Pacific oyster holobiont in the changing environment: a microbial perspective

Dissertation
in fulfilment of the requirements for the Degree "Dr. rer. nat."
of the Faculty of Mathematics and Natural Sciences
at Kiel University

submitted by

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Kiel 2015

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Date of the oral examination: 23 July 2015

Approved for publication:

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CONTENTS

ZUSAMMENFASSUNG

Die Fitness eines Tieres hängt nicht nur von seinen eigenen Merkmalen ab - praktisch jeder Aspekt der Biologie von Tieren wird tiefgreifend von mit ihnen assoziierten Mikroorganismen beeinflusst. Mikroorganismen spielen unter anderem eine wichtige Rolle für den Gesundheitszustand: sie schützen ihren Wirt vor Infektionen, sind gleichzeitig aber auch opportunistische Pathogene. Letzteres tritt vor allem dann auf, wenn die Immunabwehr des Wirtstiers durch biotischen oder abiotischen Stress geschwächt ist. Der globale Klimawandel kann solche Verschiebungen zu pathogenem Verhalten begünstigen, da steigende Temperaturen Stress für den Wirt bedeuten und gleichzeitig die Virulenz von Mikroorganismen erhöhen können. Ein Beispiel für solche umweltabhängigen, durch Bakterien hervorgerufenen Krankheitsausbrüche ist die Sommersterblichkeit einer Muschel, der Pazifischen Auster (*Crassostrea gigas*). Auch wenn der Klimawandel die Ausbreitung dieser Art in Küstenökosystemen weltweit unterstützt hat, breiten sich auf Grund steigender Temperaturen gleichzeitig die sommerlichen Austernsterben immer weiter nach Norden aus. Hervorgerufen wird das Austernsterben durch kommensalische, opportunistisch pathogene Bakterien der Gattung *Vibrio*, die einen starken Selektionsdruck ausüben und eine schnelle Anpassung der Austern an lokale *Vibrio*-Populationen bewirken. Als eine bedeutende Art in der Aquakultur und wird die Pazifische Auster aus kommerziellen Gründen häufig in neue Habitate eingeführt, wo sie neuen Mikroorganismen ausgesetzt ist. Wie mit den Austern assoziierte Mikroorganismen-Gemeinschaften auf solche neuen biotischen Umgebungen und auf Veränderungen der abiotischen Bedingungen reagieren, ist weitgehend unbekannt. Zu verstehen, wie Mikroorganismen in Austern durch (a)biotische Störungen beeinflusst werden, ist jedoch eine Voraussetzung für die Abschätzung ihrer Auswirkungen auf die Fitness von Austern.

In dieser Arbeit habe ich die Reaktionen von Mikroorganismen der Pazifischen Auster auf Stress durch Translokationen (Kapitel I und II), durch starke Temperaturschwankungen und durch Infektionen mit externen Pathogenen (Kapitel III) untersucht. Kurzzeit-Translokationsexperimente (Kapitel I) zeigten die besondere Bedeutung von Mikroorganismen in der Hämolymphe für die Fitness von Austern: Eine durch Interaktionen zwischen den etablierten und neuen Mikroorganismen hervorgerufene Destabilisierung der Bakteriengemeinschaften in der Hämolymphe führt zu potenziell tödlichen *Vibrio*-Infektionen bei umgesiedelten Austern. In ähnlicher Weise kann eine Destabilisierung durch höhere Temperaturen (22°C) zu erhöhter Austernsterblichkeit nach einer Infektion bei (Kapitel III) beitragen. Insbesondere die Kombination von Temperatur- und Infektionsstress ruft eine starke

Störung der Gemeinschaftsstruktur und die Vermehrung opportunistischer Pathogene hervor, während in anderen Fällen die Mikrofauna in der Hämolymphe eine hohe Resistenz gegenüber externen *Vibrio*-Infektionen bietet. Neben ihrer Rolle bei der Infektionsabwehr weisen kurzfristige Veränderungen der Zusammensetzung der Bakteriengemeinschaften als Reaktion auf abiotische Bedingungen (Kapitel II und III) auf die Bedeutung der Mikrofauna in der Hämolymphe bei der Akklimatisierung von Austern hin. Abgesehen von der schnellen Reaktion auf plötzlich veränderte Umweltbedingungen und dem starken Einfluss von Seewasser und nicht-stationären Mikroorganismen auf die Zusammensetzung der Bakteriengemeinschaften, werden die etablierten Mikroorganismen nach einer Translokation nur allmählich durch neue lokale Arten ersetzt. Auch wenn diese Beständigkeit der Mikrofauna Stress durch abrupt auftretende Umweltveränderungen verringern kann, führt sie nach unvorhersehbaren Störungen der Bakteriengemeinschaften zu langanhaltenden Effekten, die negative Auswirkungen auf die Fitness von Austern haben können.

Durch die Untersuchung der mit der Pazifischen Auster assoziierten Mikrofauna unter kontrollierten und natürlichen Bedingungen über multiple zeitliche und räumliche Skalen konnte ich wichtige Faktoren identifizieren, die ihre Zusammensetzung und Stabilität bestimmen. Außerdem konnte ich die besondere Rolle in der Hämolymphe lebender Mikroorganismen bei Interaktionen mit biotischen und abiotischen Umweltbedingungen zeigen. Die Ergebnisse deuten darauf hin, dass, neben dem Nutzen der Hämolymphe-Mikrofauna bei Akklimatisierungsprozessen und der Pathogenabwehr, durch den Klimawandel hervorgerufener Stress zu Veränderungen der Bakteriengemeinschaften mit nachteiligen Folgen für die Austern führen kann.

SUMMARY

Animal's fitness does not depend only on its own traits, as almost every aspect of animal's biology is profoundly affected by its associated microbiota. Among other, the microbiota play an important role in health and disease: they protect their host against infections as well as act as opportunistic pathogens. The latter usually occurs if host's immune defense has been compromised due to abiotic or biotic stress. Global climate change can promote such shifts towards pathogenicity, as rising temperatures impose stress on the host and at the same time may cause increase in virulence of microbes. One example of such environmentally-dependent disease outbreaks involving opportunistic pathogens are the summer mortalities of Pacific oysters (*Crassostrea gigas*). Although the climate change supported the spread of this invasive intertidal bivalve to coastal habitats all over the world, it also caused the mortality events to ocurr always farther north. Involved in these mortalities are the oyster commensals and opportunistic pathogens of the genus *Vibrio* sp., which impose strong selection pressure and thus cause rapid adaptation of Pacific oysters to their local *Vibrio* populations. The Pacific oyster is also an important aquaculture species that is being routinely translocated for commercial purposes to new habitats, where it is exposed to foreign microbes. Response of the oyster-associated microbial communities to this new biotic environment or to changes in abiotic conditions is largely unknown. However, understanding how the oyster microbiota are shaped by (a)biotic disturbances is a prerequisite for predicting their impact on oyster fitness.

In this thesis, I examined how Pacific oyster microbiota respond to the stress imposed by translocation (Chapter I, Chapter II), as well as by infection with external pathogens and drastic changes in temperature (Chapter III). Short-term translocation experiment (Chapter I) revealed the special role of the hemolymph microbiota for oyster fitness, since destabilization of the hemolymph communities, likely caused by interactions between the resident and new microbes, lead to potentially fatal systemic *Vibrio* infections in translocated oysters. Similarly, destabilization caused by high temperatures (22°C) probably contributed to oyster mortality following infection (Chapter III). However, the disruption of community structure and proliferation of opportunistic pathogens occurred mainly after the exposure to combined temperature and infection stress, while otherwise the hemolymph microbiota provided resistance against the external *Vibrio* pathogen. Apart from the role in oyster defense, rapid shifts in community composition in response to abiotic conditions (Chapter II, Chapter III) without the effect on community structure, indicate that hemolymph microbiota are important also for oyster acclimation. Despite this quick response to instant environmental conditions and strong influence of seawater and transient microbes on the community composition, the

resident microbiota are only gradually replaced by local bacteria after the translocation (Chapter II). Although such persistence can diminish stress caused by abrupt changes in environmental conditions, it also results in long-persisting effects after unpredictable community perturbations, which could negatively affect oyster fitness.

In summary, by studying the Pacific oyster microbiota in controlled and natural conditions over multiple spatiotemporal scales, I identified some important determinants of its composition and stability and unveiled special role of the hemolymph microbiota in the interactions with abiotic and biotic environment. My results suggest that, despite the beneficial role of hemolymph microbiota in acclimation and defense against pathogens, the combined stress imposed by the climate change could lead to detrimental community shifts.

INTRODUCTION

The holobiont

Microbiota affects host

Microbiota are nowadays recognized as an integral part of animal organisms. The holobiont concept, establishing a host together with its associated microbiota as a unit of evolution, has gained widespread acceptance (Rosenberg *et al.*, 2007; Mcfall-Ngai *et al.*, 2013). The microbiota affect developmental processes and growth of their hosts (Sison-Mangus *et al.*, 2015; Thompson *et al.*, 2015) and have been associated with aging processes (Rehman, 2012; Muller *et al.*, 2013) and longevity (Erkosar and Leulier, 2014); they are essential for the nutrition and metabolism of both invertebrate and (Ridley *et al.*, 2012; Wong *et al.*, 2014) vertebrate animals (O'Connor, 2013; Ramakrishna, 2013) and influence the development of metabolic disorders such as adiposity (Ridaura *et al.*, 2013; Dogra *et al.*, 2015); they produce and secrete hormones and thus modulate the host endocrine system (Clarke *et al.*, 2014; Neuman *et al.*, 2015) and they can even influence their host behavior (Cryan and Dinan, 2012) or affect the specificity of host-pathogen interactions (Koch and Schmid-Hempel, 2012).

One of the vital beneficial services of microbiota is their contribution to the immune defense of the host (Gross *et al.*, 2009; Hooper *et al.*, 2012; Abt and Pamer, 2014; Belkaid and Hand, 2014). Microbiota provide protection against pathogens through community traits such as resistance against colonization (Shade *et al.*, 2012; Buffie and Pamer, 2013; Kamada *et al.*, 2013) or production of antimicrobial compounds (Defer *et al.*, 2013; Desriac *et al.*, 2014), as well as through direct effects on the host (Chung *et al.*, 2012; Broderick *et al.*, 2014). Proper functioning of the immune system depends on the stimulation by microbiota (Olszak *et al.*, 2012), and disturbed or insufficiently developed microbial communities have been linked to ever-increasing incidence of allergies in humans (Bancroft *et al.*, 2012; Inoue and Shimojo, 2015; West *et al.*, 2015).

Host affects microbiota

Just as the microbiota affect their hosts, the hosts shape their associated microbiota. Species specificity of microbial communities in closely related, ecologically similar species reveals the active role of the host for the community assembly (Fraune and Bosch, 2007; Erwin *et al.*, 2012b; Naim *et al.*, 2014). At within-species level, the diversity, composition and function of microbial

communities are influenced by genotype (Rausch *et al.*, 2011; Spor *et al.*, 2011; Wegner *et al.*, 2013; Dobson *et al.*, 2015) and sex (Zouache *et al.*, 2011; Bolnick *et al.*, 2014), as well as by variable traits, including life stage (Wang *et al.*, 2011; Minard *et al.*, 2013; Hammer *et al.*, 2014; Thompson *et al.*, 2015), reproductive state (Phillips *et al.*, 2012), health condition (Green and Barnes, 2010; Salonen *et al.*, 2012; Lozupone *et al.*, 2013; Ransome *et al.*, 2014), diet and lifestyle (Robinson *et al.*, 2010; David *et al.*, 2014; Wang *et al.*, 2014; Xia *et al.*, 2014).

In addition, the microbiota are affected by the within-host habitat variability, since each body site offers a unique set of conditions to colonizing microbes (Spor *et al.*, 2011). These tissue-specific communities can exhibit different levels of stability in the face of disturbances, for example following an infection (Belzer *et al.*, 2014) or change in diet (Sudakaran *et al.*, 2012), and thus distinctly affect the host fitness. However, our limited knowledge of within-host biogeography is mostly descriptive and - with few exceptions (Antunes *et al.*, 2010; King *et al.*, 2012a; Sudakaran *et al.*, 2012; Givens *et al.*, 2013) - restricted to humans and other enothermic animals (Costello *et al.*, 2009; Dominguez-Bello *et al.*, 2010; Faust *et al.*, 2012; Huse *et al.*, 2012; Zhou *et al.*, 2013; Belzer *et al.*, 2014; Jemielita *et al.*, 2014; Lu *et al.*, 2014).

Holobiont and the environment

While the microbiota inhabiting the outer surfaces of endothermic animals can be strongly influenced by external abiotic factors (Rosenthal *et al.*, 2011), the communities associated with the inside-body habitats such as gut are more likely to be influenced by diet, health condition or life-style (Looft and Allen, 2012; Lozupone *et al.*, 2012; David *et al.*, 2014). For example, highly specialized and stable mammalian gut microbiome (Ley *et al.*, 2008; David *et al.*, 2014) reflects relatively stable abiotic conditions within the gut as well as its relative independence from the external abiotic environment. In contrast, all microbial communities within an ectothermic animal are likely to be strongly affected by changes in abiotic conditions. While ectothermic holobionts can be well adapted to the normal range of environmental fluctuations, they may not be prepared for the extreme challenges imposed by climate change. Although the climate change affects both endotherms and ectotherms (Buckley *et al.*, 2012), the tight connection between the ectotherm-associated microbiota and the environment may contribute to the higher susceptibility of ectothermic holobionts.

Response to challenges of (a)biotic environment: the threats of climate change

One of the basic features of the ongoing climate change is the increase in temperature (IPCC, 2014). Temperature is a major determinant of physiological and metabolic processes and it subsequently affects all levels of biological organization i.e. (Ratkowsky *et al.*, 1982; Willmer, 1991; Angilletta *et al.*, 2002; Angilletta *et al.*, 2004; Cano and Nicieza, 2006; Bozinovic *et al.*, 2011; de Mendoza, 2014). In addition to direct physiological consequences of the abiotic stress imposed by the rising temperatures (Bernardo and Spotila, 2006; Huey *et al.*, 2012), the ectothermic holobionts may also be challenged by altered biotic interactions, resulting in the increased risk of disease. Climate-change related disease outbreaks have already been recorded in both terrestrial and marine environment (Harvell *et al.*, 2002; Harvell *et al.*, 2009; Baker-Austin *et al.*, 2012; Elderd and Reilly, 2014). Understanding the mechanisms and processes involved in these evolutionary and ecologically important events is necessary to predict future dynamics in the affected ecosystems (Altizer *et al.*, 2013).

Diseases are usually a result of a complex interplay between the involved organisms and environmental factors (Casadevall, 2003; Altizer *et al.*, 2013) (Li *et al.*, 2010; Wedekind *et al.*, 2010; Engering *et al.*, 2013; Wendling and Wegner, 2013; Méthot, 2014; Petton, 2015), rather than a fixed outcome determined by the identity of the host and the pathogen (Casadevall, 1999; Casadevall and Pirofski, 2000; Méthot, 2014). The temperature directly affects immunological competence of ectothermic hosts (Hégaret *et al.*, 2004; Paillard *et al.*, 2004; Gagnaire *et al.*, 2006; Mydlarz *et al.*, 2006; Kortet and Vainikka, 2008; Luna-Acosta *et al.*, 2011; Dang *et al.*, 2012; Matozzo *et al.*, 2012) as well as the pathogen virulence (Mekalanos, 1992; Lemes-Marques and Yano, 2004; Kimes *et al.*, 2012). Therefore, it also shapes the outcome of host-pathogen interactions, although not necessarily in a straightforward manner (Blanford *et al.*, 2003; Thomas and Blanford, 2003). Severe shifts in environmental conditions can thus compromise host immune defenses and result in opportunistic infections (Burge *et al.*, 2013).

Despite their beneficial role in defense against external pathogens, the host-associated microbiota are also an important source of opportunistic pathogens. Diseases are often caused by common members of a healthy microbiome, which harm only the hosts weakened by abiotic or biotic stress (Bauer and Agerter, 1994; Garnier *et al.*, 2007; Cogen *et al.*, 2008; Higuchi *et al.*, 2013; Rivas *et al.*, 2013; Musharrafieh *et al.*, 2014; Wendling *et al.*, 2014). In addition, many diseases are not caused by a single pathogen, but result from polymicrobial infections or from complex, environmentally-induced shifts in host-associated communities (Cooney *et al.*, 2002; Cerf-Bensussan and Gaboriau-Routhiau, 2010; Mao-Jones *et al.*, 2010; Hamdi *et al.*, 2011; Altizer *et al.*, 2013; Fan *et al.*, 2013; Murray *et al.*, 2014; Olson *et al.*, 2014; van de Wijgert *et al.*,

2014; Zaragoza *et al.*, 2014; Carding *et al.*, 2015). On the other hand, shifts in community composition may contribute to successful acclimation of a holobiont to new environmental conditions (Reshef *et al.*, 2006). The microbiota can thus benefit, but also harm their hosts in the changing environment. Exploring the abiotic and biotic factors that affect the composition and dynamics of host-associated microbiota is therefore an important step towards understanding the impact of the climate change on ectothermic holobionts.

Holobiont in a new environment

Climate change and rising temperature also affect the species distribution (Perry *et al.*, 2005; Pickles *et al.*, 2013; VanDerWal *et al.*, 2013; Gonzalez *et al.*, 2014) and facilitate the spread of invasive species following the unintentional and intentional introductions by humans (Naylor *et al.*, 2001; Monney, 2009; Sorte *et al.*, 2010). The invaders may strongly affect invaded communities and ecosystems (Wallentinus and Nyberg, 2007; Vila *et al.*, 2010), but they as well are challenged by the new abiotic and biotic environment (Shea and Chesson, 2002). Especially the initial period of colonization can represent a significant stress for a holobiont: even if abiotic conditions are similar to the habitat of origin, the holobiont will be exposed to a novel biotic environment, including foreign microbes (Jones and Gomulkiewicz, 2012). Due to the impact of biological invasions on the affected ecosystems, much research has been focused on the factors influencing success of the colonization, ranging from genetic to environmental and community characteristics (Lee, 2002; Shea and Chesson, 2002; Theoharides and Dukes, 2007; Keller and Taylor, 2008; Bates *et al.*, 2013). However, virtually nothing is known about the stability and dynamics of microbiota during the establishment period and potential consequences for the holobiont.

Community response to disturbances and spatiotemporal dynamics

The ability of microbiota to benefit their host is linked to their stability (Shade *et al.*, 2012) in response to disturbances. Holobionts are constantly exposed to abiotic and biotic disturbances of variable intensity, predictability, area and duration (Bender *et al.*, 1984; Sousa, 1984). While controlled experiments are indispensable for quantifying the effects of individual disturbances, the results may not directly translate to microbial dynamics in natural conditions (Paine *et al.*, 1998). Studying the spatial and temporal dynamics in natural environment provides an opportunity to assess the relative effects of individual disturbances, but also to unveil other

potentially important determinants of community asssembly and stability (Borer *et al.*, 2013; David *et al.*, 2014; Stenuit and Agathos, 2015).

