glass vials containing growth media, it rapidly diversifies into a range of morphotypes. This diversification is driven by intense competition for the limiting resource oxygen. Initially, oxygen is well distributed throughout the microcosm and at this stage the smooth morphotype (SM), which grows rapidly in the broth is favoured. However, due to the metabolic activity of the smooth types, oxygen is rapidly depleted and soon it is only available at the air to liquid interface (AL interface). At this stage, the wrinkly spreader (WS) evolves. This morphotype overproduces cellulose, forms a mat which at the AL interface ,and therefore monopolizes all the available oxygen in the vial. However, smooth types rapid reappear as “cheaters” in the mat, in that they are able to insert themselves within the mat structure and obtain a share of the available oxygen at the AL interface. These types do not overproduce cellulose and therefore contribute nothing to mat strength. Cheaters increase to high frequency, as they do not pay the cost of cellulose production, until mat strength is substantially weakened it ultimately collapses (Rainey and Rainey, 2003). After the collapse of the mat, the AL interface is free once again and a new type
arises to take advantage of this newly reopened niche space, the fuzzy spreader (FS) (Ferguson et al, 2013). These types form rafts at the AL interface, however the structures formed by these types are weak and readily collapse within the broth. They are also inferior colonizers of the AL interface as compared to the WS so are unable to retain this niche after its return (Ferguson et al, 2013; Ibelings et al, included in this report). These dominant morphotypes engage in a cyclic competition as all of the oxygen strategies are fallible, so no morph can monopolize the oxygen supply at all of the time points in the adaptive radiation.

For this reason, we can say that the dominant morphs occupy different temporal niches. So, whilst in the case of Darwin`s finches, coexistence is achieved by utilizing different resources, coexistence in this system is maintained by utilizing the same resource (oxygen) at different times. We have argued previously that the adaptive radiation of Pseudomonas fluorescens fits well with the dissipative systems theory first put forward by Prigogine (1955) (Loudon et al, 2015). We suggest that in line with the theoretical predictions of Nicolis and Prigogine (1977) the Pseudomonas system develops complex forms and strategies which allow for the maximal dissipation of the resource gradients available to them. In this case, we believe that Pseudomonas fluorescens undergoes an adaptive radiation which generates species with strategies that can maximally dissipate the oxygen present in a static glass vial. This leads to an increase in the production of entropy in the system which is in line with the second law of thermodynamics as carbon dioxide, which results from the bacterial respiration, has a lower partial pressure in the atmosphere than does oxygen, in fact it has been postulated that the change from oxygen to carbon dioxide may produce the highest entropy change during bacterial cell division (England, 2013). Nicolis and Prigogine (1977) also predicted that for a bacterium growing on a single resource if a new morph arose they would either occupy a new niche or they would competitively replace an existing population by more efficiently dissipating the resource gradient utilized by the incumbent population.
Here we introduce a morphotype that arises within the *Pseudomonas fluorescens* adaptive radiation which appears to demonstrate this prediction of Nicolis and Prigogine, it is defined by a mutation in nlpD and has a distinctive ecology relative to the ancestral strain. We discuss this morph within the context of the rock paper scissors dynamics described above (and in greater detail by Ibelings *et al*, submitted) and within the context of a dissipative ecosystem.

### 5.2 Materials and methods

#### 5.2.1 Bacterial strains

The ancestral *Pseudomonas fluorescens* SBW25 strain was isolated from the leaf of a sugar beet plant from Oxford University farm, Wytham in 1989 (Rainey and Bailey, 1996). The marked strains used for competition assays were SBW25 –*lacZ*, the chromosomal *lacZ* maker is neutral (Zhang and Rainey, 2007).

#### 5.2.2 Adaptive radiation experiments

These experiments followed the standard protocol for the adaptive radiation of *Pseudomonas fluorescens* in a static microcosm (Rainey and Travisano, 1998). Briefly, microcosms containing 6ml of Kings medium B (King *et al*, 1954), were inoculated using single colonies of the ancestral SBW25 strain. These microcosms were then incubated at 28°C with loose lids to allow the inflow of oxygen. In these static conditions, the growth and metabolic activity of the bacteria creates a heterogeneous environment which promotes the adaptive radiation. Each day 3 microcosms were destructively sampled to establish the community dynamics.
5.2.3 Motility assays

The colony morphology of the nlpD mutant is very similar the ancestor in that it also appears smooth. However, when stabbed into semi solid agar with a concentration of 0.3%, the nlpD mutant is easily identified as it does not swim through the agar and produces a characteristic crystalline growth within the stab. To determine the population dynamics of the nlpD mutant, 100 colonies per microcosm were chosen at random from the plates generated from the adaptive radiation experiments. These colonies were stabbed into semi solid agar in order to determine if they contained the nlpD mutation.