The assessment of community stability inevitably depends on the sampling intensity and duration of the study (Shade *et al.*, 2013) and it can be challenging to determine the temporal scale relevant for disturbance response. Exploring the community dynamics over multiple temporal scales can thus significantly improve our understanding of the community stability in the natural environment. However, studies concerning any aspect of dynamics of the host-associated microbiota at all are still relatively rare and almost exclusively limited to a handfull of model organisms (Thompson *et al.*, 2008; Grice *et al.*, 2010; Peterfreund *et al.*, 2012; Fink *et al.*, 2013; Franzenburg *et al.*, 2013; Belzer *et al.*, 2014; Giatsis *et al.*, 2014; Jemielita *et al.*, 2014; Liang *et al.*, 2014; Marino *et al.*, 2014; Koenigsknecht *et al.*, 2015) and to humans - where high-resolution and/or long-term longitudinal studies focusing on the microbial colonization and community assembly (Trosvik *et al.*, 2010; Koenig *et al.*, 2011; Morowitz *et al.*, 2011; Costello *et al.*, 2013; El Aidy *et al.*, 2013; La Rosa *et al.*, 2014), the recovery after disturbances (Jernberg *et al.*, 2007; Dethlefsen and Relman, 2011; Perez-Cobas *et al.*, 2013), as well as the natural temporal variability (Roger and McCartney, 2010; Caporaso *et al.*, 2011; Gajer *et al.*, 2012; David *et al.*, 2014) are available. Very little is known about the temporal dynamics in other animals (Erwin *et al.*, 2012a; Björk *et al.*, 2013; Pratte *et al.*, 2015). Although the temporal component was included in a few other studies, these did not address dynamics, but focused mainly on qualitative questions: they assessed the differences between the developmental or life-stages (Trabal *et al.*, 2012; Trabal Fernandez *et al.*, 2013; Hammer *et al.*, 2014; Kueneman *et al.*, 2014; Hroncova *et al.*, 2015), examined the seasonal patterns, often in order to identify core micriobiota (Wilson *et al.*, 2008; Zurel *et al.*, 2011; Carlos *et al.*, 2013; La Riviere *et al.*, 2013; Hardoim and Costa, 2014; Li *et al.*, 2014; Ransome *et al.*, 2014) or determined the effect of disturbances (Thurber *et al.*, 2009; Robinson *et al.*, 2010; Pita *et al.*, 2013; Wegner *et al.*, 2013; Xia *et al.*, 2014) in a before-after-control-impact design (Smith *et al.*, 1993).

Studying the spatial variation also contributes to unveiling of environmental and host factors that shape microbial communities (Mihaljevic, 2012). Moreover, the ability of a community to recover after a disturbance depends, among other, on the available species pool and thus on the metacommunity structure, since re-colonization from the environment represents an important aspect of disturbance response (Leibold *et al.*, 2004; Baho *et al.*, 2012; Shade *et al.*, 2012). Comparisons over large distances or at dissimilar sites can serve to identify core microbiota (King *et al.*, 2012b; Trabal *et al.*, 2012; Larsen *et al.*, 2013; Wong *et al.*, 2013; Dishaw *et al.*, 2014) and assess their stability over a range of environmental conditions (Wilson *et al.*, 2008; Ransome *et al.*, 2014). However, to estimate relative contributions of the host, environmental

and distance related factors for community assembly by examining spatial variation (Green and Bohannan, 2006; Caruso *et al.*, 2011; Martiny *et al.*, 2011; Mihaljevic, 2012; Borer *et al.*, 2013; Nemergut *et al.*, 2013), it is necessary to apply spatially stratified design (U'ren *et al.*, 2012; Borer *et al.*, 2013). Most such studies focused on interspecific differences (Littman *et al.*, 2009; Zouache *et al.*, 2011; Lankau *et al.*, 2012; Morrow *et al.*, 2012; Phillips *et al.*, 2012; Trabal *et al.*, 2012; Reveillaud *et al.*, 2014) and few examined distance-dependent variation (Linnenbrink *et al.*, 2013) or considered multiple spatial scales (Moro *et al.*, 2011; Sudakaran *et al.*, 2012; Hroncova *et al.*, 2015; Karlinska-Batres and Worheide, 2015) in order to explore determinants of community composition within a species.

Pacific oyster - a model organism for the changing environment

Coastal habitats belong to the natural systems that are the most affected by biological invasions (Grosholz, 2002). One of the globally very successful invaders of coastal ecosystems is a hardy filter-feeding bivalve, the Pacific oyster (*Crassostrea gigas*). The Pacific oyster is a potent ecosystem engineer (Padilla, 2010; Troost, 2010): it changes the morphology of tidal flats through reef building (Walles *et al.*, 2015), modifies water chemistry and microbial diversity in its surroundings (Green *et al.*, 2012) and affects the structure and dynamics of invaded communities (Kochmann *et al.*, 2008; Wagner *et al.*, 2012; Wilkie *et al.*, 2013; Hollander *et al.*, 2015), including host-parasite interactions (Krakau *et al.*, 2006; Thieltges *et al.*, 2009). Indigenous to Japan, the Pacific oyster had been introduced into aquaculture facilities all over the world, from where it invaded local coastal habitats. Supported by ever-rising seawater temperatures, it spread far beyond its original latitudinal range (Grizel and Heral, 1991; Nehls and Büttger, 2007; Dutertre *et al.*, 2010; Fey *et al.*, 2010; Troost, 2010). For example, the warming has allowed the oysters to reproduce and thus escape from the local aquaculture facility in my study area, the island of Sylt in the Wadden Sea, where they now dominate former mussel beds (Drinkwaard, 1998; Diederich *et al.*, 2005; Witte *et al.*, 2010; Moehler *et al.*, 2011; Buschbaum *et al.*, 2012; Schumacher *et al.*, 2014).

Pacific oysters as sessile animals can naturally move only during the larval stage, but they are routinely translocated, at all life stages, between cultivation sites for aquaculture purposes (Muehlbauer *et al.*, 2014). As filter feeders, they are directly exposed to myriads of bacteria including the opportunistic pathogens of the genus *Vibrio*. *Vibrio* spp. impose a strong selection pressure on the oysters, which subsequently rapidly adapt to their local *Vibrio* populations (Wendling and Wegner, 2015). Translocation to a new environment could thus lead to increased risk of disease.

Vibrios (*Vibrio* spp. and related bacteria of the family *Vibrionaceae)* are efficient at evading bivalve immune defenses (Pruzzo *et al.*, 2005), which allows them to colonize bivalve tissues where they act as commensals, pathogens (Olafsen *et al.*, 1993; Garnier *et al.*, 2007; Romalde *et al.*, 2014; Wendling *et al.*, 2014) and even as beneficial symbionts (Defer *et al.*, 2013; Desriac *et al.*, 2014). Since they thrive in warm environments and represent prominent agents of environmentally-dependent diseases in marine habitats (Paillard *et al.*, 2004; Wendling and Wegner, 2013; Zaragoza *et al.*, 2014), the incidence and severity of vibrioses are likely to further increase with rising temperatures (Baker-Austin *et al.*, 2012; Vezzulli *et al.*, 2013).

Athough climate change has facilitated the spread of Pacific oysters over the globe, heat waves can have negative effect on the animals (Clark *et al.*, 2013). Despite the fact that the oysters, as intertidal ectotherms, tolerate large fluctuations in abiotic conditions (Bougrier *et al.*, 1995; Strand *et al.*, 2011), their populations often suffer mass mortalities; these are the consequence of a complex interaction of genetic, physiological, biotic and abiotic factors, which include high temperatures and proliferation of opportunistic pathogens such as *Vibrio* spp. (Garnier *et al.*, 2007; Samain *et al.*, 2007; Malham *et al.*, 2009; Samain, 2011; Wendling and Wegner, 2013; Pernet *et al.*, 2014). The mortality outbreaks are likely to occur farther north as the temperatures continue to rise. Indeed, Pacific oysters in France have been suffering from summer mortality syndrome since decades (Goulletquer, 1998), while the syndrome only recently affected the population in the Southern Wadden Sea (Watermann *et al.*, 2008; Fey *et al.*, 2010; Moehler *et al.*, 2011), and the Northern Wadden Sea populations have been spared so far.

How microbiota other than *Vibrio* sp. affect the fitness of the Pacific oyster holobiont in the face of abiotic and biotic challenges is largely unknown. In contrast to culture-dependent methods that identified few genera, mainly *Vibrio* and *Pseudomonas,* as oyster symbionts (Prieur *et al.*, 1990; Olafsen *et al.*, 1993; Garnier *et al.*, 2007), recent sequencing studies unveiled much higher diversity of the gill (Wegner *et al.*, 2013) and gut microbial communities (Trabal *et al.*, 2012; Trabal Fernandez *et al.*, 2013). Can these communities provide protection against pathogens such as *Vibrio* spp.? Do they themselves cause opportunistic diseases? The failure of a significant heat stress to induce shift towards potential pathogens in the gill microbiota would suggest that they do not (Wegner *et al.*, 2013). However, the question remains if such shift did occur in some other tissue. For example, the gill microbiota assemble according to genotype (Wegner *et al.*, 2013), while gut communities are more affected by site (Trabal *et al.*, 2012; Trabal Fernandez *et al.*, 2013), illustrating the difference in their interaction with the environment. Therefore, to understand the contribution of microbiota to the fitness of the oyster holobiont in the changing environment, it is necessary to address the tissue-specific

variability as well as to examine the response of oyster-associated microbial communities to abiotic and biotic disturbances.

THESIS OUTLINE

Aim

In this thesis, I address the questions that improve our understanding of how microbiota contribute to the host fitness under challenging abiotic and biotic conditions. Specifically, I was interested in the short-term response of Pacific oyster microbiota to translocation, temperature stress and infection, and its consequences for oyster interactions with opportunistic pathogens of the genus *Vibrio*, as well as for the oysters themselves. In addition, I examined natural spatial and temporal variation of the oyster microbiome in order to assess its stability and identify factors affecting its assembly. All analyses of Pacific oyster microbiota presented here are based on hypervariable V1-V2 region of the 16s rRNA gene.

The particular questions I aim to answer are:

Chalpter 1 **Short-term effects of translocation**	• How are diversity, structure and composition of microbial communities in different oyster tissues (hemolymph, gills, gut and mantle) affected by translocation and removal of resident microbiota? • How do these changes affect the abundance and activity of opportunstic pathogens and oyster survival?

Chapter II **Translocation and long-term dynamics**	• What are the medium-term (weeks to months) effects of translocation and removal of resident bacteria on the diversity and composition of oyster hemolymph micriobiome and on the activity of opportunistic pathogens? • How stable is hemolymph microbiome over medium temporal scales? • What does spatial variation reveal about factors and processes influencing the assembly of the hemolymph microbiome?
Chapter III **Temperature stress and infection**	• How do temperature, temperature stress and infection affect diversity, structure, composition and stability of hemolymph microbiota? • How do changes in hemolymph microbiome relate to oyster survival?

Chapter summaries

Chapter I

In the first chapter, I examined how translocation affects Pacific oyster microbiota at the scale of days, and how these changes influence the oysters and the activity of associated opportunistic pathogens from the family *Vibrionaceae.* I assumed that the microbiota inhabiting individual oyster tissues fulfilled different functions and that they varied in their response to disturbances as well as in how they affected the host fitness. In order to evaluate their role in the successful acclimation to a new environment and to estimate how interactions of resident and newly encountered microbiota influence the oyster survival, I performed a field experiment as described in Figure C1.

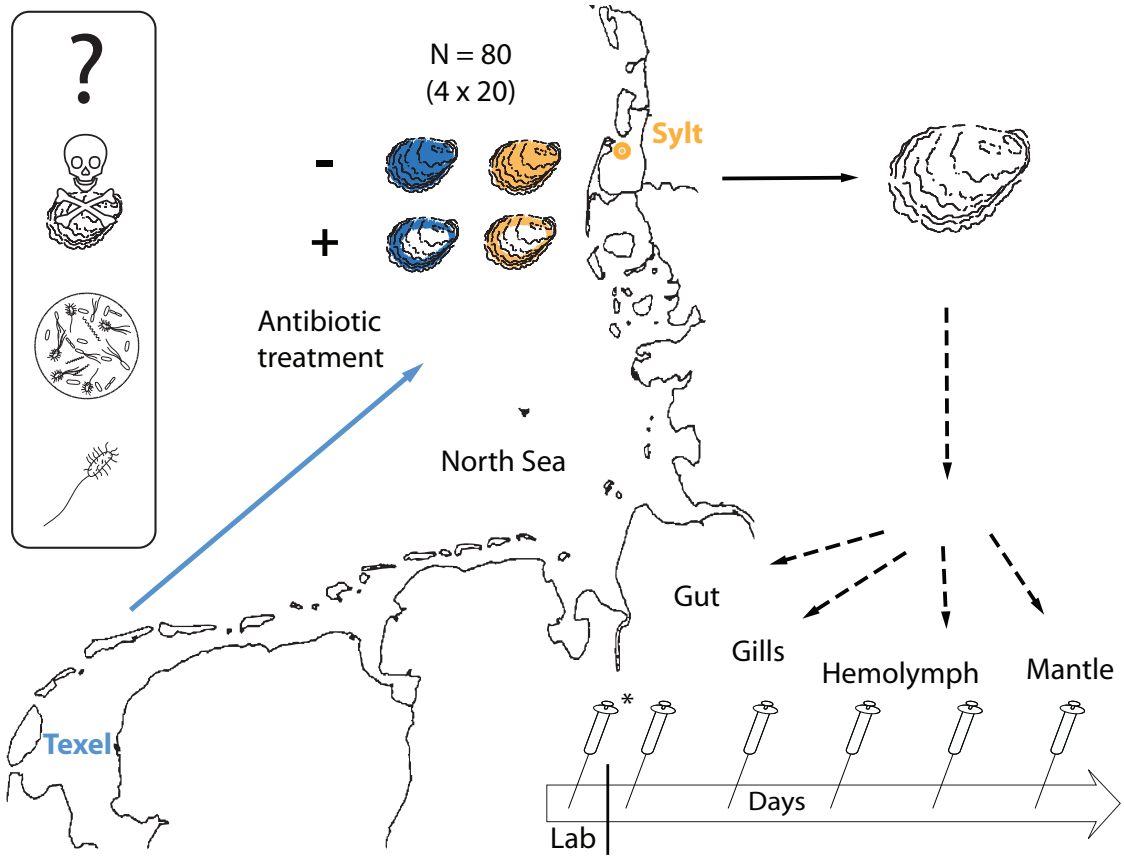


Figure C1. *Experimental design and sampling, Chapter I. Pacific oysters from Texel were transported to Sylt. Prior to the field-deployment, resident microbiota in the half of both local and translocated oysters were removed by antibiotic treatment. The oyster hemolymph was sampled prior to deployment in order to analyze immune parameters and microbiota (V1-V2 16s rDNA, MiSeq). Each day for five days following deployment, a subset of 16 oysters was sacrifised for the analysis of mantle, gill, gut and hemolymph microbiota and Vibrionaceae activity. Seawater samples were collected for microbiota analysis on three occasions. The Note: Syringes denote sampling points. * Only hemolymph sampled.*

All oysters had very active *Vibrionaceae* population in the hemolymph, regardless of origin or treatment. However, *Vibrionaceae* were rare in in the mantle, gut and gill tissues of all oysters except the translocated non-antibiotic-treated ones, which were also the only ones with significant mortality. Apart from the active *Vibrionaceae* community, the hemolymph microbiota differed from those in the solid tissues in other aspects as well: they had higher diversity and connectivity and were clearly related to ambient water communities. While not being able to link the mortality to either immune or genetic factors, I found evidence for destabilization of the hemolymph microbiota in the mortality affected, translocated non-

antibiotic treated group. I argue that the observed destabilization was caused by interactions of resident and foreign microbes and resulted in potentially fatal systemic vibriosis.

In the light of the above results, I propose that the distinctness of the hemolymph microbiome reflects its relevance for the interactions with the abiotic and biotic environment and ultimately for oyster fitness. I therefore focus on microbial communities in the hemolymph in the remaining chapters. In order to comprehensively evaluate the role of microbiota for oyster fitness, it is necessary to examine their variability and dynamics under natural conditions and over longer temporal scales (Chapter II), as well as to understand how they respond to important (a)biotic disturbances such as temperature stress and infection (Chapter III).

Chapter II

In this chapter, I focused on the long-term dynamics (months) of Pacific oyster hemolymph microbiota in response to translocation and perturbation of the resident microbial community. In addition, I explored the natural spatial variation of the hemolymph microbiota over multiple scales to gain insight in the factors important for the hemolymph community assembly. By combining the reciprocal translocation of the oysters from two genetically differentiated populations and removal of resident bacteria, I aimed to assess the long-term stability of hemolymph microbiota and to disentangle influences of population genetic makeup, environmental conditions and site (Figure C2).

I found high temporal (within-individual) and spatial (within-site) small-scale variability and low large-scale (between sites and sampling points) variability, probably reflecting similar mean abiotic conditions over the sampling period and high microenvironmental heterogeneity of the intertidal habitat respectively. However, bacteria within the hemolymph microbiome differed in their response to environmental conditions: while the transient, seawater-related bacteria were strongly influenced by immediate abiotic environment, shifts in the resident part of community were more gradual and the oyster origin signature and effects of perturbation by antibiotics were apparent for weeks. Still, the community composition eventually converged, indicating that local (a)biotic environment is more important than genetic differentiation between the oyster populations.

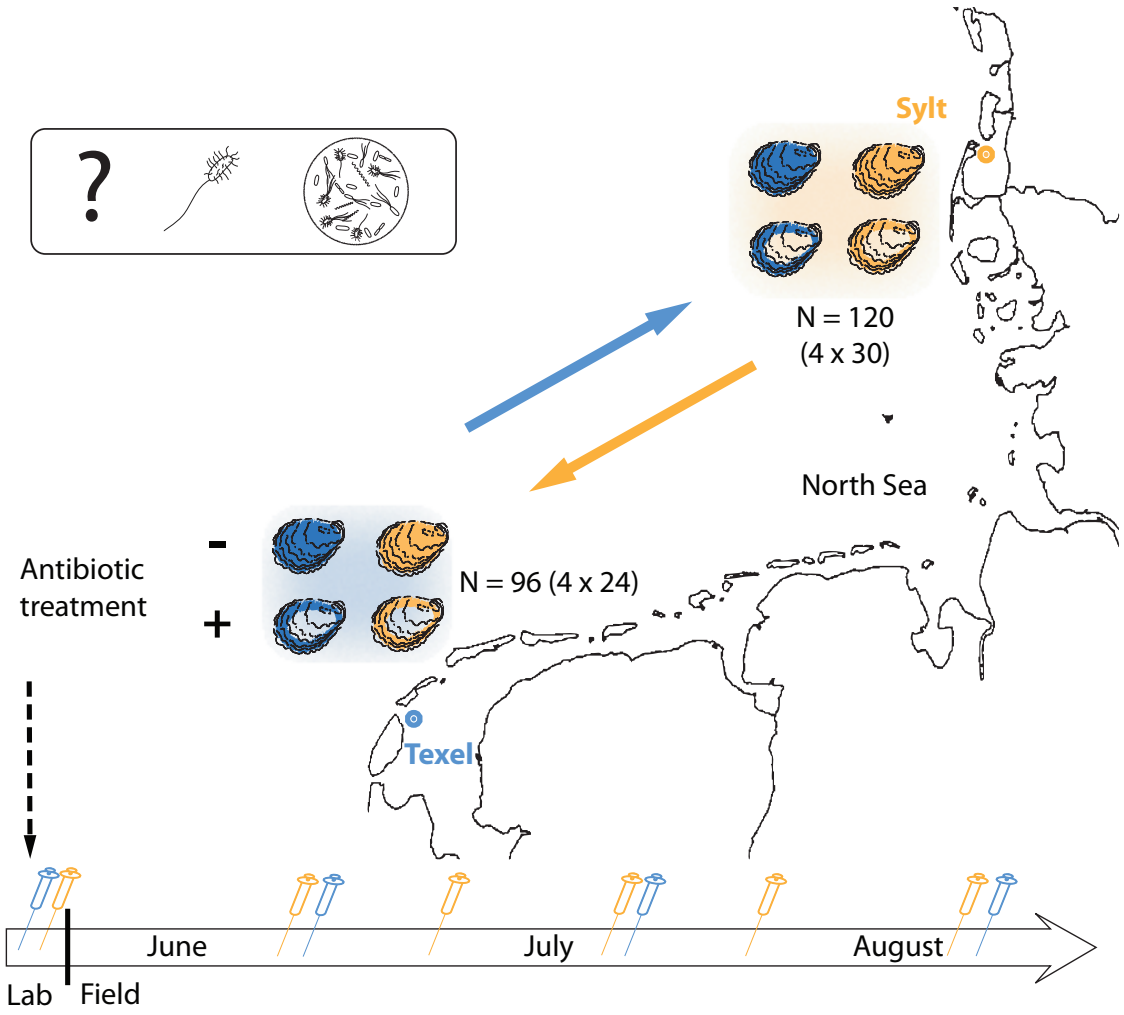


***Figure C2**. Experimental design and sampling, Chapter II. Translocation of Pacific oysters from Texel to Sylt and vice versa was performed at the beginning of June and the oyster hemolymph and seawater was sampled regularly during the next three months. Half of the oysters were treated with antibiotics prior to deployment, in order to remove resident microbiota (as described in Chapter I). The oysters were placed in mesh bags, four in each bag, and always two or three bags were placed together at randomly chosen spots on the oyster banks and their exact position was noted. In this way, it was possible to explore spatial variation over different scales. Syringes denote the sampling points, their color denotes the sampling site.*

Chapter III

In this chapter, I examined how temperature, temperature stress and infection interact to influence the hemolymph microbiota and oyster survival over a short time scale in a controlled laboratory experiment (Figure C3).

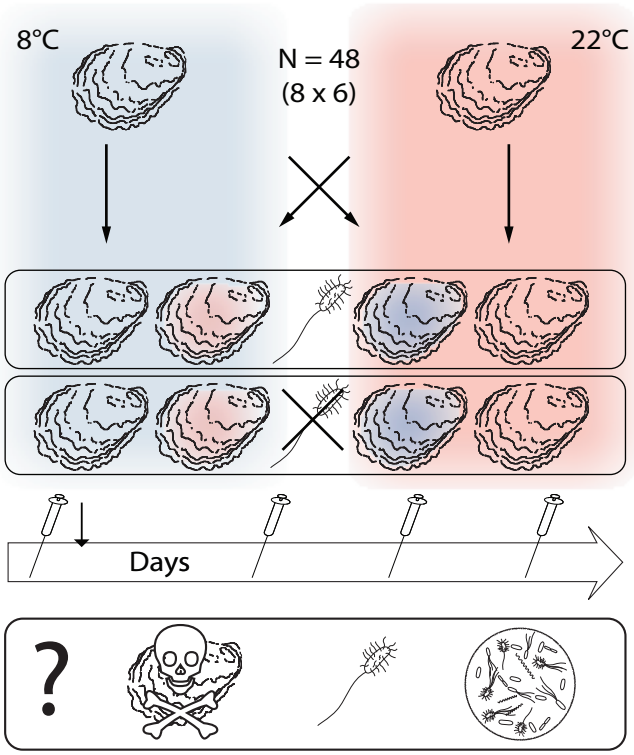


***Figure C3**. Experimental design and sampling, Chapter III. Pacific oysters acclimated to either 8°C or 22°C were temperature stressed by transfer to opposite temperature and/or infected by a Vibrio sp. The hemolymph was sampled for microbiota analysis (16s rRNA V1-V2 region, pyrosequencing) and the oysters checked for survival at three occassions during a week-long period.*

Vibrio infection caused significant mortality, especially of the warm-stressed oysters. When I examined the hemolymph microbiota shortly before the host death, I found disrupted community structure and proliferation of opportunistic pathogens. However, the oysters that survived the infection harbored diverse microbiota undistinguishable from those of the uninfected animals. On the other hand, the community responded quickly to temperature change by shifts in OTU abundances while retaining similar higher-taxon composition. Still, warm stress decreased the temporal stability of the hemolymph microbiota. I therefore propose that the hemolymph microbiota play an imprtant role in the acclimation and in the protection against pathogens. However, indirect and/or direct effects of combined stress on the oysters and their microbiota can result in the loss of community structure and have fatal consequences for the holobiont.

CHAPTER I

Clash of Colonization: Role of Tissue-specific Microbiota in Initial Establishment Success of Pacific oysters

Abstract

Translocation to a new environment imposes significant biotic and abiotic stress on animals and their associated microbiota. As microbiota positively and negatively affect host fitness, it is important to better understand the three-way environment-host-microbiota interactions. Due to differences in function, the effects of these interactions on the host will likely vary between the tissues. Here, we assess the relative importance of microbiomes associated with different tissues of Pacific oyster for its successful establishment under new environmental conditions. Pacific oysters (*Crassostrea gigas*) are routinely translocated for commercial purposes, thus providing a relevant background to investigate these interactions. In detail, we transplanted oysters from the Southern Wadden Sea (Texel, Netherlands) to the Northern Wadden Sea (Sylt, Germany) and compared their initial survival and microbial colonization to that of local oysters from the Northern Wadden Sea. Effects of the resident microbiome in both groups were controlled by administering antibiotics to half of the oysters and we followed survival and the composition of hemolymph, mantle, gill and gut microbiomes over five days. The only group with significant mortality were non-antibiotic-treated oysters from Texel, for which high titers of active *Vibrio* sp. in solid tissues indicated systemic infections. Network analyses revealed that the hemolymph microbiota had the highest within-tissue connectivity and were related to seawater communities. Since antibiotic treatment decreased modularity and increased connectivity in hemolymph microbiomes, we propose that their destabilization in non-antibiotic-treated oysters from Texel facilitated the passage of *Vibrio* sp. into solid tissues, thereby invoking systemic infections and disease. These interactions of the hemolymph microbiome with the external and internal environment may thus reflect an important role for oyster fitness.

Introduction

Exposure to novel environmental conditions can impose biotic and abiotic stress on the affected organism (Shea and Chesson, 2002; Hedge and Johnston, 2014). However, such stressors will not only affect the organism directly, but also the microbiota associated with the organism. Decoupling the fine-tuned intimate interaction between host, microbiota and the environment may result in disturbed developmental or physiological processes (Sison-Mangus *et al.*, 2015). On the one hand, stress-induced microbiome instability can shift bacterial communities towards opportunistic pathogens (Chapter III, Rosenberg *et al.*, 2007; Pita *et al.*, 2013; Bauvais, 2014). On the other hand, resident bacteria can buffer the response of the host to environmental change by maintaining homeostasis, for example, by preventing the colonization and establishment of pathogens (Kamada *et al.*, 2013b; Abt and Pamer, 2014; Desriac *et al.*, 2014). Therefore, a stable microbiome can provide vital services to the host and environmental disturbance may bear multilayered consequences beyond direct effects on the host.

One extreme form of environmental shift is translocation of organisms to new habitats beyond their normal range. This can happen either unintentionally (e.g. species invasions) or intentionally in cases such as species reintroductions or aquaculture where it is common practice (Galil *et al.*, 2014). Such habitat shifts will likely be associated with drastic changes in environmental conditions and will not only lead to new interactions of the host with the new environment, but also of its associated microbiota. Further, both hosts and their microbiota will not only be affected by changes in abiotic conditions, but will also be exposed to new microbes. In humans, for example, travelling to exotic destinations is accompanied by shifts in microbiome composition that is often associated with health problems (David *et al.*, 2014). While these new colonizers may not automatically cause harm to the organism, the interaction with the resident microbiome might lead to unforeseeable consequences that cannot be explained by examining the host in isolation. To conclusively determine the role of microbiota in colonization success, one therefore needs to investigate the three-way interactions between hosts, their microbiota and the environment.

One species that combines several aspects of novel habitat colonization is the Pacific oyster (*Crassostrea gigas*). It is common practice to transfer oysters over large distances for aquaculture purposes (Muehlbauer *et al.*, 2014), through which Pacific oysters successfully invaded coastal habitats worldwide and significantly impacted these ecosystems (Kochmann *et al.*, 2008; Wagner *et al.*, 2012). Both transfer and invasion introduce potential mismatches between hosts, their resident, co-adapted microbiota and the new environment including microbes. Despite

the applied and fundamental relevance, the consequences of mismatch are largely unknown both for the host-associated microbiota as a whole as well as for functionally relevant groups involved in such mismatches.

One important lineage of symbionts and pathogens of marine animals are the bacteria of the genus *Vibrio,* e.g. (Hoffmann *et al.*, 2010; Rowley *et al.*, 2014). In oysters, several strains have been implicated in environmentally-dependent diseases (Garnier *et al.*, 2007; Elston *et al.*, 2008), invoking rapid evolutionary responses in host populations (Wendling and Wegner, 2015), making them an ideal focus group to investigate the effects of mismatch on the dynamics of opportunistic pathogens. Another interesting group of potential oyster symbionts belong to the genus *Arcobacter* (Chapter III, Romero *et al.*, 2002). Although they have been identified as important opportunistic pathogens in laboratory studies, their prevalence in oysters might imply more beneficial effects of their presence that are interesting to explore (Chapter III).

It is also likely that microbiome disturbance will have different effects in different tissues. In mammals, for example, gut microbiota are important for immunity and certain changes are related to potentially life-threatening conditions (Kamada *et al.*, 2013a; Ferreira *et al.*, 2014), while the belly-button communities are much less likely to have such an effect (Hulcr *et al.*, 2012). In oysters, hemolymph communities seem to mirror oyster condition, as moribund animals tend to have low diversity and are dominated by few strains (Chapter III, Garnier *et al.*, 2007). How much other tissues contribute to oyster condition is far less known, because most studies focus on whole body homogenates (Beleneva *et al.*, 2007) or single tissues (Zurel *et al.*, 2011; Trabal *et al.*, 2012; Trabal Fernandez *et al.*, 2013; Wegner *et al.*, 2013). The variation between the tissues is only rarely considered (Antunes *et al.*, 2010; King *et al.*, 2012), especially in the context of host fitness.

In this study, we set out to experimentally explore how microbiota in different tissues of Pacific oysters are affected by translocation to new habitats and how the resulting microbiota mismatch affects oyster fitness in the new environment. To do so, we transplanted oysters from two invasive, genetically distinct invasive populations of the Wadden sea (Texel, Netherlands and Sylt, Germany, (Moehler *et al.*, 2011)) and monitored the short term microbiota shifts and colonization in different tissues during the initial phases of establishment in the new habitat. To manipulate the microbiome mismatch, we treated half of the oysters with antibiotics in order to minimize the interactions between resident microbiota and new colonizers, while the other half was transplanted with their natural resident microbiome. We followed oyster survival and changes in diversity, composition and abundance of oyster-associated bacterial communities as a whole, and *Vibrionaceae* and *Arcobacter* in particular, over the first five days in the new habitat.

In this way, we could estimate how the composition and diversity of microbiota in different tissues contribute to the success of oyster establishment in a new environment.

Material and Methods

Biological material

To test microbiome mismatch we transplanted oysters from the southern Wadden Sea to the Northern Wadden Sea and followed their success and changes in microbial communities over a five-day period. Southern Wadden Sea oysters were collected in de Cocksdorp, Texel, Netherlands (53° 0' N, 4° 54' E), cleaned of epibionts and transported to the AWI Wadden Sea station on Sylt. The same number of oysters was collected from the transplantation site (Oddewatt, Sylt, Germany, 55° 1' N, 8° 26' E). Initially, the oysters (N=80) were kept in pre-filtered seawater from their original location at ambient temperature (~14°C). To remove parts of the resident microbiome we added an antibiotic cocktail (100 µg/l of each ampiciline, tetratcycline, gentamycine and kanamycine, SigmaAldrich, Hamburg, Germany) to half of the oysters from each location. These concentrations were chosen based on previous tests, where we treated the oysters with the antibiotics and plated out hemolymph on marine agar until no colonies had grown. After 3 days, we took hemolymph samples from the adductor muscle with 23_1/4 gauge (0.6 dm, 30 mm) needles via notches drilled on the ventral side of the shell immediately after collection (to give the oysters time to recover). We froze ~200 µl aliquots of hemolymph for microbiota analysis at -80°C, and immediately processed the remaining sample to measure of immune parameters (total hemocyte count (THC), phagocytosis rate, hemolymph plasma protein concentration). To estimate the number of cultivable *Vibrionaceae*, we plated 5 µl of hemolymph on TCBS agar.

Experimental setup and sampling

For the field transplant, four oysters (one from each treatment group) were put into single bags with a mesh size of 1 cm, resulting in 20 bags that were brought out to the original site of collection of the northern Wadden Sea oysters (Odewatt). For the following five days we randomly collected four bags every day. We checked the survival and dissected the surviving oysters, after taking a hemolymph sample through the predrilled hole. We cut around 25 mm3 (~100 mg wet weight) of the mantle, gills and gut tissues with a sterile knife and flushed them

thoroughly with sterile PBS in order to remove transient, non-attached bacteria. We immediately froze half of the tissue for microbiota analysis at -80°C, while the other half was used to determine the number of cultivable *Vibrionaceae*. To do so, we homogenized the tissue pieces in 500 µl of sterile PBS in the Qiagen Tissue Lyzer using a single 5 mm stainless steal bead at 20 Hz for 3 min and plated 10 µl on TCBS agar.

To determine the background composition of the seawater microbial communities, we took seawater samples (100 ml) on three occasions during the sampling period. The samples were filtered onto 0.2 µm 47 mm Nucleopore Track-Etch Membrane filters, which were then used for DNA extraction.

Oyster immune parameters

In order to measure the total hemocyte counts (THC), 50 µl of hemolymph was mixed with equal amounts of 6% formaldehyde in SSW and marine anticoagulant solution (Fedders and Leippe, 2008). The resulting solution was further diluted 3x in sterile PBS and the cell count was measured with an automated cell counter (Scepter, Millipore, Darmstadt, Germany).

For phagocytosis, we followed established protocols (Wendling and Wegner, 2013). In short, 3x60 µl of hemolymph were allowed to adhere to the bottom of 96-well plates for 1 hour. The supernatant was carefully decanted, and the neutral-red-staind zymosan solution (SigmaAldrich, Hamburg, Germany) was added to hemocytes and incubated with shaking for one hour. The reaction was stopped by the addition of 6% formol in SSW. The wells were washed several times with PBS, the hemocytes with the phagocytozed particles were solubilized in acidified ethanol (1% acetic acid, 50% ethanol) and the absorbance was measured at 550 nm with Nanodrop ND-1000 spectrometer (peqlab, Erlangen, Germany). The standard curve was constructed from zymosan solution samples with known particle concentration and the results were expressed as the number of phagocytozed particles per hemocyte.

To estimate the plasma protein content, 200 µl of hemolymph was centrifuged for 5 min at 5000 g. The protein concentration in the supernatant was measured in triplicates with Quick-Start Bradford protein Bio-Assay (BioRad, Hercules, CA USA) according to manufacturer's protocol.

DNA extraction

DNA was extracted from app. 200 µl of hemolymph, or approx. 50 mg of mantle, gill and gut tissue with Wizard SV 96 Genomic DNA Purification System (Promega, Manheim Germany).

The samples were placed in pre-cooled (-20°C) TissueLyser Adapters (Qiagen, Hilden, Germany) and homogenized in a mixer mill (Retsch, Haan, Germany), using a mixture of 0.5 mm glass-zirconium beads and a single 5 mm tungsten bead (for mantle, gill and gut) or 1 mm glass beads (for hemolymph) at 30 Hz for 5 min in order to completely disrupt the tissues and bacterial cells. The standard protocol for DNA extraction from animal tissues was used for mantle, gill and tissue, while only proteinase K (20 µl of 20 mg/ml solution, SigmaAldrich, Hamburg, Germany) was added to the hemolymph for protein digestion. The samples were incubated at 55°C for at least 4 hours and extracted according to manufacturer's protocols. Blank extractions to check for bacterial contamination of reagents were also performed.

For the seawater samples, the filters were cut with sterile scissors into smaller pieces and homogenized in 2 ml tubes with a mixture of beads from PowerWater® DNA Isolation Kit (MO BIO Laboratories, Inc, Carlsbad, CA, USA) and 0.5 mm glass-zirconium beads in RLT buffer (DNeasy Blood & Tissue Kit, Qiagen, Hilden, Germany) and further treated as described in (Thomsen *et al.*, 2012). Shortly, a round of bead beating at 30 Hz for 5 minutes was followed by 10 minutes at 56°C with continuous mixing. This was repeated twice and then the proteinase K was added and the digestion mix was incubated for 2 hours. The samples were then extracted following the manufacturer's protocol with the adjusted reagent volumes.

PCR

We amplified 16s rRNA V1-V2 regions with uniquely barcoded 27f and 338r PCR primers. PCR reactions (25 µl) were set up in 96-well plates as follows: 4 µl of each forward and reverse primer (final conc.: 0.28 µM), 0.5 µl dNTPs (final conc.: 200 µM each), 0.25 µl Phusion Hot Start II High-Fidelity DNA Polymerase (0.5 unit per reaction) and 5 µl of HF buffer (7.5 mM MgCl2, Thermo Fisher Scientific, Inc., Waltham, MA, USA). We used 1 µl of undiluted hemolymph DNA, 1 µl of 10x diluted seawater DNA and 2-4 µl of solid tissue DNA per reaction. For each 96 well plate, twenty control reactions (12.5 µl) were performed: one positive control and unique combinations of all used forward and reverse primers as negative control, with water as a template.

The PCR cycling conditions were as follows: 30 sec inital denaturation at 98°C, then 30 cycles: 9 sec denaturation at 98°C, 1 min annealing at 55°C, 90s extension at 72°C, 10 min final extension at 72°C.

In order to check for the product and to estimate its amount, the reactions were analyzed immediately on a 1.5 % agarose gel. Briefly, 5 µl of loading buffer was mixed with 3 µl reaction and loaded into the gel prepared with SYBR Safe DNA Gel Stain (Life Technologies GmbH,

Darmstadt, Germany), including 3 µl of O`geneRulerTM 100 bp Plus DNA Ladder (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The separation was conducted at 120 V/35 cm for 80 minutes. The result was photographed with Gel Doc™ XR+ System and analyzed with Image Lab™ Software (BioRad, Hercules, CA USA) to estimate the absolute concentration of the PCR products using the ruler as internal standard. If there was no amplification in blank extractions, they were excluded from further analysis. Equal amounts of the products from a single gel were pooled together (25 - 100 ng per sample), run on 1.5% agarose gel and purified with MinElute Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufactuter´s protocol, including optional additional centrifugation and steps recommended for salt-sensitive applications. The concentration of DNA in the resulting subpools was measured fluorometrically with Qubit dsDNA br Assay Kit (Life Technologies GmbH, Darmstadt, Germany) in the Qubit fluorometer (Life technologies Invitrogen Gmbh, Karlsruhe, Germany). The equal amounts of subpools were then mixed together and frozen at -20°C until sequencing. The paired-end sequencing by synthesis was performed on a MiSeq platform at the Max Planck Institute for Evolutionary Biology in Plön, Germany.