5.2.4 Time lapse photography

Colonies which had been identified as containing the nlpD mutation were inoculated into microcosms and cultured overnight at 28° in a shaken environment to promote rapid growth. Of this overnight culture 6µl was then used to inoculate microcosms to be studied using time lapse photography. The microcosms were fixed to a shelf with a black background previously constructed for this purpose by Dr. Gayle Ferguson. A digital camera and the software Breeze systems PS remote were then used to set the camera to take a photograph of the assembled microcosms once every 600 seconds. These photos were then assembled into movies using “Windows movie maker.”

5.2.5 Fitness assays

To determine the relative fitness of the nlpD mutant it was competed against SBW25 – lacZ. A 1:1 ratio of the two competitors was generated in an eppendorf tube and 6µl of this mixture was used to inoculate competition microcosms, which were incubated under static conditions
for 24 and 48 hours before being destructively sampled and plated onto KB plates containing Xgal. Xgal was added to the plates as when it reacts with the lacZ marked strains it has the effect of turning the colonies blue, making them easy to identify.

5.2.6 Invasion from rare assays

To test for frequency dependent interactions the nlpD mutant was competed against WS, FS and the ancestral SM type. In each of the reactions one morphotype was dominant the other was rare, with a ratio of 100:2 respectively. The ratio was created in an Eppendorf tube and 6µl of this mixture was used to inoculate the static microcosms. The two strains were allowed to compete for 3 days under static conditions. The respective malthusian parameters of the strains were used to calculate the selection rate coefficient. Positive selection rate coefficient values were deemed to indicate that the morph could invade from rare, whilst negative values indicated that the morph could not invade the dominant from rare.

5.2.7 Microscopy

In order for cells to occupy the AL interface in the way the non-motile morphs seem to do, they would be required to produce some form of extracellular polymer (EPL) in order to stick to each other and the side of the vial as observed in the time lapse movie. In order to determine if the cells were producing EPLs we added calcofluor- white, a dye that fluoresces blue under UV light to KB plates. Non motile strains were diluted from overnight culture and plated onto these plates using sterile glass beads. After incubation for 48 hours at 28°C, colonies were scraped from these plates and spread onto a microscope slide using a sterile plastic loop, before being fixed with a cover slip. Analysis and photographs of these slides were provided by Dr. Peter Deines
5.2.8 Microsensor measurements

Oxygen microsensors with a guard cathode were constructed at MPI Bremen. Sensors were calibrated in KB media and MilliQ water daily before profiling. Sensors were placed directly at the AL interface with the assistance of custom programs and a dissection microscope. Profiles were generated using custom software. And were recorded from 250 microns above the AL interface and at increments of 50 microns until a depth of 500 microns and after this threshold reading were recorded at increments of 250 microns until a depth of 4000 microns in order to make the profiles comparable to those found in Ibelings et al.

5.3 Results

5.3.1 Population dynamics of the nlpD mutant

The nlpD mutant is found to arise early in the adaptive radiation before it seemingly completely disappears by day 5 of the adaptive radiation. However, a rapid decline in the population can be observed after the day 2 peak which coincides with the evolution of WS.

5.3.2 Competitive fitness of the nlpD mutant

The fitness of the nlpD mutant is very low in comparison to the SBW25 ancestor. The relative fitness was found to be negative after both 24 and 48 hours under static conditions. This indicates that the nlpD mutant has a low fitness in comparison to the ancestral strain during
days 1 and 2 of the experimental adaptive radiation, which are the days on which the two morphs are found to coexist.

5.3.3 Time lapse photography

The movie, WS_SM_nlpD, which is available with this report, shows the behavior of the WS, the ancestral SM morphotype and the nlpD mutant as they grow in a static microcosm over a 6 day time course. The first microcosm on the left containing the WS, as expected on the basis of Rainey and Travisano, (1998), grows mainly at the air to liquid interface. There is very little growth in the broth and the mat is much thicker than those that are usually seen when the microcosm is inoculated with the SM morphotype. The middle microcosm in the video contains the ancestral smooth morphotype, it also grows as expected, i.e. the broth first becomes cloudy as it is colonized by smooth types, after which the formation of a mat can be observed, as wrinkly spreaders arise and colonize the AL interface on approximately day 3. The mat becomes thicker as the movie progresses, until in the end it collapses on approximately day 5. This is due to the smooth type cheaters that hitchhike in the mat in order to access the limited oxygen supply at the air liquid (AL) interface. However, these smooth types do not overproduce cellulose and therefore contribute nothing towards mat strength and play a key role in its collapse (Rainey and Rainey, 2003). In the last microcosm on the right, the nlpD mutant can be seen to display behavior very different from that of the SM morphotype. It should be noted that the rapid colonization of the broth does not occur, the growth also appears weak and the green fluorescence that is so striking in *Pseudomonas fluorescens* fails to appear. Upon closer inspection of the vial, it is possible to see the nlpD mutants attempt to colonize the AL interface as early as days 1 and 2. However, they are unable to form a mat that fully covers the AL interface, they are only able to successfully colonize the very edge of the vial. The structures formed by the nlpD mutant appear to be very
weak as the structures readily collapse in strings. Each time the strings collapse, the morphs rapidly recolonize the edge of the AL interface. It appears that these morphs repeatedly use the same oxygen strategy across the 6 day time course recorded by the movie. Figure 3A shows a still photograph of the nlpD mutant occupying the novel niche at the AL interface and compares it to the superior mat formed by the wrinkly spreader types as shown in picture 3B. Although on diversity spread plates these nlpD mutants are indistinguishable from the ancestor, the video shows that by occupying a different niche within the spatial microcosm, they have a different strategy for survival than the ancestor. It is also interesting to note that as can be observed in the video, these types lack the ability to diversify into wrinkly and fuzzy spreaders.