Sequence quality control and preprocessing

All sequencing libraries were processed together. Quality control, OTU clustering and taxonomy assignment were performed in Mothur (Schloss *et al.*, 2009), following the Mothur MiSeq SOP (Kozich *et al.*, 2013). We retained only overlapping regions of the contigs and removed any sequences with ambiguous bases and/or homopolymers of 8bp or longer, in order to ensure good quality and reduce the number of spurious OTUs. The sequences were aligned to silva 119 reference alignment (Quast *et al.*, 2013) cut to V1-V2 region, and the taxonomy was assigned with 80% confidence cutoff, using the Greengenes taxonomy 13_08 (DeSantis *et al.*, 2006) and the Naïve Bayesian Classifier (Wang *et al.*, 2007) as implemented in Mothur. Unknown (i.e. sequences not assigned to any kingdom), chloroplast, Archaea and Eukaryotic sequences were removed from further analysis. We performed single-linkage pre-clustering with 2 differences allowed (Huse *et al.*, 2010), removed the chimeras and created 97% OTUs using average-linkage clustering method. Consensus taxonomy for an OTU was assigned with a 50% consensus confidence threshold. We calculated rarefaction curves of diverse α diversity metrics in Qiime (Caporaso *et al.*, 2010), in order to estimate the effect of sampling effort and to determine sequencing depth for the final analysis. We subsampled the dataset to 8000 reads per sample for the final analysis. Because the abundant OTUs (>100 reads) in the single positive blank control were rare (< 1%) in the remaining samples, we simply excluded them from

further analysis. In order to calculate a tree needed for phylogenetic measures of diversity, we picked a representative set of sequences using the distance method in Mothur and calculated the tree using fasttree (Price *et al.*, 2010). The final dataset is comprised of 3 seawater, 141 hemolymph, 68 gill, 61 gut and 62 mantle samples (335 in total).

Statistical analysis

All statistical analyses were performed in R (R Core Team, 2013). For α diversity, we used a complete rarified dataset (8000 reads per sample) to analyze the differences in evenness and species richness. We first tested for differences between the seawater and oysters using non-parametric tests, subsequently for differences between the tissues using robust analysis of variance (Wilcox and F.D., 2014) and finally we analyzed hemolymph and solid tissues separately with linear mixed models (Alexandra Kuznetsova, 2014; Bartoń, 2014; Bates D, 2014).

CFU counts were analyzed with negative-binomial generalized models (package MASS, (Ripley, 2002)). Because not only *Vibrio* sp., but also other *Vibrionaceae* grow on TCBS agar and, in addition, classification of short reads to low taxonomic levels can be unreliable, we decided to focus the analyses on the whole *Vibrionaceae* family, and not just the genus *Vibrio*.

For beta diversity, we kept only the OTUs with relative abundance higher than 0.1% in at least 10 samples to reduce the dataset complexity. We calculated Bray-Curtis distances and weighted UniFrac distances (Hamady *et al.*, 2010) using the phyloseq package (McMurdie and Holmes, 2013) and the results were further analyzed by nonmetric multidimensional scaling (NMDS) and Permanova (non-parametric permutational multivariate analyis of variance (Anderson, 2001)) as implemented in the Adonis function in the vegan package (Oksanen *et al.*, 2013). We first compared the tissues, and then analyzed the beta diversity in each tissue separately.

We statistically examined the variation at the class-level taxonomical composition between the tissues and the changes in the abundances of OTUs and genera in hemolymph microbiota according to origin and treatment by multivariate generalized mixed models (mvabund package (Wang *et al.*, 2012)). This method enabled us to identify taxa responsible for the observed differences without potential confounding of location and dispersion effects inherent to distance-based methods (Warton *et al.*, 2012).

We included time as an ordered factor in the models to check for temporal trends in the data. However, we could not disentangle individual variability from true time effects due to our experimental design. For this reason we do not further discuss the temporal trends, although we included them in results for completeness.

To explore both positive and negative associations of the OTUs within and between the tissues, we constructed an association network using the sparcc algorithm (Friedman and Alm, 2012) implemented in Mothur. We performed 10000 permutations and kept only correlations >0.4 with p value < 10-4 to exclude as many spurious correlations as possible (Marino *et al.*, 2014). The input matrix was organized similar to (Faust *et al.*, 2012): OTUs in tissues in the rows and individual oysters in columns. Only the oysters with available data for all four tissues and the OTUs that appeared in at least 1/3 of the samples were analyzed (Berry and Widder, 2014). We statistically determined the significance of observed connectivity within/between the tissues by comparison to connectivity between the random subsets of nodes of equal size as the tested group (Faust *et al.*, 2012). Additionally, to asses the effect of treatment and origin on microbial associations in the hemolymph, we constructed a network for each experimental group of oysters including only hemolymph samples and calculated their descriptive statistics including clustering coefficient (Newman *et al.*, 2002) and modularity. The networks were visualized using igraph package (Csardi G, 2006). Raw demultiplexed sequence data are available at European Nucleotide Archive under the study accession number PRJEB8492.

Results

Oyster survival and immune parameters

Non-antibiotic treated control oysters from Texel showed the highest mortality (30% vs. 0-5%, χ^2=12.222, df=3, p=0.007, Figure I-1). This mortality could not be linked to genetic differences between the populations nor to differences in immune system activity, as neither the total hemocyte count (Anova: $F_{3,56}$=1.085, p=0.363) nor phagocytosis rate per hemocyte (Anova: $F_{3,55}$=0.579, p=0.632) differed between the oyster groups. However, control oysters had higher plasma protein concentration, which could indicate reduced protein turnover in antibiotic-treated animals (Anova: $F_{3,63}$=4.565, p=0.006, adj. R^2 = 0.140; treatment: F1 = 12.900, p=0.001, effect size = 0.159).

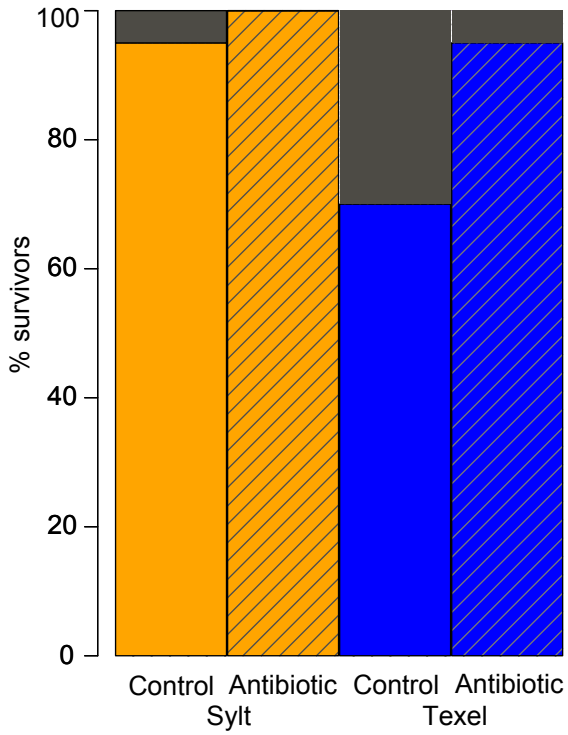


***Figure I-1**. Survival of local and translocated oysters after antibiotic treatment. Survival is shown for all oysters after a period of five days on the oyster reef.*

Differences between bacterial communities of seawater and oyster tissues

Bacterial assemblages from seawater differed substantially from those found in oyster tissues. Although the microbial communities of seawater (sw) and oyster samples had similar diversity and distribution (evenness: median: sw = 0.549, all oyster samples = 0.641, Asymptotic Wilcoxon Mann-Whitney Rank Sum Test: p = 0.334, Figure I-2A), overall taxonomic diversity was much higher in seawater (median: sw = 873, all oyster samples = 310: p = 0.008, r = -0.142, Figure I-2B). In contrast, the relative OTU richness of *Vibrionaceae* was much higher in oyster tissues (median: sw = 0.006, oyster = 0.079, Asymptotic Wilcoxon Mann-Whitney Rank Sum Test: p = 0.003, r = 0.159), mainly owing to high *Vibrio* diversity in the hemolymph (Figure I-2C). The activity of *Vibrionaceae* - estimated as CFU count on TCBS agar – confirmed this pattern with much higher numbers in hemolymph than in seawater (median: hemolymph = 4062.5, sw = 500, p = 0.004, r = 0.166, Figure I-2D). Similarly, the relative OTU richness of *Arcobacter* was also significantly higher in the oyster tissues (median: sw = 0.006, oyster = 0.036, Asymptotic Wilcoxon Mann-Whitney Rank Sum Test: p = 0.010, r = 0.141). Seawater communities were rather homogenous over time and were dominated by a few α-Proteobacteria and Flavobacteriaceae (Figure I-3A, Figure I-3B). A subsample of the 14 most frequent OTUs dominating seawater communities (mean relative abundance >= 0.01) were also found in the majority of oyster samples (85%) albeit in lower abundances, establishing seawater

as a source of bacteria for oyster microbiome, especially for the hemolymph (mean, median and range: hemolymph = 0.059, 0.009 (0, 0.433); solid tissues = 0.005, 0.002 (0, 0.083)). However, oyster microbiota displayed substantially higher within-tissue variability and were clearly differentiated from seawater communities in terms of community composition (Figure I-3A, Figure I-3B).

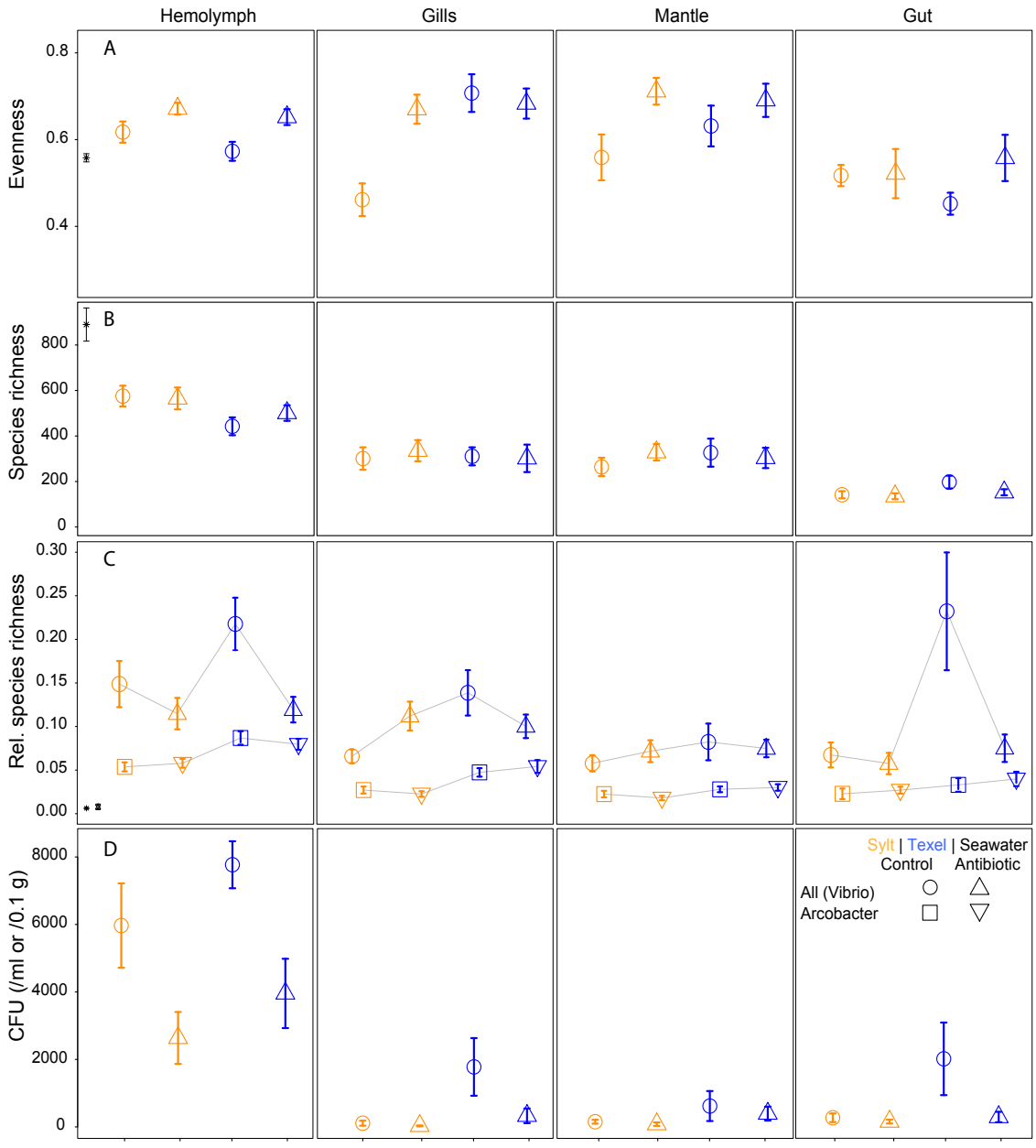


***Figure I-2**. α-diversity within oyster tissues grouped according to oyster origin and treatment: A) Evenness and B) species richness including all OTUs; C) relative species richness of Vibrionaceae and Arcobacter. D) Cultivable Vibrionaceae in 1 ml of hemolymph or app. 100 mg of solid tissue. Seawater samples are shown in the hemolymph plot for reference.*

***Table I-1**. Linear mixed model for for effects of oyster origin and treatment on overall evenness (clear) and species richness (shaded) in the hemolymph (Upper) and solid tissues (Lower).*

			DF	*F*[1]	*Significant contrasts*	*Estimate*	*SE*	*2.5% CI*	*97.5% CI*
Hemolymph	*Main effects*	Origin	1, 98.237	0.002					
			1, 115.947	1.596					
		Treatment	1, 99.089	6.343*	Antibiotic vs control	0.032	0.013	0.007	0.057
			1, 84.596	4.704*	Antibiotic vs control	1.210	0.558	0.116	2.303
		Time	5, 91.95	1.277					
			5, 23.474	1.363					
		Survival	1, 114.134	8.147**	Died vs survived	-0.066	0.023	-0.112	-0.021
			1, 118.609	1.345					
	Interaction terms	Origin x Treatment	1, 66.48	0.012					
			1, 118.131	0.784					
		Origin x Time	5, 92.221	1.012					
			5, 116.06	1.134					
		Treatment x Time	5, 92.679	0.632					
			5, 17.966	1.623					
	Random variation	Oyster				0.061		0.025	0.085
						0.000		0.000	1.889
		Cage				0.000		0.000	0.027
						1.817		0.000	2.479
Solid tissues	*Main effects*	Tissue	2, 154.823	11.274****	Mantle & gills vs gut	0.040	0.009	0.024	0.057
			2, 110.027	29.572****	Mantle & gills vs gut	1.531	0.200	1.139	1.922
		Origin	1, 154.983	3.709					
			1, 48.221	1.278					
		Treatment	1, 154.835	13.801****	Antibiotic vs control	0.044	0.012	0.021	0.067
			1, 47.878	0.011					
		Time	4, 52.298	0.424					
			4, 24.652	3.180					
	Interaction terms	Tissue x Origin	2, 154.658	2.852					
			2, 109.428	0.411					
		Tissue x Treatment	2, 154.009	0.310					
			2, 109.434	1.299					
		Origin x Treatment	1, 156.437	2.308					
			1, 48.967	3.475					
		Origin x Time	4, 154.888	1.038					
			4, 48.415	1.647					
		Treatment x Time	4, 155.397	2.714*	(Antibiotic vs control) vs cubic trend	-0.068	0.026	-0.120	-0.016
			4, 47.871	0.830					
		Tissue x Time	8, 154.434	1.235					
			8, 108.87	2.150					
		Tissue x Treatment x Origin	2, 154.265	4.678*	(Mantle & gills vs gut) vs (Sylt vs Texel) vs (Antibiotic vs control)	0.023	0.008	0.007	0.040
			2, 110.155	0.166					
	Random variation	Oyster				0.000		0.000	Inf
						1.617		0.000	2.116
		Cage				0.039		0.000	0.083
						0.721		0.000	1.811

[1]significance levels: * ≤ 0.05, ** < 0.01, *** < 0.001

***Table I-2**. Linear mixed model for for effects of oyster origin and treatment on relative species richness of Vibrionaceae (clear) and Arcobacter (shaded) in the hemolymph (Upper) and solid tissues (Lower).*

			DF	*F*[1]	*Significant contrasts*	*Estimate*	*SE*	*2.5% CI*	*97.5% CI*
Hemolymph	*Main effects*	Origin	1, 82.174	1.190					
			1, 97.694	12.081***	Sylt vs Texel	-0.020	0.006	-0.031	-0.009
		Treatment	1, 86.039	5.951*	Antibiotic vs control	-0.042	0.017	-0.076	-0.008
			1, 99.303	2.270					
		Time	5, 70.55	2.783*	Linear trend	-0.091	0.033	-0.155	-0.027
			5, 91.129	21.187***	Linear trend	-0.078	0.012	-0.102	-0.054
		Survival	1, 99.267	2.869					
			1, 117.749	0.250					
	Interaction terms	Origin x Treatment	1, 56.625	0.294					
			1, 54.911	0.209					
		Origin x Time	5, 74.851	1.988					
			5, 91.307	0.659					
		Treatment x Time	5, 75.507	0.919					
			5, 92.208	2.527					
	Random variation	Oyster				0.099		0.063	0.133
						0.019		0.000	0.034
		Cage				0.035		0.000	0.092
						0.000		0.000	0.014
Solid tissues	*Main effects*	Tissue	2, 110.837	5.927**	Gills vs mantle	0.029	0.008	0.013	0.046
			2, 116.343	6.421**	Gills vs mantle	0.017	0.005	0.007	0.027
		Origin	1, 48.861	8.218**	Sylt vs Texel	-0.028	0.010	-0.048	-0.009
			1, 56.786	25.600****	Sylt vs Texel	-0.024	0.005	-0.034	-0.015
		Treatment	1, 48.344	1.521	Antibiotic vs control				
			1, 56.421	0.643					
		Time	4, 18.617	1.138					
			4, 57.065	1.924					
	Interactions terms	Tissue x Origin	2, 110.327	2.460					
			2, 115.74	2.118					
		Tissue x Treatment	2, 110.475	5.236**	(Mantle and gills vs gut) vs (Antibiotic vs control)	0.016	0.005	0.006	0.026
			2, 115.609	0.874					
		Origin x Treatment	1, 49.798	5.737*	(Sylt vs Texel) vs (Antibiotic vs control)	0.024	0.010	0.004	0.043
			1, 56.94	0.125					
		Origin x Time	4, 48.99	0.985					
			4, 57.022	0.962					
		Treatment x Time	4, 48.698	0.872					
			4, 56.045	1.612					
		Tissue x Time	8, 109.887	2.002					
			8, 115.156	0.762					
		Tissue x Treatment x Origin	2, 111.067	1.574					
			2, 116.34	0.055					
	Random variation	Oyster				0.058		0.026	0.069
						0.020		0.000	0.026
		Cage				0.016		0.000	0.048
						0.000		0.000	0.011

[1]significance levels: * ≤ 0.05, ** < 0.01, *** < 0.001

Effects explaining bacterial community differences within oysters: Tissue and oyster identity

Within oysters, α-diversity of the host associated microbial communities significantly differed between the studied tissues (Figure I-2A, Figure I-2B; Robust Wilcox bootstrapped ANOVA: evenness: $F_{3,81.56} = 9.088$, $p < 0.001$, effect size = 0.368; species richness: $F_{3,98.493} = 68.052$, $p = <0.001$, effect size = 0.747), with substantially lower diversity observed in the gut and the highest species richness in the hemolymph. A similar pattern was observed for the proportion of species assigned to *Arcobacter* ($F_{3,126.746} = 39.886$, $p < 0.001$, effect size = 0.673) - and *Vibrionaceae* ($F_{3,127.508} = 7.767$, $p < 0.001$, effect size = 0.376, Figure I-2C). Species richness of *Vibrionaceae* was positively correlated with density of active bacteria cultured from solid tissues (Spearman's ρ: 0.280, $p < 0.001$, CI = (0.143, 0.427)), but not in the hemolymph (Spearman's ρ: 0.126, p= 0.137), indicating that the increase in *Vibrio* diversity in solid tissues could be a sign of systemic infection. On the other hand, the evenness of the *Vibrionaceae* community was negatively correlated with cultivability in both hemolymph (Spearman's ρ: -0.196, p = 0.020, CI = (-0.239, -0.021)) and solid tissues (Spearman's ρ: -0.208, p = 0.004, CI = (-0.347, -0.080)), suggesting that high activity of *Vibrionaceae* was associated with loss of diversity, and probably due to the proliferation of a few, potentially pathogenic OTUs.

Tissue also explained a significant portion of variance in the community structure, especially when phylogenetic relatedness was taken into account (Figure I-4A), indicating substantial ecological differences between the tissues. The hemolymph communities were clearly distinguished from solid tissues by smaller within-group variance (ANOVA testing the homogeneity of multivariate dispersions: $F_{3,328} = 52.824$, $p = 0.001$) and by higher relative abundance of ε-Proteobacteria (*Arcobacter*), Flavobacteria, Fusobacteria (*Psychriliobacter*) and γ-Proteobacteria (*Oceanospirillaceae*, *Vibrionaceae*) and fewer Spirochaetes (Brachyspirae) and β-Proteobacteria (Table I-S1). Among the solid tissues, the most conspicuous difference was the high abundance of Mollicutes (*Mycoplasma*) in the gut, while the mantle and gill microbiomes were in general quite similar to each other.

In addition to the large effect of tissue, a considerable amount of variability was explained by community similarity between individual oysters (Figure I-4). In this case, however, the explained variability was higher when phylogenetic relatedness was disregarded. This suggests that the shared basic phylogenetic structure is further shaped by genotype, condition or simply spatial autocorrelation, resulting in the fine-scale individual variation between the oysters.

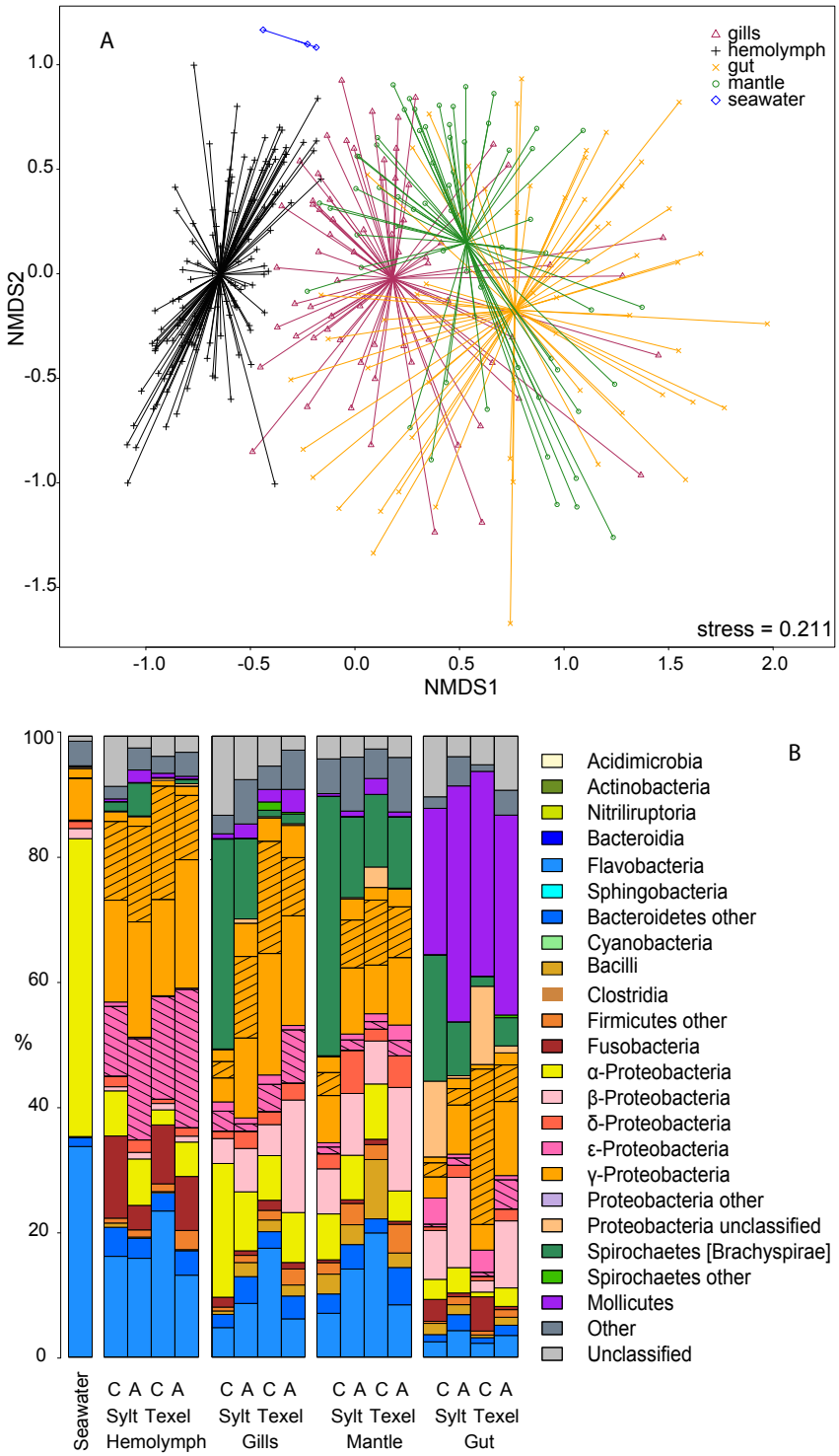


Figure I-3. *β-diversity and composition of seawater and oyster microbiota. Above: NMDS plot of Bray-Curtis distances between the samples showing differences between the tissues and seawater samples. Below: mean relative abundances of classes in the seawater and within tissues, grouped according to oyster origin and treatment. Shading lines show mean relative abundances of Vibrionaceae (within γ-Proteobacteria) and Arcobacter (ε-Proteobacteria).*

Effects explaining bacterial community differences within oysters: experimental translocation and antibiotic treatment

We examined the effects of population origin and antibiotic treatment on α-diversity separately for the hemolymph and solid tissues (Figure I-2A, 2B, Table I-1). In the hemolymph, both the species richness and evenness were higher in the treated animals, regardless of their origin (evenness model: dAIC = -110.293, marginal R2 = 0.211, conditional R2 = 0.438; sp. richness model: dAIC = 34.214, mar. R2 = 0.222, cond R2 = 0.308). Interestingly, low evenness of hemolymph microbiota before the transfer to the field correlated with oyster survival, indicating that disrupted community structure associated with few dominating OTUs may have played a role in the mortalities. Local and transplanted oysters differed significantly in their response to antibiotics: the treatment increased the evenness of the mantle and gill, but not of the gut microbiota in Sylt oysters, while the opposite was true for the Texel oysters (evenness: dAIC = -169.253, mar. R^2 = 0.310, cond. R^2 = 0.348; sp. richness: dAIC = 46.934, mar. R^2 = 0.361, cond R^2 = 0.479, Table I-2). Looking at the taxa of special interest (Table I-2, Figure I-2C, Figure I-2D), the proportion of species richness assigned to the genus *Arcobacter* was higher in all tissues of Texel oysters and was not influenced by the treatment (hemolymph: dAIC = -59.474, mar. R^2 = 0.526, cond. R^2 = 0.590; solid tissues: dAIC = -247.668, mar. R^2 = 0.288, cond R2 = 0.365). In contrast, relative species richness of *Vibrionaceae* showed a more complex pattern and was affected by the antibiotic treatment with different tissue specific responses among local and translocated oysters (hemolymph: dAIC = -95.295, mar. R^2 = 0.211, cond. R^2 = 0.577; solid tissues: dAIC = -206.342, mar. R^2 = 0.278, cond R^2 = 0.496). In detail, antibiotics reduced *Vibrionaceae* diversity in the hemolymph regardless of the origin, while the effect was small in solid tissues of Sylt oysters. In Texel oysters, on the other hand, antibiotics had a strong effect, especially in the gut. This pattern was partly reflected for active cultivable *Vibrionaceae*. Here, cultivable *Vibrionaceae* in hemolymph from both oyster sources were strongly reduced by antibiotic treatment (Anova of quasipoisson glm: treatment: $F_{1,135}$ = 13.344, p < 0. 001, origin: $F_{1,135}$ = 2.509, p = 0.116, treatment x origin: $F_{1,135}$ = 0.110, 0.740). In the solid tissues, on the other hand, the cultivable *Vibrionaceae* were much more numerous in Texel oysters (origin, mantle: $F_{1,57}$ = 6.327, p = 0. 015, gills: $F_{1,60}$ = 18.754, p < 0. 001, gut: $F_{1,62}$ = 10.333, p = 0. 002) with the strong response to the antibiotic treatment in the gills and the gut (treatment, mantle: $F_{1,57}$ = 0.119, p = 0. 732, gills: $F_{1,60}$ = 9.669, p = 0. 003, gut = $F_{1,62}$ = 9.105, p = 0. 004).

In order to examine the effects of population origin and antibiotics on β-diversity, we analyzed each tissue separately (Figure I-3, Figure I-4). Despite high temporal/individual variability, the antibiotic effects and population signature were apparent throughout the

experiment. Although there were significant differences in abundance of some minor genera (mainly assigned to Flavobacteria, α- and γ-Proteobacteria) in the hemolymph, neither *Vibrionaceae* nor *Arcobacter* showed significant variation (Table I-S2). Differences between oyster groups resulted mostly from shifts on the OTU-level within genera. For example, different *Arcobacter* OTUs characterized the hemolymph communities of Texel and Sylt oysters (Table I-S3). Higher-level taxonomical composition was very similar regardless of treatment or origin.

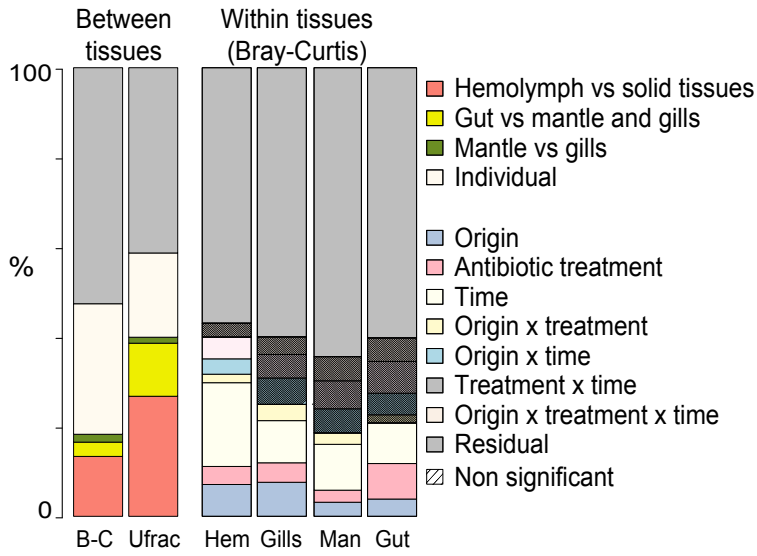


Figure I-4. *Effect sizes of experimental factors on bacterial community dissimilarities expressed as the percentage of explained variance in the underlying distance matrices by A) tissues and individuals and B) oyster origin, antibiotic treatment, time and their interactions in single tissues. Since separate individuals were sampled every day, time is partially confounded with between individual variation.*

Association networks

The vast majority (89.7%) of associations in the whole-oyster network occurred within tissues (Figure I-5). Only the hemolymph, however, had higher connectivity than expected by chance (compared to 1000 random node subsets, $p < 0.05$). We therefore focused on hemolymph for further analysis, and constructed a network for each treatment x origin combination to examine microbial associations in response to treatment (Figure I-6). All resulting networks shared a densely inter-connected area, whose core consisted of the same OTUs that were also abundant in the seawater samples and establish the connection between hemolymph and the environment. Other motifs, dominated by *Vibrionaceae* or *Arcobacter* and some anaerobes such as Fusobacteria and Clostridia, were recovered in each oyster group and

they tended to be negatively associated with the first environmental subcluster. The recurrence of these subclusters in all treatment groups indicates that the composition of hemolymph microbiota was similar in all groups and largely determined by factors not controlled for in our design. However, while antibiotic treatment did not affect the composition of hemolymph microbiomes, it significantly affected the co-occurrence among these OTUs, i.e. the network structure. Antibiotics strongly reduced modularity and increased the connectivity density, which may reflect more intense between-OTU interactions in antibiotic treated communities (Table I-3, Supplementary Table I-S5). Since these networks also showed a higher proportion of negative correlations, a shift to more competition-based interactions could be observed (Table I-3). On the other hand, the network based on the non-antibiotic-treated oysters from Texel had a pronounced modular structure accompanied by a lower clustering coefficient, reflecting more sparsely connected modules.

	Sylt		Texel	
	Control	Antibiotic	Control	Antibiotic
# samples	35	36	32	38
# nodes	149	211	129	190
# edges	721	2348	362	2742
% positive edges	82.1	61.1	85.4	56.7
Average degree	9.678	22.256	5.612	28.863
Max degree	49	94	24	93
# clusters	12	5	7	2
Connectance	0.065	0.106	0.044	0.153
Average path length	3.331	2.693	3.604	2.417
Average betweenness centrality	0.011	0.008	0.015	0.007
Modularity	0.224	0.053	0.599	0.036
Global clustering coefficient	0.625	0.540	0.459	0.565

***Table I-3**. Properties of the networks depicted in Figure I-6. Detailed comparison with random networks can be found in Supplementary Table I-S5.*

Discussion

Intentional (transport, cultivation) and unintentional (invasion) translocation exposes plants and animals to a variety of novel abiotic and biotic conditions. One example is exposure to new microbiota colonizing hosts, where they interact not only with the host but also with the resident microbiome. The high mortality of translocated oysters in our study could suggest such interactions, since resetting the microbial communities by administering antibiotics prior to deployment in the field significantly reduced mortality in translocated hosts. Although this effect might have been due to mitigation of a transport-stress induced bacteriosis, we did not record any mortality in the initial lab based rearing and could not detect any signs of previous

disease or significant differences in immune response. Additionally we never observed systematic mortalities of transported oysters in previous lab experiments (Wendling and Wegner, 2015). Genotypes were randomly distributed between antibiotic treatments (Moehler *et al.*, 2011), suggesting that it is not the direct interactions between hosts and new microbiota, but more likely the interactions between the resident bacteria and the new environment that might have played a role in mortalities.

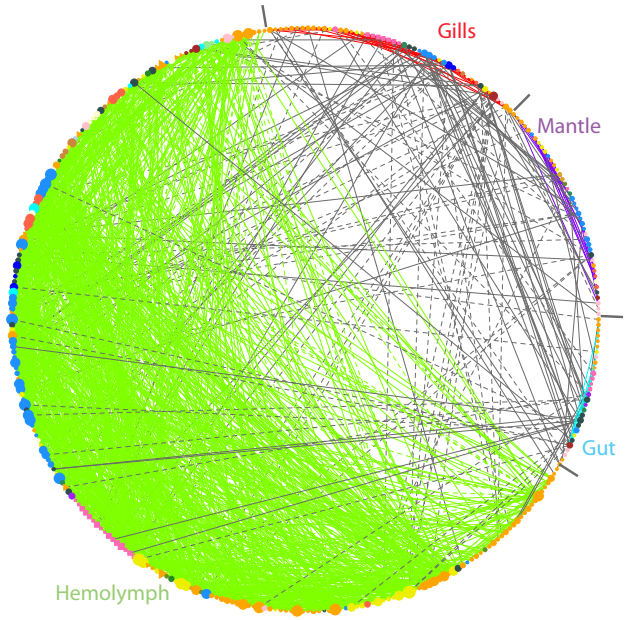


Figure I-5. *Association network showing relationships within and across the tissues. Nodes are colored by taxonomy, as in Figure I-3B and the size is proportional to degree. Full line denotes co-occurrence, the dashed line denotes co-exclusion; edge weight is proportional to correlation strength. Color of edges connecting OTUs within the tissue correspond to color of that tissue, inter-tissue edges are grey.*

The oysters that eventually died had less even hemolymph community structure prior to deployment, underlining the importance of evenness for a successful response to environmental challenges (Wittebolle *et al.*, 2009). In conjunction with results from Chapter III, such distribution anomalies of hemolymph microbiota may help predict oyster disease prior to its visible signs (Ransome *et al.*, 2014). Similarly, allowing oysters to partially recover their microbiota after antibiotic treatment prior to deployment resulted in higher bacterial diversity, which may have prevented potentially deleterious effects of depleted microbial communities. Moreover, the higher degree of connectivity in co-occurrence High loads of active *Vibrionaceae* in solid tissues of translocated non-antibiotic treated oysters from Texel contrasted the low baseline *Vibrionaceae* activity observed in the other groups of oysters and could represent signs of systemic infections and thus a proximate cause of mortality. Unlike the solid tissues, the *Vibrionaceae* community in the hemolymph was species rich and active in all

healthy oysters, underlining the importance of taking a tissue-specific analysis to understand the effects of microbiome structure and composition on the host.

The hemolymph microbiome was different in many ways from the communities in solid tissues. In general, all hemolymph communities had similar higher-level taxonomical composition. Fine scale differences could be observed only on the lower taxonomic levels (e.g. *Arcobacter* OTUs), suggesting that hemolymph is characterized by a relatively stable microbiome that is fine-tuned depending on the environment or oyster condition (Chapter III). Microbiome stability became especially obvious by recurrent core OTU assemblages in all groups. These assemblages might represent alternative community states, possibly related to changes in oxygen concentration due to tidal-cycle associated valve closing (Sow *et al.*, 2011; Faust *et al.*, 2012), as the prevalence of aerobic, seawater-deduced bacteria in one subcluster and dominance of microaerobic or anaerobic species (Levican *et al.*, 2014) in the other might suggest. Switching microbial activity between these two states could represent a healthy microbiome responding to predictable recurring environmental conditions such as tides (Relman, 2012). Alternatively, the dominance of an anaerobic subcluster - also containing *Vibrio* and Shewanella - could be associated with deteriorated health (McHenery and Birkbeck, 1986). This may suggest a close connection to changes in the internal and external oyster environment important for acclimation and maintenance of homeostasis.

networks likely reflects community stability, and with this the successful establishment of oysters in the new environment (Estrada, 2007; Scheffer *et al.*, 2012). However, the interpretation of the co-occurrence network topology is not straightforward (Faust and Raes, 2012), and its ecological implications vary widely depending on the sort of input data and network building criteria (Faust *et al.*, 2012; Widder *et al.*, 2014; Williams *et al.*, 2014; Peura *et al.*, 2015). In addition, the relationship between stability and structure can differ substantially between the network types (Thebault and Fontaine, 2010) and depending on kind of disturbance (Holme, 2011). High modularity and lower clustering coefficients in non-treated oysters from Texel could indicate decreased compensation capacity and thus lower ability to respond to disturbance (Yachi and Loreau, 1999). Although the exact processes behind the observed changes in community structure remain unclear, oysters as filter feeders are in constant contact with a multitude of bacteria, and intense interactions of the resident microbiota with the novel biotic environment could have affected community stability. Such destabilization of the hemolymph microbiome might explain the spillover of *Vibrionaceae* into solid tissues, resulting in systemic disease and higher mortality of translocated oysters.