5.3.4 Invasion from rare assays

As can be seen in Figure 3, both the WS and FS were easily able to invade the mutant nlpD strain from rare. However, the nlpD strain cannot invade either of these types from rare, a result that would seem to support the theory that both FS and WS are superior colonizers of the AL interface, as compared to the nlpD strain. The nlpD strain and the ancestral smooth morphotype can invade each other from rare, albeit with much lower selection rate constants. This supports the hypothesis that these two morphotypes can coexist as the nlpD mutant avoids direct competition with the ancestor by occupying the niche at the edge of the air to liquid interface whilst the ancestor is able to survive in the broth phase. Unlike WS mats, which completely seal off the AL interface, the feeble surface structures built by the nlpD mutant seem to allow some oxygen penetration to the broth. The results from the invasion from rare assays would suggest that in the case of the nlpD mutant there is no advantage to being rare.
5.3.5 Microscopy

When studied under the microscope the nlpD mutant was found to form chains of cells as compared to the randomly distributed cells that can be observed in the ancestral strain. The difference can be seen in figure 5A (nlpD) and B (SBW25).

5.3.6 Microelectrode profiles

As can be seen in figure 6A the steep oxygen gradients that we associate with the growth of all the other morphotypes in the pseudomonas fluorescens radiation are not found for the nlpD mutant. In fact, the nlpD mutant never manages to dissipate the resource and reach anoxia above a depth of 500 microns which leads to the assumption that the nlpD mutant is a poor dissipater of the oxygen gradient in static glass vials.

5.4 Discussion

Nicolis and Prigogine, (1977), postulated that competitive replacement may occur if another morph arises that is a more efficient dissipator of the resource gradient. The population dynamics of the nlpD mutant offer an interesting insight into coexistence and competitive replacement. The nlpD mutant typically arises on day 1-2 of the experimental adaptive radiation and it’s population density peaks on day 2-3 before the numbers decrease to an undetectable level by day 5. It is interesting that the highest population density for the nlpD mutant, coincides with a time period when the ancestral smooth type dominates the microcosm population. This is surprising as the fitness assays carried out for this study have shown this nlpD mutant to be highly unfit compared to the ancestral strain with which it seems to coexist. These morphotypes must then have a strategy that allows them to exist
with the ancestor without directly competing with it. Studying the behavior of the nlpD mutant using time lapse photography has revealed that instead of competing with the ancestral strain to grow in the broth phase, the nlpD mutant cells stick together and attempt to colonize the AL interface, which at this point in the radiation is a novel and unoccupied niche. It had been previously thought that the wrinkly spreader was the first occupant of a niche at or close to the AL interface in the adaptive radiation of *Pseudomonas fluorescens* but our results find that the nlpD mutant is able to do this already from days 1 – 2 onwards. This colonization of the edge of the AL is associated with mutation in nlpD as this behavior has been found in both natural isolates from the adaptive radiation and a mutant previously created in the Rainey laboratory which possesses a reconstruction of the nlpD mutation. The mechanism by which the nlpD mutant occupies this niche at the AL interface is as yet unknown, however we speculate that it may be linked to “cell chains” which are formed by the nlpD morphotype, (see figure 4.5). These chains of cells which can be observed when the nlpD mutant is viewed under the microscope may explain how the cells can stick together and collapse in the strings that are visible in the video. It is also clear from the video that this niche at the very edge of the AL interface has a low carrying capacity. With increasing colonization of this niche the structures built at the edge of the AL interface collapse and fall to the base of the microcosm. The cells rapidly recolonize when no other morphotypes are present, as can be observed in the movie, but the structures collapse repeatedly suggesting that the numbers of this morphotype will always be modulated by the low carrying capacity of the niche at the edge of the glass vial. This may explain the low fitness observed for the morph in the 50:50 fitness assays as although the nlpD mutant starts at the same concentration as the ancestor in this case, the restriction placed on population size by the limited niche means that the population must inevitably drop and a negative fitness will be recorded, whilst as the ancestral strain can grow without competition in the broth its population size will greatly increase. The case of the nlpD is interesting as whilst the coexistence of the other main
morphs appears to be maintained by frequency dependent selection, it would appear that in the case of the nlpD mutant there is no advantage to being rare and its temporary coexistence is purely facilitated by the ability to occupy the novel niche.