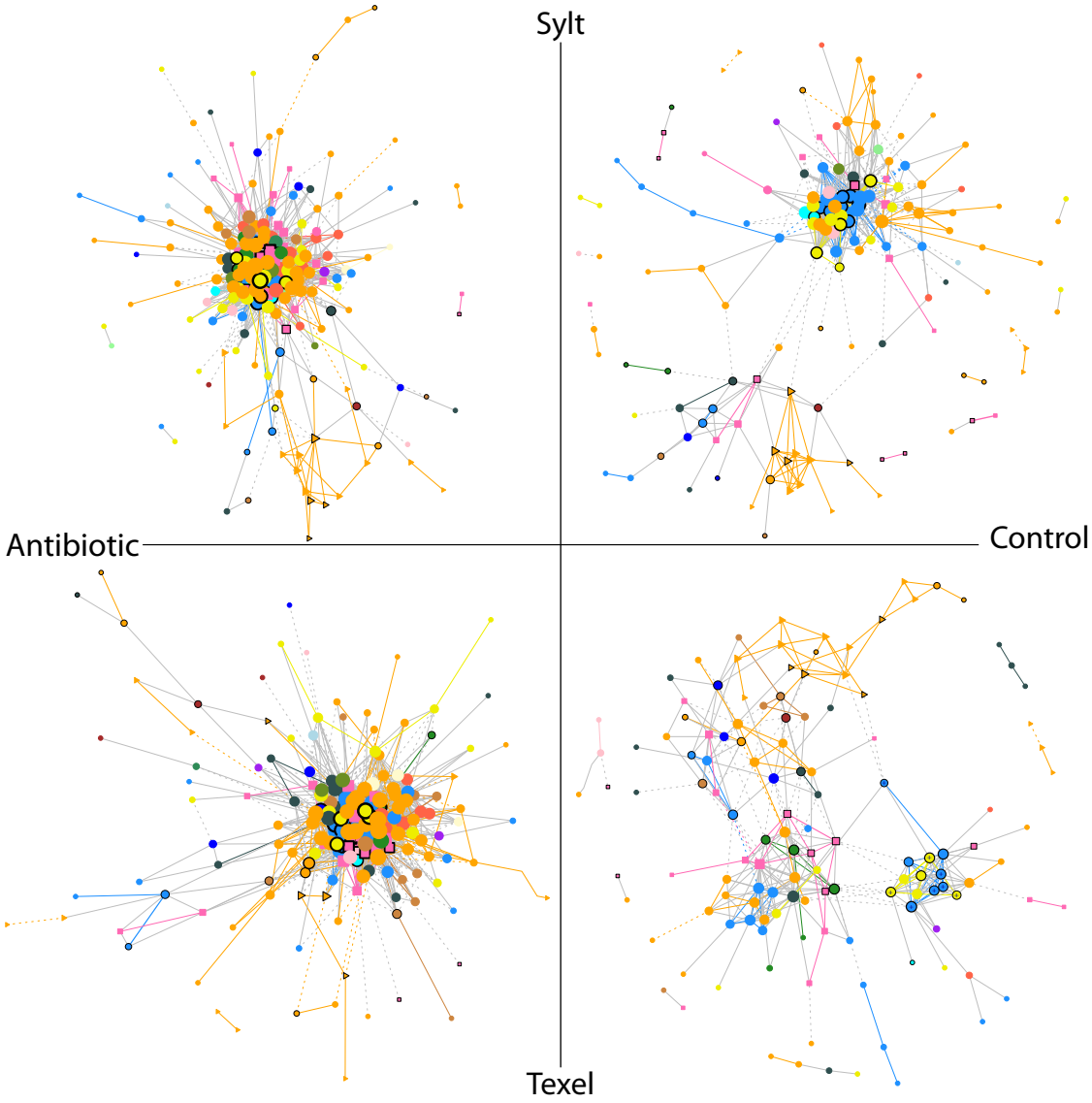


***Figure I-6**. Association networks of hemolymph OTUs grouped by oyster origin and treatment. Black frame denotes OTUs found in all four networks. Nodes are colored by taxonomy, as in Figure I-3 and the size is proportional to degree. Full line denotes co-occurrence, the dashed line denotes co-exclusion; edge weight is proportional to correlation strength. Edges connecting OTUs within the same class are colored by taxonomy, else they are grey. □ Arcobacter; △Vibrionaceae. In the last network, "+" denotes OTUs that are abundant in the seawater.*

While the high diversity of hemolymph microbiota may reflect its dynamic role in the interaction with the environment, the lower diversity of gut or gill microbiota may be due to more intimate relationship between the host and the microbiota fulfilling specific roles in target tissues, such as nutrition in the gut (Duperron *et al.*, 2007; Fraune and Zimmer, 2008; Rodrigues

et al., 2010; Fernandez-Piquer *et al.*, 2012; Trabal Fernandez *et al.*, 2013) or other important physiological processes. For example, increased abundance of *Mycoplasma* was previously found in the gills and the hemolymph of stressed and dead oysters (Chapter III, Wegner *et al.*, 2013). Mycoplasmas have been reported from the guts of crabs (Givens *et al.*, 2013), as well as from the oyster stomachs (King *et al.*, 2012) and the gut goblet cells (Harshbarger and Chang, 1977). Sydney rock oysters actually lost *Mycoplasma*-like symbionts from digestive glands following infection (Green and Barnes, 2010). Tanaka et al. (Tanaka *et al.*, 2004) proposed that Tenericutes might act as amino-acid producers, and they have been shown to increase survival of terrestrial isopods when they are starved or fed with low quality food (Fraune and Zimmer, 2008). The high numbers of these bacteria in the gut in the current study could thus also indicate a role in oyster nutrition, highlighting the tissue specific context in which bacterial roles need to be considered.

While it is highly likely that the initial microbiomes differed between locations, it is currently unresolved how persistent those differences are over time. We observed the strongest differences between gill microbiota of Texel and Sylt oysters throughout the initial phases of colonization. Gills show high bacterial activity in Pacific oysters (Hernández-Zárate and Olmos-Soto, 2006) and many bivalve bacterial symbionts are situated here (Duperron *et al.*, 2007; Dubilier *et al.*, 2008; Rodrigues *et al.*, 2010). While gill community differences could be attributed purely to carry over effects of the original site (Trabal Fernandez *et al.*, 2013; Wegner *et al.*, 2013) that could gradually disappear (Wendling *et al.*, 2014), previous studies showed that gill microbiomes can exhibit long-term stability (Zurel *et al.*, 2011) and correlate with their host genotype (Wegner *et al.*, 2013). Long term stability of these differences could therefore also reflect differences in the genetic structure and history of both oyster populations (Moehler *et al.*, 2011).

In conclusion, we present a comprehensive, experimental study that manipulated the oyster microbiota and examined the between-tissue variation in the context of translocation. Our data highlight the importance to consider microbiota in a tissue–specific context to understand the interaction of the holobiont with a new environment. Specifically, the high abundance of active *Vibrionaceae* in the hemolymph of healthy oysters is in stark contrast to low *Vibrionaceae* loads in solid tissues where high loads can cause systemic disease. Although the microbiota in the solid tissues are likely important for oyster physiology and metabolism, the stability and quick response to environmental changes indicates the prominent role of the hemolymph microbiome for oyster homeostasis, which is rather relying on community structure than taxonomic composition. In order to elucidate the processes behind the observed changes in community diversity and structure and to better understand function of microbiota in different

tissues, more studies focusing on the metabolism and physiology of functionally important symbionts and of the whole community are needed.

CHAPTER I SUPPLEMENTS

Table I-S1. *Multivariate generalized linear model (negative binomial) showing significant differences at the class level between the oyster tissues, origin and treatment.*

Multivariate scores	*df*	*Score*	*p*
Tissue	3, 328	740.74	0.001
Origin	1, 327	127.93	0.001
Treatment	1, 326	130.94	0.001
Time	5, 321	500.43	0.001
Tissue x Origin	3, 318	159.80	0.001
Tissue x Treatment	3, 315	128.85	0.001
Origin x Treatment	1, 314	116.16	0.001
Tissue x Origin x Treatment	3, 311	125.39	0.001

Significant univariate scores	*Tissue*	*Origin*	*Treatment*	*Time*	*Tissue x Origin*	*Tissue x Treatment*	*Origin x Treatment*	*Tissue x Origin x Treatment*
Acidimicrobiia	23.15	1.19	9.29	27.45	4.96	3.97	3.40	5.89
Actinobacteria	48.81	0.93	17.95	80.52	3.41	6.49	0.00	0.10
Nitriliruptoria	18.08	2.27	0.39	18.98	1.60	2.54	4.13	0.00
Bacteroidia	32.25	0.01	0.23	9.41	0.65	2.66	0.38	2.33
Flavobacteriia	81.12	9.30	4.42	29.48	2.68	10.41	27.29	9.98
Sphingobacteriia	8.27	0.79	0.65	26.36	2.90	5.40	2.45	3.84
Bacteriodetes other	7.77	0.04	3.12	46.52	2.20	9.85	0.28	6.91
Synechococcophycideae	4.83	0.95	5.56	21.18	0.70	1.09	0.61	3.49
Cyanobacteria other	17.22	0.25	6.66	20.32	8.90	3.89	0.85	4.48
TG3	8.87	0.53	1.86	24.44	26.35	6.50	0.05	0.72
Bacilli	72.43	2.21	3.82	18.20	3.11	6.42	0.16	5.75
Clostridia	20.12	25.99	15.94	5.52	2.86	0.67	0.31	3.29
Fusobacteria	66.50	0.62	18.62	4.14	0.19	10.60	0.16	6.45
BD1-5	52.99	9.99	7.32	35.11	9.70	6.11	24.16	14.67
α–Proteobacteria	74.81	20.62	0.74	64.20	5.81	13.06	11.80	3.47
β–Proteobacteria	75.11	3.38	12.23	13.19	10.21	6.50	0.34	9.44
δ–Proteobacteria	62.22	1.73	25.45	35.98	2.89	3.35	0.12	4.21
ε–Proteobacteria	108.77	18.58	0.81	46.41	3.97	10.06	9.18	4.73
γ–Proteobacteria	42.53	20.55	3.59	4.80	24.21	5.67	23.05	13.21
Proteobacteria other	86.79	5.53	1.05	23.06	12.31	8.93	0.09	14.60
[Brachyspirae]	38.80	34.22	3.38	22.07	7.25	3.23	7.83	0.95
Spirochaetes	12.51	1.91	1.16	3.85	1.04	1.78	0.58	1.83
Mollicutes	207.27	0.05	5.18	78.37	6.69	2.02	3.47	0.66
unclassified	21.12	4.56	15.38	22.54	10.74	1.10	11.80	5.70

Coefficients	*Tissue*			*Origin*	*Treatment*	*Tissue x Origin*		*Origin x Treatment*
	(Mantle, gills, gut) - Hemolymph	*(Mantle, Gills) - Gut*	*Gills-Mantle*	*Sylt-Texel*	*Treated-Control*	*(Mantle, gills, gut) - Hemolymph : Sylt-Texel*	*(Mantle, Gills) - Gut : Sylt-Texel*	*Texel-Sylt : Control-Treated*
Acidimicrobiia	0.14				0.32			
Actinobacteria	0.24	0.34			0.47			
Nitriliruptoria	-4.00							
Bacteroidia	-0.33							
Flavobacteriia	-0.41	0.35		-0.18				0.29
Sphingobacteriia								
Bacteriodetes other	-0.19							
Synechococcophycideae								
Cyanobacteria other								
TG3							1.91	
Bacilli	0.54							
Clostridia		0.41	-0.47	-0.49	0.37			
Fusobacteria	-0.55				-0.48			
BD1-5	-0.48	0.30	0.62	-0.53				0.61
α–Proteobacteria	-0.18	0.41	0.51	0.38				-0.19
β–Proteobacteria	0.49				0.33			
δ–Proteobacteria		0.35			0.44			
ε–Proteobacteria	-0.22		0.36	-0.25				
γ–Proteobacteria	-0.16			-0.31		-0.09		0.24
Proteobacteria other		-0.83						
[Brachyspirae]			-0.62	0.99				
Spirochaetes								
Mollicutes	0.43	-1.16						
unclassified			0.62		-0.41			-0.33

***Table I-S2**. Multivariate generalized linear model (negative binomial) showing effects of oyster origin and treatment on the abundances of genera within the hemolymph. Only genera with significant differences are listed.*

Multivariate scores	*df*	*Score*	*p*
Origin	1, 139	179.376	0.001
Treatment	1, 138	180.330	0.001
Time	5, 133	722.795	0.001
Survival	1, 132	67.462	0.774
Origin x Treatment	1, 131	132.710	0.001
Origin x Time	5, 126	364.293	0.843
Treatment x Time	5, 121	348.940	0.976

Significant univariate scores								***Coefficients***		
Class	*Order*	*Family*	*Genus*	*Origin*	*Treatment*	*Time*	*Origin x Treatment*	*Texel-Sylt*	*Control-Treated*	*Texel-Sylt : Control-Treated*
Acidimicrobiia	Acidimicrobiales	C111	NA	0.94	9.90	23.18	6.78			
Actinobacteria	Actinomycetales	NA	NA	0.35	2.58	24.05	0.40			
Nitriliruptoria	Nitriliruptorales	Nitriliruptoraceae	Nitriliruptor	1.69	0.87	21.74	3.48			
Flavobacteriia	NA	NA	NA	1.09	2.70	40.25	0.46			
Flavobacteriia	Flavobacteriales	Flavobacteriaceae	NA	8.71	10.32	2.66	16.06			0.32
			Lutimonas	0.69	8.05	31.64	5.96			
			Olleya	26.22	1.01	21.86	21.97	0.76		0.58
			Polaribacter	4.09	4.83	53.74	0.04			
			Sediminicola	1.09	1.87	42.68	3.98			
			Tenacibaculum	3.92	7.31	21.46	2.64			
			Winogradskyella	11.77	1.13	5.59	0.04	-0.34		
			unclassified	7.12	0.95	22.41	1.36			
		unclassified	unclassified	3.47	0.10	22.43	0.43			
Sphingobacteriia	Sphingobacteriales	Balneolaceae	NA	1.64	2.71	26.92	2.03			
		Ekhidnaceae	NA	2.75	6.56	33.53	2.50			
Bacteroidetes	unclassified	unclassified	unclassified	1.44	1.02	29.58	8.69			
Synechococcophycideae	Synechococcales	Synechococcaceae	Prochlorococcus	1.15	1.82	32.47	3.49			
Cyanobacteria	unclassified	unclassified	unclassified	1.03	5.27	65.41	0.22			
TG3	TG3-2	NA	NA	1.50	2.53	55.62	0.15			
Clostridia	Clostridiales	Clostridiaceae	NA	11.89	13.92	11.41	0.60	0.79	-0.14	
BD1-5	NA	NA	NA	5.69	1.98	38.65	10.72			
α-Proteobacteria	Rhodospirillales	NA		12.69	3.36	5.34	1.43	0.39		
	Rickettsiales	Pelagibacteraceae	NA	2.82	4.15	31.74	2.45			
	Rhodobacterales	Rhodobacteraceae	Octadecabacter	1.01	3.27	39.76	0.12			
			Phaeobacter	27.32	2.10	9.23	5.39	-0.64		
			Rhodobaca	0.03	0.01	20.76	3.91			
			Thalassobius	3.57	2.83	50.77	3.62			
			unclassified	11.22	1.82	11.52	6.88	-0.29		
	Rhodospirillales	Rhodospirillaceae	NA	1.37	4.13	34.48	0.04			
	Rhizobiales	unclassified	unclassified	6.27	18.60	8.57	2.36		-0.85	
δ-Proteobacteria	Syntrophobacterales	Desulfobacteraceae	Desulfococcus	0.29	2.77	33.28	1.99			
	Desulfobacterales	Desulfobulbaceae	NA	0.16	5.80	49.58	5.37			
	Desulfobacterales	Desulfobulbaceae	Desulfotalea	1.74	11.63	19.14	0.43		-1.31	
	Desulfuromonadales	Desulfuromonadaceae	NA	0.03	3.24	32.71	1.07			
ε-Proteobacteria	Campylobacterales	Campylobacteraceae	*Arcobacter*	4.45	4.06	39.48	1.39			
γ-Proteobacteria	Chromatiales	NA	NA	1.60	4.99	39.79	9.04			
	NA	NA	NA	2.95	5.34	43.99	6.10			
	Alteromonadales	NA	NA	4.37	15.78	36.00	0.09		-0.60	
	Alteromonadales	Alteromonadaceae	HTCC2207	0.90	2.77	35.31	4.00			
	Alteromonadales	Colwelliaceae	Thalassomonas	10.91	2.13	6.87	20.28	-0.63		-0.57
	Alteromonadales	Colwelliaceae	unclassified	14.45	24.77	10.93	2.18	-0.23	0.42	
	Oceanospirillales	Halomonadaceae	CandidatusPortiera	0.84	3.53	31.27	1.63			
	Pseudomonadales	Moraxellaceae	Psychrobacter	3.76	0.02	26.37	1.01			
	Oceanospirillales	Oceanospirillaceae	Marinomonas	16.03	4.96	27.53	4.11	0.74		
		Oceanospirillaceae	Neptunomonas	14.03	2.38	16.63	1.09	-0.51		
		Oceanospirillaceae	Oceanospirillum	15.81	4.70	26.70	0.10	0.91		
		Oleiphilaceae	NA	25.57	0.09	5.59	4.59	-0.45		
	Alteromonadales	OM60	NA	0.00	4.70	35.57	6.59			
		Pseudoalteromonadaceae	Pseudoalteromonas	4.13	10.45	28.42	0.47			
			unclassified	17.21	9.03	2.96	10.28	0.65		
		Psychromonadaceae	Psychromonas	4.34	12.98	20.67	0.70		-0.49	
		Shewanellaceae	Shewanella	7.11	3.16	32.00	3.80			
	Thiotrichales	Thiotrichaceae	Leucothrix	15.62	0.84	14.48	2.23	-0.42		
	unclassified	unclassified	unclassified	0.02	0.45	22.07	5.03			
Proteobacteria	unclassified	unclassified	unclassified	14.76	0.00	9.47	0.05	0.29		
[Brachyspirae]	[Brachyspirales]	Brachyspiraceae	NA	15.41	12.85	8.17	1.32	-1.24	0.17	
Mollicutes	Mycoplasmatales	Mycoplasmataceae	Mycoplasma	1.28	2.01	23.79	0.02			
Bacteria	unclassified	unclassified	unclassified	7.02	15.45	21.49	16.02		0.42	-0.39

Table I-S3. Multivariate generalized linear model (negative binomial) showing effects of oyster origin and treatment on the OTU abundances within the hemolymph. Only OTUs with significant differences are listed.

Multivariate scores	*df*	*Score*	*p*
Origin	1, 139	207.979	0.001
Treatment	1, 138	226.704	0.001
Time	5, 133	946.829	0.001
Survival	1, 132	99.475	0.977
Origin x Treatment	1, 131	145.320	0.269
Origin x Time	5, 126	540.110	1.000
Treatment x Time	5, 121	511.804	1.000

Significant univariate scores									***Coefficients***	
		Class	Order	Family	Genus	Origin	Treatment	Time	Texel-Sylt	Control-Treated
Otu000749	Actinobacteria	Actinobacteria	Actinomycetales	NA	NA	0.46	2.39	20.49		
Otu000136		Bacteroidia	Bacteroidales	NA	NA	1.09	2.30	46.66		
Otu000033		Flavobacteriia	NA	NA	NA	0.28	1.61	27.19		
Otu000019			Flavobacteriales	Flavobacteriaceae	NA	13.71	1.59	13.82	0.59	
Otu000044						1.42	3.70	28.95		
Otu000085						0.64	1.94	20.58		
Otu000131						4.78	5.79	23.55		
Otu000164						0.44	0.12	10.24		
Otu000192						0.87	2.71	20.86		
Otu000228						0.46	4.32	21.65		
Otu000350						12.85	0.07	8.02	-0.75	
Otu000052					Lutimonas	0.72	5.35	29.42		
Otu000024					Olleya	26.13	0.79	20.77	0.79	
Otu000106					Polaribacter	0.92	1.72	24.20		
Otu000172					Polaribacter	0.73	1.27	25.41		
Otu000006					Sediminicola	1.09	1.87	42.68		
Otu000146					Tenacibaculum	2.42	5.50	28.64		
Otu000041					unclassified	15.06	2.04	1.47	-1.29	
Otu000144					unclassified	0.49	3.25	24.29		
Otu000209					unclassified	11.38	9.14	38.82		
Otu000420					unclassified	0.34	1.15	25.17		
Otu000438					unclassified	0.22	0.64	21.28		
Otu001719	Cyanobacteria	unclassified	unclassified	unclassified	unclassified	0.62	3.56	51.59		
Otu000094	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	NA	6.47	6.11	20.15		
Otu000178						10.82	15.82	20.94		-1.91
Otu000129	GN02	BD1-5	NA	NA	NA	0.34	1.62	28.67		
Otu000174						14.26	6.89	25.42	0.81	
Otu000233						8.12	0.02	24.01		
Otu000007	Proteobacteria	α–Proteobacteria	Rhodobacterales	Rhodobacteraceae	Octadecabacter	0.78	2.15	29.84		
Otu000050						0.59	3.93	42.49		
Otu000047					Phaeobacter	20.46	2.19	6.97	-0.96	
Otu001157					Rhodobaca	0.05	0.01	20.59		
Otu000060					Thalassobius	2.04	1.47	42.50		
Otu000119					unclassified	14.28	0.00	5.45	-0.61	
Otu000049			Rhodospirillales	Rhodospirillaceae	NA	0.35	2.61	21.74		
Otu000016			Rickettsiales	Pelagibacteraceae	NA	2.88	4.07	30.18		
Otu000032		δ–Proteobacteria	Desulfobacterales	Desulfobulbaceae	NA	0.01	3.94	32.70		
Otu000011		ε–Proteobacteria	Campylobacterales	Campylobacteraceae	*Arcobacter*	8.42	26.88	31.79		-0.72
Otu000013						16.99	0.87	29.18	-1.41	

Otu000015						7.14	5.76	39.27		
Otu000022						25.68	1.97	31.46	0.48	
Otu000027						15.64	0.40	25.46	0.68	
Otu000030						16.70	0.73	25.72	-0.70	
Otu000031						0.00	2.99	28.69		
Otu000053						5.22	23.43	17.15		-0.57
Otu000064						2.05	12.48	30.06		-0.33
Otu000068						3.54	21.29	39.67		-0.81
Otu000080						7.26	0.71	33.38		
Otu000110						13.25	0.10	16.21	0.61	
Otu000126						1.16	4.89	32.00		
Otu000128						17.18	4.68	14.15	1.19	
Otu000251						0.84	4.61	22.12		
Otu000271						0.45	7.14	21.98		
Otu000310						3.16	16.71	25.26		-0.15
Otu000088		γ–Proteobacteria	NA	NA	NA	0.67	3.69	24.21		
Otu000028			Alteromonadales	NA	NA	4.17	16.05	36.11		-0.65
Otu000195				Alteromonadaceae	HTCC2207	0.82	2.95	36.70		
Otu000048				Colwelliaceae	NA	16.43	1.31	11.12	-0.53	
Otu000165					Thalassomonas	14.12	0.12	13.79	-2.87	
Otu000104					unclassified	4.84	24.02	4.28		0.70
Otu000026				OM60	NA	0.09	3.59	31.78		
Otu000029						0.04	2.64	27.32		
Otu000004				Pseudoalteromonadaceae	Pseudoalteromonas	2.88	13.39	36.14		-0.58
Otu000008						4.06	3.10	19.64		
Otu000023						0.59	0.00	55.02		
Otu000102					unclassified	16.96	8.83	2.90	0.65	
Otu000098				Psychromonadaceae	Psychromonas	17.53	22.61	15.57	0.67	-0.68
Otu000039				Shewanellaceae	Shewanella	5.11	0.60	21.05		
Otu000046						2.31	0.12	25.38		
Otu000059						4.90	6.88	34.55		
Otu000034			Chromatiales	NA	NA	0.14	6.54	30.37		
Otu000036			Oceanospirillales	Halomonadaceae	CandidatusPortiera	0.84	3.50	31.21		
Otu000191				Oceanospirillaceae	NA	4.88	13.60	7.41		0.79
Otu000079				Oceanospirillaceae	Marinomonas	12.53	7.72	18.81	1.01	
Otu000107				Oceanospirillaceae	Marinomonas	12.67	6.13	14.50	3.08	
Otu000234				Oceanospirillaceae	Marinomonas	17.71	4.21	20.47	1.26	
Otu000307				Oceanospirillaceae	Marinomonas	4.51	0.90	8.98		
Otu000108				Oceanospirillaceae	Oceanospirillum	16.07	10.36	22.93	1.32	
Otu000175				Oleiphilaceae	NA	21.94	0.01	12.54	-1.87	
Otu062536			*Vibrio*nales	*Vibrionaceae*	*Vibrio*	2.73	17.67	10.56		-1.39
Otu001371		unclassified	unclassified	unclassified	unclassified	18.30	0.87	5.93	0.87	
Otu000012	Spirochaetes	[Brachyspirae]	[Brachyspirales]	Brachyspiraceae	NA	15.38	12.92	8.08	-1.28	0.16
Otu000002	Bacteria	unclassified	unclassified	unclassified	unclassified	4.29	15.67	28.96		0.85
Otu000487	unclassified	unclassified	unclassified	unclassified	unclassified	7.70	1.89	19.95		

***Table I-S4**. Multivariate generalized linear model (negative binomial) showing effects of oyster origin and treatment on the genera abundances in solid tissues. Only genera with significant differences are listed.*

Gills					**Gut**				**Mantle**			
Multivariate scores		*df*	*Score*	*p*		*df*	*Score*	*p*		*df*	*Score*	*p*
Origin		1, 66	153.105	0.001	Origin	1, 59	47.191	0.851	Origin	1, 60	124.361	0.001
Treatment		1, 65	116.822	0.001	Treatment	1, 58	62.953	0.054	Treatment	1, 59	92.733	0.001
Time		4, 61	381.637	0.001	Time	4, 54	198.860	0.998	Time	4, 55	270.413	1.000
Origin x Treatment		1, 60	79.873	0.999	Origin x Treatment	1, 53	42.034	1.000	Origin x Treatment	1, 54	54.725	1.000
Origin x Time		4, 56	267.637	1.000	Origin x Time	4, 49	138.046	1.000	Origin x Time	4, 50	197.930	1.000
Treatment x Time		4, 52	231.915	1.000	Treatment x Time	4, 45	566.184	0.003	Treatment x Time	4, 46	202.872	1.000
Significant univariate scores												
Class	*Genus*	*Origin*	*Treatment*	*Time*	*Class*	*Genus*	*Treatment x Time*					
α–Proteobacteria	unclassified	11.91	1.56	1.35	Mollicutes	Mycoplasma	361.25					
Clostridia	uncl. Clostridiaceae	14.30	4.88	7.23								
ε–Proteobacteria	*Arcobacter*	11.59	0.74	22.01								
γ–Proteobacteria	uncl. *Vibrionaceae*	13.22	0.01	20.31								
γ–Proteobacteria	Pseudoalteromonas	12.59	6.74	13.18								
unclassified	unclassified	12.04	7.40	13.82								
Coefficients												
		Texel-Sylt					*(Control - Treated):linear trend*					
α–Proteobacteria	unclassified	-0.87			Mollicutes	Mycoplasma	12.56					
Clostridia	uncl. Clostridiaceae	0.90										
ε–Proteobacteria	*Arcobacter*	0.86										
γ–Proteobacteria	uncl. *Vibrionaceae*	0.67										
γ–Proteobacteria	Pseudoalteromonas	1.02										
unclassified	unclassified	-0.35										

***Table I-S5**. Properties of original hemolymph networks (clear) and average of 1000 random networks (shaded) with same number of nodes and edges.*

	Sylt				Texel			
	Control		Antibiotic		Control		Antibiotic	
# samples	35	NA	36	NA	32	NA	38	NA
# nodes	149	149	211	211	129	129	190	190
# edges	721	721	2348	2348	362	362	2742	2742
% positive edges	82.1	NA	61.1	NA	85.4	NA	56.7	NA
Average degree	9.678	9.68	22.256	22.26	5.612	5.61	28.863	28.86
Max degree	49	18.39	94	35.32	24	12.35	93	43.03
# clusters	12	1.01	5	1	7	1.41	2	1
Connectance	0.065	0.07	0.106	0.11	0.044	0.04	0.153	0.15
Average path length	3.331	2.44	2.693	1.98	3.604	2.98	2.417	1.86
Average betweenness centrality	0.011	0.01	0.008	0	0.015	0.02	0.007	0
Modularity	0.224	0.17	0.053	0.05	0.599	0.35	0.036	0.04
Global clustering coefficient	0.625	0.07	0.540	0.11	0.459	0.04	0.565	0.15

CHAPTER II

Scale-dependent spatiotemporal variation and long-term disturbance response of Pacific oyster hemolymph microbiota

Abstract

The composition and dynamics of microbiota are important for defense against pathogens but also for acclimation to new environments. To secure resistance against pathogens, a stable, compact microbiome is advantageous, while acclimation requires prompt changes of microbiome composition in response to environmental conditions. These opposing forces are particularly relevant for marine bivalves including oysters, because these important aquaculture species are routinely translocated around the world and have repeatedly experienced disease associated mass mortality events. Bivalves, and oysters in particular, harbor a diverse and functionally important microbial community within the hemolymph. However, the role of environmental and host genetic factors for the assembly of hemolymph microbiomes is largely unknown, and the few investigations of natural temporal and spatial variation of the hemolymph microbiome have been limited to few taxa (e.g. *Vibrio* spp.). In this study, we examined the seasonal dynamics of diversity and composition of the complete hemolymph microbiome of oysters that were reciprocally transplanted between two locations in the Wadden Sea (Sylt and Texel), characterized by two independent and genetically differentiated invasions. To control for the effect of previous bacterial colonization prior to field deployment, we additionally treated half of the oysters with antibiotics and investigated community similarity differentiating between the complete microbiome and its resident part respectively. With our spatially stratified experimental design we could identify important processes and factors affecting the composition of hemolymph microbiota at various scales. We found similar phylotype composition in all oysters and considerable large-scale spatiotemporal stability. Small-scale variability was probably associated to variation in microenvironmental conditions. Despite of this high variability we found persisting effects in the composition of resident microbiota in both of our treatments. The antibiotic treatment had a long persisting effect on within and between host diversity, whereas differences between oyster origins gradually disappeared in both locations, indicating that in the long run environmental factors outweighed

the genetic differences between the oyster populations. We can thus conclude that environmental influences on oyster hemolymph microbiota are dampened by its stable structure but that a profound disturbance can have long-term consequences.

Introduction

Pacific oysters (*Crassostrea gigas*) are hardy intertidal animals that have invaded coastal habitats throughout the world, following the intentional translocations for aquaculture purposes (Ruesink *et al.*, 2005). They can strongly impact invaded ecosystems (Kochmann *et al.*, 2008; Thieltges *et al.*, 2009; Padilla, 2010) and therefore the factors that affect the spread of Pacific oysters can have far-reaching consequences. One important aspect of animal biology in general are the effects of associated microbiota (McFall-Ngai *et al.*, 2013) and understanding the factors and processes that shape the oyster microbiota are of considerable ecological relevance. Moreover, as translocations are an ongoing practice in aquaculture, studying response of the oyster holobiont to new environments over extended periods of time - including the changes in the microbiome itself - also addresses commercial interests (Muehlbauer *et al.*, 2014).

Hemolymph is the blood analogue of invertebrates with important immune functions. Unlike blood, however, hemolymph abounds with microbial life also in healthy animals, and parts of these hemolymph microbiota have recently been shown to fulfill immune function for the defense of the host by producing antimicrobial compounds (Defer *et al.*, 2013; Desriac *et al.*, 2014). In addition, the hemolymph community structure is an indicator of host condition (Chapter III). While the hemolymph communities prevent establishment of external pathogens, their composition responds quickly to changes in abiotic environment, thus contributing to environmental acclimation (Chapter III). Despite the pivotal role of hemolymph microbiome for oyster fitness, the studies considering its natural variation have been limited to cultivable and potentially pathogenic bacteria, mostly of the genus *Vibrio* (Garnier *et al.*, 2007; Wendling *et al.*, 2014). Vibriosis imposes a strong selection pressure on oyster populations (Wendling and Wegner, 2015), but *Vibrio* sp. are also commonly isolated from healthy oysters (Prieur *et al.*, 1990; Garnier *et al.*, 2007; Wendling *et al.*, 2014). Moreover, unlike transient microbiota, oyster-associated *Vibrio* spp. persist under depuration conditions (that is, in the absence of source population) and are thus considered to be a part of resident oyster microbiome.

The prompt adjustments to environmental conditions, few barriers between the open oyster circulatory system and its surroundings, and high filtration rates (Troost, 2010) would suggest that the hemolymph microbiome is shaped mainly by immigration and that new colonizers quickly replace resident bacteria. However, transplant experiments demonstrated the persistence of indigenous *Vibrio* populations even months after translocation (Wendling *et al.*, 2014). As the resistance to colonization reflected by high community stability represents an

important functional aspect of the host-associated microbiota (Shade *et al.*, 2012), it is important to also examine how the microbiome as a whole responds to a new environment (Chapter I).

Although marine animals can have species-specific microbiomes with core phylotypes shared over large distances, the strongest determinant of community composition is often geography (Wilson *et al.*, 2008; Trabal *et al.*, 2012; Burgsdorf *et al.*, 2014; Dishaw *et al.*, 2014). The distance-decay relationship is a universal biogeographical pattern that has been demonstrated for microbial communities in both marine and terrestrial habitats (Bell, 2010; Martiny *et al.*, 2011; Zinger *et al.*, 2014; Nguyen and Landfald, 2015). The drivers behind this relationship can differ over spatial scales (Martiny *et al.*, 2011). Moreover, these drivers can differ for different taxa, especiallly in a host-associated community, where host genotype might affect resident, co-adapted, but not transient bacteria. Thus, examining the distance-decay relationship at different spatial scales within an oyster reef can shed additional light on processes and conditions that affect the assembly of the oyster microbiome.

To combine all these aspects of oyster hemolymph microbiome composition in one comprehensive study, we performed a reciprocal translocation experiment with two genetically differentiated oyster populations from two sites in the Wadden Sea (Texel and Sylt) (Moehler *et al.*, 2011) and repeatedly sampled the same individuals to follow the changes in their microbiota over longer time periods (i.e. a summer season). To further disentangle host genotype from prior colonization effects, we removed large parts of resident microbiome by antibiotics before the deployment in half of the oysters. Moreover, our field deployment allowed us to assess spatial variation over small (< 1 m) and medium scales (10^1-10^2 meters, within site) for the microbiome as a whole and for resident microbiota respectively. With this spatially and temporally stratified sampling we can thus identify important processes and differentitate between host and environmental factors determining the assembly of oyster microbiome at various spatial and temporal scales.

Material and Methods

Biological material

To examine the natural spatial and temporal variation of Pacific oyster hemolymph microbiota and effects of population origin, we reciprocially transplanted oysters from the southern Wadden Sea to the Northern Wadden Sea and repeatedly sampled hemolymph from the same individuals througout the summer of 2012. The Southern Wadden Sea oysters were

collected in de Cocksdorp, Texel, Netherlands (53° 0' N, 4° 54' E) and the Northern Wadden Sea oysters were collected at Oddewatt, Sylt, Germany (55° 1' N, 8° 26' E). The oysters were then cleaned of epibionts and transported to the AWI Wadden Sea station on Sylt, respectively to the NIOZ on Texel. A total of 96 oysters were used in experiment on Texel, and 120 oysters were used on Sylt. The animals were treated identically at both sites. Initially, the oysters were kept in pre-filtered seawater from their original location at the ambient temperature (~14°C). The oysters were pretreated as described in Chapter I. Briefly, half of the oysters were treated with a mix of four antibiotics (ampiciline, tetratcycline, gentamycine and kanamycine, SigmaAldrich, Hamburg, Germany) to remove resident microbiota and perturb the community structure. Three days later, prior to deployment, the hemolymph samples were take from the adductor muscle: 200 µl was stored at -80°C for microbiota analyis and 5 µl was plated on TCBS agar to determine the abundance of active *Vibrionaceae*.

Experimental setup and sampling

For the field transplant, always four oysters (one from each treatment group) were put in a single bag with mesh size of 1 cm, resulting in 20 (Sylt) or 16 (Texel) bags that were brought out to the original site of collection on Texel or Sylt. Two bags on Sylt or three bags on Texel were always put together on one sampling spot, with distances between the spots being in range of tens of meters. In this way, we could estimate how spatial scales affect similarity in microbiota composition. The hemolymph was sampled biweekly on Sylt and once a month on Texel. The samples were immediately placed on ice. On Sylt, 5 µl of hemolymph was plated on TCBS agar immediately upon return to the laboratory. The rest of the sample was frozen at -80°C for DNA extraction. We did not perform estimation of *Vibrionaceae* activity on Texel for logistic reasons. We also took 100 ml of seawater at each sampling occasion to determine the composition of environmental microbial communities.

DNA extraction

DNA extraction, PCR and the sequence quality control were performed as described in Chapter I and are therefore only briefly described here. DNA was extracted from app. 200 µl of hemolymph with Wizard SV 96 Genomic DNA Purification System (Promega, Manheim Germany). The samples were homogenized in a mixer mill (Retsch, Haan, Germany), lysed with proteinase K (20 µl of 20 mg/ml solution, SigmaAldrich, Hamburg, Germany) and extracted

according to manufacturer's protocols. To check for bacterial contamination of reagents, we included blank extractions as well.

The seawater samples were filtered and the filters were extracted with DNeasy Blood & Tissue Kit, Qiagen, Hilden, Germany as described in (Thomsen *et al.*, 2012). The samples were exposed to three rounds of bead beating and and cell lysis at 56°C, followed by protein lysis by proteinase K and subsequent DNA purification.

PCR

PCR (25 µl, 30 cycles, 1 min annealing at 55°C) of the 16s rRNA gene V1-V2 regions was performed with equal concentrations of uniquely barcoded 27f and 338r PCR primers, using 0.5 unit of Phusion Hot Start II High-Fidelity DNA Polymerase per reaction. In addition to hemolymph (1 µl/reaction) and seawater (1 µl of 10x diluted/reaction) samples, we performed positive and negative control (water as template) reactions for each 96well plate.

We estimated the amount of PCR product by the image analysis (Image Lab™ Software, BioRad, Hercules, CA USA) of agarose gel photographs. We pooled equal amounts (25-100 ng/sample) of the products run on a single gel and purified them with MinElute Gel Extraction Kit (Qiagen, Hilden, Germany). We measured the DNA concentration in the obtained subpools fluorometrically (Qubit dsDNA br Assay Kit, Life Technologies GmbH, Darmstadt, Germany) and pooled equal amounts together. The libraries were then sequenced on a MiSeq platform at the Max Planck Institute for Evolutionary Biology in Plön, Germany.

Sequence quality control and preprocessing

Sequencing libraries were processed together with the samples from Chapter I. We performed the quality control (removal of ambiguous bases, homopolymers, chimeras and sequencing errors (Huse *et al.*, 2010)), OTU picking (97% identity) and taxonomy assignment (Greengenes taxonomy 13_08 (DeSantis *et al.*, 2006)) as described in Mothur MiSeq SOP (Schloss *et al.*, 2009; Kozich *et al.*, 2013). Based on rarefaction curves, we decided to subsample the dataset to 10000 reads per sample. The final dataset comprised of 712 samples in total: 8 seawater, 704 hemolymph (167 laboratory and 537 field). An additional sample from Chapter I, representing the seawater community on Sylt at the beginning of June was included.