Gause’s principle of competitive exclusion states that “two species cannot coexist with exactly the same set of requisites.” (Gause, 1934). This would certainly appear to be the case for the nlpD mutant and the WS, which arises later in the adaptive radiation of *Pseudomonas fluorescens* as the survival strategy of both requires the colonization of the AL interface. The population density of the nlpD mutant rapidly declines after day 3, which coincides with the evolution of the WS morphotype; the WS also colonizes the AL interface but does so more effectively than the nlpD mutant. This type is able to cover the entirety of the AL interface by overproducing cellulose. The role of the WS type as a more effective colonizer of the AL interface is supported by the results of the invasion from rare assays, which found that the WS type could easily invade and establish itself within a dominant community of the nlpD mutant but the nlpD mutant was unable to reciprocally invade. Although the WS mat is also found to collapse, this is due to a surplus of cheating smooth types, (Rainey and Rainey, 2003) rather than a surplus of cells of their own morphotype, as is the case for the nlpD mutant. This would suggest that over excretion of cellulose is a more effective means of colonizing the AL interface than the, as yet unknown, mechanism of the nlpD mutant. The WS is so effective at colonizing the AL interface that by day 5 of the adaptive radiation the nlpD mutant has dropped to undetectable frequencies and has possibly gone extinct. This would suggest that with its niche now occupied by a higher fitness competitor the nlpD mutant cannot effectively compete against the SM in the broth or the WS at the AL interface, and therefore disappears. We note that the nlpD mutant did not have a fitness benefit over the ancestral strain in our fitness assays after 24h or 48h in static conditions whilst it’s niche is available and put forward two possible reasons for this; the first and most likely reason is that the fitness
assays are based on colony numbers, the carrying capacity of the niche at the edge of the glass vial limits the number of nlpD mutants in such a way that the number of nlpD mutants will always be lower than the number of ancestral colonies which can densely populate the broth at this stage. The second possible reason for this low competitive ability is that the nlpD mutants arise rapidly in the adaptive radiation, even within the short timescale of the fitness assays, in this case it is possible that the unmarked mutant strain must compete for their niche with new mutants arising from the marked ancestral strain.

The dominant morphs in the *Pseudomonas fluorescens* radiation engage in a cyclic competition for oxygen, in which the fallibility of each oxygen strategy leads to the stable maintenance of diversity in the system. Previous studies have created time lapse videos of the growth of the FS, (Ferguson et al, 2013), these show the fuzzy spreader’s strategy for obtaining oxygen at the AL interface is to form flimsy rafts of cells at the AL interface which readily collapse to the bottom of the vial. This strategy is actually very similar to that which is utilized by the nlpD mutant in that both strategies take advantage of a vacancy at the AL interface but neither results in the construction of a stable structure, such as the mat of the wrinkly spreader.

However, it has been shown that the FS can engage in cyclic competition with the other dominant morphs, as it’s rafts are too weak for the SM to “hitchhike” on, (Rainey and Travisano, 1998; Ferguson et al, 2013). FS cannot compete with WS in the absence of smooth cheaters, which episodically open up the AL interface for the FS. This raises an interesting question regarding the seeming extinction of the nlpD mutant, if the nlpD mutant utilizes a similar niche to the fuzzy spreader, why is it then that this type cannot recolonize its preferred niche after the collapse of the wrinkly spreader mat, why is it that it cannot compete with the fuzzy spreader. The answer to these questions may be revealed by the results from our invasion from rare assay. We found that the fuzzy spreader could easily invade an abundant nlpD mutant population from rare but the nlpD mutant could not
reciprocally invade the fuzzy spreader. This suggests that the FS too – like the WS – is a superior colonizer of the AL interface as compared to the nlpD mutant. It is possible that the extinction of the nlpD mutant may be explained by the fact that it is vulnerable to two of the dominant morphotypes, both of which have a superior strategy for colonizing its preferred niche than the one it possesses itself.

In addition to the low fitness values which may lead to the extinction of the nlpD mutant, the microelectrode profiles suggest that it is also a very poor dissipator of the oxygen gradient. Whilst the nlpD communities do manage to fully break down the gradient to anoxia this never occurs at a depth of above 500 microns which reduces the selective advantage of growth at the AL interface which may explain the fact that WS do not evolve from the nlpD type. These results are also consistent with our hypothesis that Pseudomonas fluorescens mutants vary in their utilization strategies and their abilities to dissipate the oxygen gradient as the nlpD mutant which has an inferior strategy for gradient dissipation is rapidly competitively replaced by a superior oxygen dissipator.

5.5 Concluding remarks

Our results describe the evolution of a new morphotype within the *Pseudomonas fluorescens* adaptive radiation. This morph takes advantage of a novel niche and manages to persist for a short time despite a low fitness compared to the ancestral strain by avoiding direct competition with the ancestor. The morph is competitively replaced most likely to the extent of extinction by the WS which is a superior coloniser of the AL interface. If our theory that *P. fluorescens* evolves strategies that allow for the maximal dissipation of the oxygen gradient is correct then the fact that nlpD is such a poor dissipator of the gradient may explain why it is so rapidly eradicated by the system.
References


Figure 5.1: This figure shows the population dynamics of the nlpD mutant. The mutant arises quickly before peaking and rapidly declining until they can no longer be detected in the population. The graph shows the average number of colony forming units (cfu) per ml in the original culture for the 3 microcosms sampled.

Figure 5.2
**Figure 5.2:** This figure displays the relative fitness of the nlpD mutant when competed against the ancestral strain under static conditions for either 24 hours or 48 hours respectively.

**Figure 5.3**

![Image A](image1.png)

*Figure 5.3: Picture A shows an nlpD mutant attempting to colonize the AL interface, whilst picture B shows the more efficient WS colonizing the same niche.*
Figure 5.4

Figure 5.4: Results of the invasion from rare assays. The arrows are positioned such that the arrow begins at the circle representing the rare morph in each competition and finishes at the circle representing the dominant morph in each competition. The values associated with each arrow represent the selection rate constant, the difference in the Malthusian growth parameter $r_i$ of a rare morph invading a dominant resident morph ($s_{ij} = r_i - r_j$). Zero values indicate negative selection rate constant values, meaning that the rare morph was unable to invade.
Figure 5.5

A
Figure 5.5: Compares the nlpD mutant which in picture A can be seen to stick together in chains of cells to form large “snakes”. This is compared to the ancestral SBW25 strain, which in picture B can be shown to have randomly distributed cells.
Figure 5.6

A

Day 1
Day 2
Day 3
Day 4
Day 5

NlpD MUTANT

B

Day 1 SBW25
Day 2 SBW25
Day 3 SBW25
Day 4 SBW25
Day 5 SBW25

SBW25 CONTROL
**Figure 5.6** A shows the oxygen profiles obtained when glass vials were inoculated with the nlpD mutant, the steep profiles seen for other types never form for this mutant. B shows the steep profiles quickly obtained for the SBW25 control vials.
6.1 *Pseudomonas fluorescens* SBW25 can be used as an effective model for dissipative systems theory

The main aim of this thesis was to explore the possibility that the bacterium *Pseudomonas fluorescens* SBW25 could be used as a model system to test the predictions of dissipative systems theory in a real world system. In this concluding discussion I will argue that the results presented in this thesis offer compelling evidence in support of this theory.

Two of the main predictions of dissipative systems theory were tested in this thesis, they were as follows:

1. In order to maximally dissipate a free energy gradient a system should develop complex structures that dissipate energy and strategies for maintaining energy dissipation over time.
2. To ensure continued energy dissipation, feedbacks should exist between the system and the external environment.

I will consider the evidence reported here for each of these main predictions separately. For the first, it was important in the first instance to establish that a gradient of free energy could have some impact on the diversity and functioning of the bacterium *Pseudomonas fluorescens* SBW25. The free energy gradient in question throughout this thesis is an oxygen gradient. In chapter 2, the effects on the oxygen gradient on *Pseudomonas fluorescens* community
dynamics were studied in detail. The work presented in this chapter established that under natural air conditions (20% oxygen, 80% nitrogen) the *Pseudomonas* system exhibits rock-paper–scissors like dynamics. The bacteria are able to coexist as they each have a strategy for obtaining the oxygen (free energy) in the system, however each of these strategies is fallible and leaves each of the morphotypes susceptible to invasion from one of the others. Wrinkly spreader (WS) types can invade the ancestral smooth type (SM) by building a biofilm and monopolizing the available oxygen, SM types can reciprocally invade the WS type by arising as cheats in the mat, the fuzzy spreader (FS) is susceptible to the WS as the latter is a far more efficient colonizer of the AL interface and it has been shown previously (Rainey and Travisano, 1998; Ferguson et al, 2013) that the FS type can reduce the growth of the SM type as the rafts formed by the former are too flimsy to support SM cheats. The results presented in chapter 2 show that these dynamics are highly dependent on the oxygen concentration in the microcosm and in line with dissipative systems theory the strategy which is the most efficient at using the oxygen available at each temporal point prevails. For example, when oxygen is only available in the first few microns from the AL interface and the gradient is exceptionally steep, the WS type that can best intercept the oxygen diffusing in from the air has a clear fitness advantage but when there is a less steep gradient in the microcosm and oxygen is plentiful the SM type has an advantage in the rock paper scissors like competition, on the basis of its superior metabolism and growth rate. Interestingly and also in line with the predictions of DST these RPS dynamics are substantially altered by changing the concentration of oxygen (free energy) in the system. For example in the invasion from rare assays we carried out under pure oxygen, the SM type had a much greater fitness advantage over the WS than under air, ecologically this makes sense as when oxygen penetrates deeper into the broth - as is the case when incubated under pure oxygen - there is not the same desperate need to grow at the very surface of the AL interface so the energy expenditure of overproducing cellulose and forming a biofilm are not as advantageous in this case. This also
fits well with dissipative systems theory as the smooth type (which is the more efficient oxygen user due to an increased metabolic rate) has a selective advantage when there is more oxygen to use meaning that the system is gravitating towards the most efficient strategy for oxygen dissipation. At the other extreme when the invasion from rare is carried out under pure nitrogen, the WS has a much greater selective advantage than it does under air or pure oxygen. This is because oxygen is limited in the broth immediately after inoculation under this condition so the only way for the bacteria to survive is by forming a biofilm at the AL interface. Again this result is in line with the DST prediction as the system favours the best strategy to ensure maximal oxygen consumption.

Perhaps most relevant to DST of the results reported in chapter 2 is the finding that the diversity present in the system was highest when the adaptive radiation was carried out under pure (100%) oxygen conditions as compared to the air or pure nitrogen conditions. The fact that the most extensive adaptive radiation occurred when the levels of free energy were highest is in line with the predictions of Nicolis and Prigogine (1977) that complexity will develop in order to dissipate higher levels of free energy. This observation was used as a building block for the research reported in chapter 4, in fact the stage 1 experiment described in this chapter (labeled “origins”) is a repeat of this key result. The results reported in chapter 4 seem to suggest that the first energy conditions that are faced by a bacterium and the species interactions that result may have a significant effect on the evolutionary trajectory that they take regardless of the conditions they face later. This offers some support to the theory that *Pseudomonas fluorescens* function as dissipative structures as in this experiment it was found that the bacteria that had previously been exposed to the highest energy conditions were the best able to deal with such an influx of oxygen when they were faced with it again. The doubling time of *Pseudomonas fluorescens* is approximately 20 minutes and in the four days that the bacteria were incubated under the oxygen conditions described in the stage 1
experiment in chapter 4 it is expected that they would acclimate to their current conditions but for this effect to be heritable and to last for the duration of another adaptive radiation in a new environment is highly surprising. However, the interaction effect we report in chapter 4 lasted for the duration of a five day experiment in new conditions, this is a result that merits greater study because although this development in response to high energy conditions supports the prediction from DST that the community should develop to be capable of utilizing high resource conditions when exposed to them, the mechanisms by which this effect is obtained are far from clear. It is possible that the observed patterns are due to epigenetic effects, these have been observed in other bacteria, for example *Pseudomonas aeruginosa* which has been found to have an epigenetic switch for virulence gene expression (Turner et al, 2009). It has also been predicted by Klironomos and colleagues (2013) that epigenetic effects may have an effect on mutation rates and their importance in the process of adaption may have been greatly underestimated. Epigenetic effects can be heritable across several generations so they offer a possible explanation for the continued effect of pre exposure observed in chapter 4. Another possible explanation is that the in the initial stages of the second adaptive radiation the acclimated bacteria have different effects on the environment similar to priority effects. Priority effects have previously been found to be important in the *Pseudomonas* system, with Fukami and colleagues, (2007), finding that diversity in the system could be reduced by the early arrival of the WS. A recent study has also found support for the hypothesis that the influence of priority effects are stronger in less stressful environments, (Leopold, 2015), whilst unclear if this is the case in this experiment it could be argued that the air pre exposure constitutes the least stressful of the origin conditions so it is possible that priority effects were magnified in this case. A final possibility is that although the colonies picked for use in the stage 2 experiments were phenotypically similar, it is possible that there were genetic differences unrelated to the colony morphology present in these
bacteria and these may have led to sustained differences between the treatments. Further work is required in order to fully understand this result.

The early energy conditions were found to have a significant effect on the complexity (measured here as species diversity) that the system could obtain when moved into completely independent conditions. In fact as we report in chapter 4, only the origin conditions had an effect on the diversity reached by the bacteria the new conditions had no significant effect on diversity. The highest diversity was found in bacteria that had been pre exposed to either completely anoxic (0% oxygen) conditions or the high energy (100% oxygen) conditions and a significantly lower diversity was obtained in colonies that had been pre exposed to the natural air conditions. From these results it is clear that bacteria which have been exposed to extreme conditions are capable of generating the highest complexity. That a higher level of complexity was found under the 100% oxygen conditions supports the prediction of DST that dissipative structures can become more complex in order to more efficiently dissipate the available oxygen. The higher diversity found in the bacteria that had been incubated under nitrogen is not immediately explained by DST but it does make sense when the ecological theory of adaptive radiation is considered, colonies that had been grown under very low oxygen conditions would have experienced an increase in the available oxygen in all of the conditions in the stage 2 experiments reported in chapter 4. This may have been analogous to ecological opportunity (Yoder et al, 2010) and have driven an adaptive radiation as the radiation, which occurred under pure nitrogen was so restricted compared to those found under aerobic conditions, (see chapter 2 and 4 results). However, why the diversity found when the bacteria had been previously exposed to air was so low is not apparent. It is interesting to note that the bacteria previously exposed to air were also the most abundant. Although there is no apparent explanation for this result, there is reason to believe it is a consistent trend as it can also be seen in the results presented in chapter 3 that when diversity is lowest, the abundance of bacteria is highest. It is also worth noting that
when diversity is low the population is almost completely comprised of the more metabolically efficient SM types. When considered in a DST light, it may be possible that when the bacteria cannot dissipate the oxygen by diversifying into complex communities with different strategies to ensure maximal oxygen use, the system attempts to counteract the shortfall of oxygen utilization strategies by increasing the number of cells that are contributing to the metabolism. Further work is required to understand this fascinating result.

Nicolis and Prigogine (1977) predicted that to conform with this prediction of dissipative systems theory that the system will develop in order to maximally dissipate a free energy gradient, morphotypes that arose should either occupy a new niche or be a superior colonizer of an existing niche. This is similar to the competitive exclusion principle first put forward by Gause (1934). The DST interpretation of this result manifests in an expectation that poor dissipaters (in the context of this thesis poor oxygen users) would be rapidly competitively replaced. Evidence that suggests this prediction is accurate for *Pseudomonas fluorescens* SBW25 and is presented in chapter 5 of this thesis. The nlpD mutant described here exhibits both types of the behaviour predicted by Nicolis and Prigogine (1977), when it first arises it occupies a novel niche at the very edge of the AL interface. This is a niche that is unavailable to the ancestral smooth type and therefore the nlpD mutant can use oxygen in a new spatial location, which contributes to overall oxygen use. However, as can be seen in the microelectrode profiles shown in chapter 5, the nlpD mutant is a very poor dissipater of oxygen, it never manages to achieve the steep gradients characteristic of the system. It is then driven to extinction by the arrival of the WS which is a superior colonizer of the niche at the AL interface and leads to more efficient use of the oxygen than nlpD. The nlpD mutant also cannot radiate into any other morphotypes and as diversity (complexity) is essential for
efficient use of oxygen (exemplified by the results presented in chapter 3), which may explain why the nlpD mutant is eradicated from the system.

The second prediction of dissipative systems theory that is explored in this thesis using the bacterium *Pseudomonas fluorescens* SBW25 is that feedbacks must exist between the organism and its environment to ensure continued gradient dissipation. Such feedbacks are also predicted in ecology and may manifest themselves in the process of niche construction. In chapter 2, evidence was presented that niche construction is present in the *Pseudomonas* system and there are two main types to consider. The first being the initial metabolism of the smooth type which creates a steep gradient, which in turn creates the niche at the AL interface, and the second is the formation of the biofilm mat at the AL interface which limits the access to oxygen to the WS type until the smooth type develops a cheating strategy to compete with the WS in their own niche. In chapter 3 we explore the prediction from dissipative systems theory that inhibiting the feedback mechanisms of a system would result in a loss of complexity in the system and reduced gradient dissipation. This trend is exemplified by the results in chapter 3, inhibiting both of the forms of niche construction in *Pseudomonas fluorescens* leads to a complete loss of complexity in the system. The loss of feedbacks also leads to an inability to fully use the oxygen in the system. This supports the prediction from DST that feedbacks are essential to the functioning of a dissipative ecosystem.

The results presented above demonstrate that the bacterium *Pseudomonas fluorescens* SBW25 can be successfully used to test the main predictions of dissipative systems theory. Indeed they also demonstrate that *Pseudomonas fluorescens* responds to an oxygen gradient in the way that DST predicts a living system will respond to a free energy gradient. This suggests that *Pseudomonas fluorescens* may be used for further study to test the implications of free energy gradients on living systems. It also indicates that bacteria in general can be used as a
model ecosystem to test the validity of physical sciences theories when applied to the living world.

6.2 Importance of the results for models of life

The results of this thesis which support the hypothesis that *Pseudomonas fluorescens* SBW25 can be used as a model system for dissipative systems theory has important implications for models of early life and the emergence of diversity. Many of the studies that have attempted to apply physics theory to the study of biological systems have been mathematical (England, 2013; Sneppen, 2014) or data analysis studies (Aoki, 2006). While these studies draw interesting conclusions about the origins of life, their application to living systems has always been speculative. This thesis demonstrates that when properly interpreted in a biological context it is entirely possible to test the predictions from a highly theoretical discipline in a real life biological system. Simplifying the system to a microcosm containing a simple environment and one, easily manipulated free–energy gradient, along with a well understood living species (a bacterium) allowed the predictions of DST to be tested. The theoretical predictions were confirmed in a way that is compatible with the already well-studied ecology of the species. These approaches could be adapted to test other theories on the origin of life that have been reliant so far on purely theoretical approaches. One such example would be the Gaia theory put forward by James Lovelock (1979). This theory which suggests that Earth self regulates to create conditions that are amiable to life, (Lovelock, 1979; Lenton, 1998) offers a fascinating picture of a dynamic earth. However, it has attracted much criticism from evolutionary biologists who argue that the idea of living organisms working together towards a common goal is contradictory to the “survival of the fittest” (Spencer, 1864) and “selfish gene” (Dawkins, 1976) theories that are central to evolutionary theory. Indeed one of the most famous criticisms of the theory was that there was nothing in the
nature of living organisms to create the feedbacks postulated to exist by Lovelock (Doolittle, 1981). A similar criticism could be levered at dissipative systems theory, why should a complex bacterial community function in a way that also maximally utilizes a free energy gradient and why would the feedbacks predicted by Nicolis and Prigogine (1977) develop in a selfish evolutionary system. The answer to this paradox can be seen in the results presented in this thesis. The bacteria grow in a selfish manner, when the oxygen environment is suited to their strategy for obtaining oxygen they grow as much as possible, in each case it is a change in the oxygen environment brought on by the growth and development of the bacteria that leads to a transformation in the most advantageous growth strategy (e.g. from WS mats to SM cheats to FS rafts). The RPS dynamics described in chapter 2 are maintained by competitive coexistence are in line with evolutionary theory. The feedbacks between the bacteria and the abiotic oxygen environment develop naturally in the Pseudomonas fluorescens system and as we show in chapter 3 of this thesis, they are crucial for the development of complexity and for efficient resource use. The feedbacks develop in line with the expected behaviour of the bacteria. Bacteria such as Pseudomonas fluorescens and perhaps even Pseudomonas fluorescens itself could potentially be used to test predictions from the Gaia theory. Watson and Lovelock (1983) developed a model of self regulation in a living world called the daisy world model. Daisy world is an imaginary world that is pushed out of equilibrium by a star similar to the sun, it gets warmer over time. There are two types of life possible on the planet a black daisy and a white daisy. The pale white daisies cool the environment, whilst the black daisies warm the environment. At the start of the experiment only the black daisies can grow in the temperature present on the planet, as they grow they warm the planet until it is warm enough for the white daisies to grow. (Watson and Lovelock 1983; Lenton, 1998). Although the principle purpose of this thesis was to establish Pseudomonas fluorescens as a model system for DST it is possible to imagine it being used as a real life model for the predictions of daisy world. That the growth of the black daisies creates
an environment conducive to the growth to the white daisies is similar to *Pseudomonas fluorescens* in that the growth of the smooth creates the oxygen atmosphere that allows for the growth of the wrinkly spreader. However, more work is required to establish the suitability of *Pseudomonas fluorescens* to study the predictions of the daisy world model and other models of early life.

### 6.3 Future directions

This thesis establishes *Pseudomonas fluorescens* as a model system to test the predictions of dissipative systems theory. However, this system has a great deal more to offer in aiding our understanding of how this bacterium might function as a dissipative system. In particular, mathematical models of *Pseudomonas fluorescens* as a dissipative system are required as these will help to further understand the results and how they are comparable to the original physical theory. The results presented in chapter 4 are incredibly interesting but there is still much that we don’t fully understand about these results, in particular it is not clear why the complexity that develops after the bacteria has been pre exposed to air (20% oxygen) is so low in comparison to either no or high oxygen. In depth experiments on community structure in response to exposure to varying oxygen gradients for varying periods of time and repeated exposures are needed to better understand these results. It would also be advantageous to see if the patterns observed for *Pseudomonas fluorescens* SBW25 could be emulated in another bacterial system. One possible system in which this could be studied would be the metal leaching activities of thermophilic bacteria. Bioleaching is another process which takes in high free energy and producing entropy as the leaching is an irreversible process. Previous work has shown that multi species (more complex) communities are more efficient at this process than single species communities (Maezato et al, 2012).
6.4 Concluding statement

Understanding how and why life evolved on planet earth and why in particular it is so complex may guide the hunt for life on other planets. Dissipative systems theory predicts that complex dissipative structures evolve strategies that can maximally break down a free energy gradient. This theory makes compelling predictions for the emergence of biodiversity but has to date proved difficult to test experimentally. This thesis explores the possibility of using the bacterium Pseudomonas fluorescens SBW25 as a real life model system to test the predictions of dissipative systems theory. The results presented offer support for this hypothesis and the general conclusion of the thesis is that the system is an appropriate model. The thesis provides an important first step for finding more experimental tests for dissipative systems theory and contributes to a growing field of model systems for early life.
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First and foremost, I would like to thank my supervisor Bas Ibelings for giving the opportunity to do my PhD in his lab, for always letting me explore the research avenues that interested me most and offering advice and support for the entirety of the last 3 years. I would also like to say a particular thank you to Blake Matthews for helping me every step of the way in developing the perspective that was explored in this thesis, to Patrick Venail for helping me to get my head around all the data, to Katrin Hammerschmidt for helping me get started and to Duygu Sevilgen for teaching me how to use microsensors.

I would also like to thank Paul Rainey for allowing me to join his lab group for 6 months, I learned a lot from him and his feedback substantially improved the work presented in this thesis. I would like to thank Dirk De Beer, similarly for allowing to join his group at the Max Planck institute for Marine Microbiology.

I have had the benefit of being a member of three great research groups during my PhD and I have learned from everyone that I’ve worked with but I would like to thank in particular Irene Gallego, Ena Suarez, Evi Mantzouki, Xue- Xing Zhang, Andy Farr, Peter Lind, Yeserin Yeldrim, Peter Dienes, Gayle Ferguson, Jenny Herzog and Artur Fink.

I would like to thank my family and friends for emotional support, particularly Jabin, Sheryl, Tinneke, Ellen, Sue, Chris and Elsa.

This thesis is dedicated to my mother and grandmother for never ending support.