Statistical analysis

Statistical analyses were performed in R (R Core Team, 2013). The α-diversity analysis was based on the Shannon's H index, calculated from the complete subsampled dataset. We first tested for differences between the seawater and oysters within a site, and then between the oysters from two different sites using non-parametric tests. We tested the differences between the pre-deployment communities separately from the field samples, due to specific environment (laboratory conditions) and to explicitly determine the initial differences between the groups. In order to assess the effects of oyster origin and antibiotic treatment on α-diversity, as well as its temporal variability in the field, we fitted a separate linear mixed model for each site (Alexandra Kuznetsova, 2014; Bartoń, 2014; Bates D, 2014).

We employed negative-binomial generalized models (package MASS, (Ripley, 2002)) to analyze CFU counts. Because not only *Vibrio* sp., but also other *Vibrionaceae* grow on TCBS agar and classification of short reads to low taxonomic levels can be unreliable, we decided to focus the analyses on the whole *Vibrionaceae* family, and not just the genus *Vibrio*.

In our previous network analysis of OTU associations within hemolymph (Chapter I), we identified two main OTU clusters: one consisting of OTUs that were abundant in the seawater and the other dominated by *Vibrionaceae* and *Arcobacter*. We performed the same anaylsis with the hemolymph samples here and again identified these clusters (Figure II-1). We then analyzed β-diversity and distance-decay relationship including all OTUs or excluding the "seawater" OTUs (based on the network).

For β-diversity, we removed rare OTUs (<0.1 % relative abundance) in order to reduce the dataset complexity (Gobet *et al.*, 2010). We calculated Bray-Curtis distances of hellinger-transformed OTU tables and analyzed the resulting distance matrices by unconstrained and constrained analysis of principal coordinates (CAP, (Anderson and Willis, 2003)) using the *capscale* function and by Permanova (non-parametric permutational multivariate analyis of variance (Anderson, 2001)), using the *adonis* function, both implemented in the *vegan* package (Oksanen *et al.*, 2013). In order to examine how oyster origin, antibiotic treatment and site affected the β-diversity throughout the summer, we analyzed the hemolymph communities at four time-points: before deployment, and once in June, July and August. Although the oysters at Sylt and Texel were not sampled simultaneously, the difference in the field was at most ten days and the samples were analyzed together. Two additional time-points were sampled and analyzed

on Sylt. In order to determine if communities from the same oyster are more similar to each other than to other oysters, we compared the within-and among-oyster Bray-Curtis distances. In addition, we calculated the bacterial turnover within oysters as a proportion of OTUs shared between the initial and subsequent sampling points (Gobet *et al.*, 2012). We also calculated average Bray-Curtis distances of composite communities between the time points in order to assess the bacterial turnover at a large spatiotemporal scale.

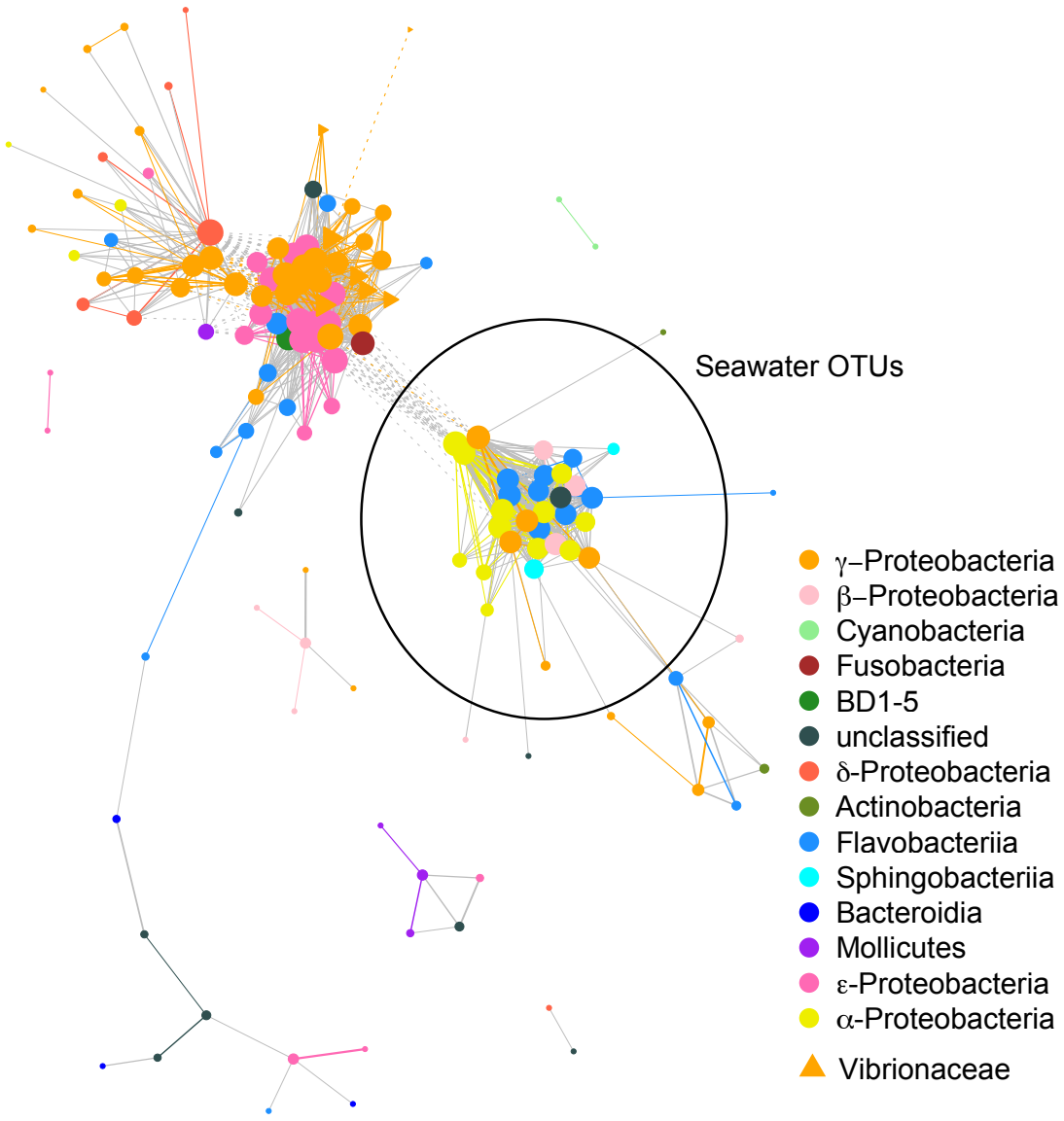


***Figure II-1**. Association network showing cluster with the "seawater" OTUs.*

We analyzed the distance-decay relationship as described in (Martiny *et al.*, 2011). Briefly, we used 1- Bray-Curtis distance as a measure of similarity and calculated all pairwise distances

between the samples. We then calculated linear models for overall distance-decay relationship (between sites), as well as for within-spot (up to 1 m) and between spots (tens of meters) distance ranges separately. In order to estimate how this relationship was affected by transient OTUs, we performed the analysis excluding the seawater OTUs and compared the resulting slopes to the original ones.

Temperature data were obtained from the Sylt time-series (courtesy of Tatyana Romanova, Wadden Sea Station Sylt, Germany) and from NIOZ Jetty, Texel, Netherlands (Aken 2008).

Results

α-diversity (Figure II-2)

In general, both seawater and hemolymph communities on Texel were more diverse than their counterparts on Sylt (Asymptotic Wilcoxon Mann-Whitney Rank Sum Test for Shannon's H, seawater: median Sylt = 4.468, median Texel = 6.186, Z = -2.558, p = 0.011, effect size = -0.81; hemolymph: median Sylt = 3.859, median Texel = 4.435, Z = -7.195, $p < 10^{-6}$, effect size = -0.271). The difference between the sites could be partially due to higher temperatures on Texel (mean, min. and max. temp.: Texel = 17.61 (15.13, 20.35), Sylt = 16.96 (14.91, 19.24)), as α-diversity positively correlated with temperature (Kendall's $\tau = 0.167 \pm 0.026$). Interestingly, the hemolymph communities on Texel had lower diversity than the local seawater microbiota (Z = -2.6293, p = 0.009, effect size = -0.153), while no significant trend could be detected for Sylt samples (Z = -1.219, p = 0.228).

To explicitly determine the initial effects of the treatments in the laboratory conditions, we tested the pre-deployment diversity separately from the field diversity dynamics.

Antibiotic treatment significantly affected the hemolymph communities prior to deployment, but it initially decreased diversity at the Texel site and increased diversity on Sylt (Anova, treatment x site: $F_{1,158} = 15.775$, $p < 10^{-4}$, linear coefficient (antibiotic-control : Sylt-Texel) = 0.206 ± 0.052, $t_{158} = 3.962$, $p < 10^{-4}$, effect size = 0.302). In addition, we observed higher diversity in the local oysters on Sylt, but no effect of origin on Texel (origin x location: $F_{1,158} = 1.165$, p = 0.055; linear coefficient ((oSylt-oTexel):(sSylt-sTexel)): 0.097 ± 0.052, $t_{158} = 1.860$, p = 0.065, effect size = 0.146). Nevertheless, oyster hemolymph microbiota responded consistently to antibiotic treatment irrespective of origin at both sites (treatment x origin: $F_{1, 158} = 0.03$, p = 0.812; treatment x origin x site: $F_{1, 158} = 0.00$, p = 0.938).

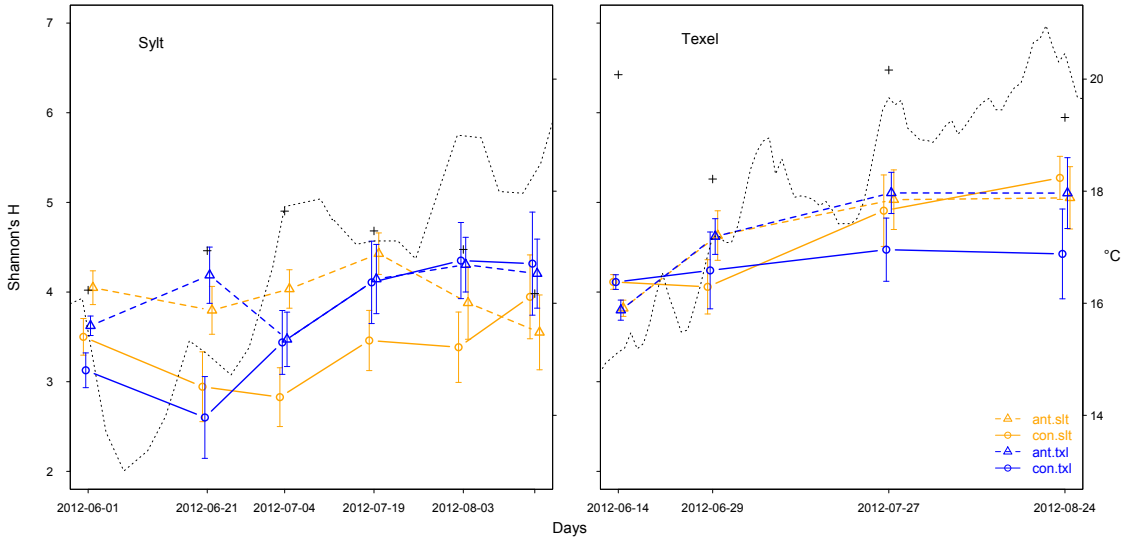


***Figure II-2**. α-diversity of hemolymph and seawater (+) microbiota on Sylt and Texel throughout the experiment. Dotted line represents temperature.*

Analysis of α-diversity in the field after deployment (Table II-1) showed that the increased α-diversity after antibiotic treatment persisted for at least three weeks on Sylt with similar, although not significant, trends observed in antibiotic treated oysters on Texel. While oyster origin had no significant overall effect on α-diversity, the initial sorting by treatment was reversed towards the end of the sampling period when α-diversity grouped according to oyster origin with higher diversity found in translocated oysters from Texel (Figure II-2, Origin x Treatment x Time interaction in Table II-1). While communities of translocated treated and control Texel oysters quickly converged after this 3 week period, the effect of antibiotics persisted much longer in local Sylt oysters where convergence could be observed only by August. Such sorting could only partly be observed or was incomplete at the Texel site where higher diversity towards the end oft he experiment was observed mainly in control oysters whith translocated oysters from Sylt showing higher diversity than oysters from Texel. This could probably be explained by persistent parts of the microbial communities carried over from the original site that were over time complemented by newly aquired taxa at the new site adding up to higher overall diversities. Persistence of individual microbial communities could be observed by better fits of statistical models containing random effects (oyster, cage and spot) that explained substantially more variation in the data (marginal and conditional R^2, Table II-1). This indicates that the structure of hemolymph microbiota might have been strongly affected by

the oyster genotype, but also by microenvironmental conditions at the individual sampling spots (such as oyster and algal density, imersion time etc.).

	Fixed effects	*Sum Sq*	*Mean Sq*	*df*	*F*	*p*	*Significant contrasts*	*Estimate*	*2.5% CI*	*97.5%CI*
Texel	Origin	0.46	0.46	1, 58.5	0.32	0.57				
	Treatment	3.94	3.94	1, 61.89	2.74	0.10	*Antibiotic-Control*	0.18	-0.03	0.40
	Time	10.14	5.07	2, 121.78	3.52	0.03	Linear trend	0.35	0.02	0.68
	Origin x Treatment	0.86	0.86	1, 61.85	0.60	0.44				
	Origin x Time	1.56	0.78	2, 122.4	0.54	0.58				
	Treatment x Time	0.44	0.22	2, 122.15	0.15	0.86				
	Origin x Treatment x Time	1.41	0.70	2, 122.01	0.49	0.61				
	Random effects									
	Oyster							0.60	0.00	0.94
	Cage							0.22	0.00	0.67
	Spot							0.41	0.00	0.85
Sylt	*Fixed effects*									
	Origin	0.84	0.84	1, 66.16	0.60	0.44				
	Treatment	5.69	5.69	1, 64.06	4.11	0.05	Antibiotic-Control	0.22	0.01	0.43
	Time	17.64	4.41	4, 236.23	3.18	0.01	Linear trend	0.40	0.06	0.73
	Origin x Treatment	0.52	0.52	1, 66.64	0.37	0.54				
	Origin x Time	3.59	0.90	4, 237.35	0.65	0.63				
	Treatment x Time	14.91	3.73	4, 237.27	2.69	0.03	(Antibiotic-Treatment) x Linear trend	-0.54	-0.87	-0.21
	Origin x Treatment x Time	12.37	3.09	4, 237.62	2.23	0.07	(Sylt-Texel):(Antibiotic-Control):Quadratic trend	-0.39	-0.70	-0.07
	Random effects									
	Oyster							0.71	0.52	1.03
	Cage							0.35	0.00	0.70
	Spot							0.21	0.00	0.62

Table II-1. Linear mixed models for Shannon's H of hemolymph microbiota on Texel (dAIC=-31.31, logLik=-358.40 (df = 16), R2 marginal=0.06, R2 conditional =0.32) and Sylt (dAIC=-36.55, logLik=-576.43 (df = 24), R2 marginal=0.10, R2 conditional =0.39) during the summer 2012.

β-diversity

The seawater communities were comparatively homogenous and showed less spatiotemporal variation than the hemolymph communities (Analysis of multivariate homogeneity of group dispersions: $F_{1,\ 708} = 123.85$, $p < 10^{-6}$; average Bray-Curtis distance within the groups with standard deviation: seawater = 0.567 ± 0.147, oysters = 0.857 ± 0.119). However, only 1% variability was explained by type of sample, indicating that the seawater represents an important

source of hemolymph microbiota and that the seawater and oyster hemolymph communities can be very similar in some cases (Permutational MANOVA seawater vs. hemolymph: $F_{1,551}$ = 5.356, R^2 = 0.010, p = 0.001, Figure II-S1). Close relationship between the seawater and oyster microbiota is further reflected in the association network: one of the major clusters is composed primarily of seawater OTUs (Figure II-1).

Pre-deployment communities were significantly affected by oyster origin and antibiotic treatment, as well as by their interaction (Figure II-S2, Table II-2). The community composition (Figure II-S3) was stable at the class level and differed from the field samples by higher relative abundances of α- and γ-Proteobacteria, mainly *Arcobacter* and *Vibrionaceae* and the absence of the seawater OTUs. The field communities had similarly uniform higher-taxon composition at both sites throughout the sampling period, but, unlike the laboratory samples, they were characteized by high relative abundances of Tenericutes and of an unclassified bacterium related to Spirochaetes. These abundant bacteria were previously reported from oysters from Australia and Japan indicating that they might represent ubiquitous oyster symbionts.

Following the deployment, the influence of oyster origin was still apparent two weeks later in the field, but disappeared afterwards (Table II-2, Figure II-3). The effect of antibiotic treatment persisted for a longer period until the end of July. It is important to mention, however, that the variability explained by both factors was generally small, indicating that other factors like between individual variation determined the bulk of the observed community variation. Exclusion of OTUs representing the typical seawater cluster only slightly affected the variability explained by origin or antibiotic treatment, while it mostly reduced the variability explained by distance (Table II-2).

The rate of bacterial turnover within oysters did not increase with time (Figure II-S4) and although the within-oyster temporal variability was high, it was smaller than the variation observed among the oysters (within Bray-Curtis: 0.818±0.140, among: 0.876±0.105, Wilcoxon Mann-Whitney Rank Sum Test: Z= -9.283, $p < 10^{-6}$). On the other hand, the average distance between the composite communities representing the sampling points within sites was much lower (Sylt Bray-Curtis= 0.454±0.049, Texel Bray-Curtis = 0. 467±0.063,), probably reflecting the similarity in mean environmental conditions.

	All OTUs					Without "seawater" OTUs				
Pre-deployment	*Df*	*SS*	*MS*	*pseudo F*	R^2	*Df*	*SS*	*MS*	*pseudo F*	R^2
Origin	1	1.76	1.76	10.74	0.05***	1	1.74	1.74	10.63	0.04***
Treatment	1	1.37	1.37	8.35	0.04***	1	1.35	1.35	8.25	0.03***
Site	1	4.81	4.81	29.30	0.12***	1	4.83	4.83	29.57	0.12***
Origin x Treatment	1	0.86	0.86	5.22	0.02***	1	0.85	0.85	5.21	0.02***
Origin x Site	1	1.73	1.73	10.57	0.04***	1	1.72	1.72	10.55	0.04***
Treatment x Site	1	1.57	1.57	9.55	0.04***	1	1.57	1.57	9.61	0.04***
Origin x Treatment x Site	1	0.59	0.59	3.60	0.02***	1	0.58	0.58	3.54	0.01***
Residuals	160	26.26	0.16		0.67	160	26.14	0.16		0.67
Total	167	38.95			1.00	167	38.77			1.00
June										
Origin	1	0.54	0.54	1.85	0.01*	1	0.64	0.64	1.96	0.01**
Treatment	1	0.80	0.80	2.77	0.02***	1	0.85	0.85	2.59	0.01**
Distance	2	4.19	2.09	7.23	0.08***	2	3.27	1.63	4.98	0.06***
Origin x Treatment	1	0.21	0.21	0.72	0.00	1	0.26	0.26	0.79	0.00
Origin x Distance	2	0.48	0.24	0.83	0.01	2	0.59	0.29	0.89	0.01
Treatment x Distance	2	0.62	0.31	1.07	0.01	2	0.61	0.31	0.94	0.01
Origin x Treatment x Distance	2	0.73	0.37	1.27	0.01	2	0.72	0.36	1.09	0.01
Residuals	156	45.16	0.29		0.86	156	51.24	0.33		0.88
Total	167	52.73			1.00	167	58.18			1.00
July										
Origin	1	0.29	0.29	0.95	0.01	1	0.35	0.35	1.02	0.01
Treatment	1	0.47	0.47	1.51	0.01'	1	0.47	0.47	1.35	0.01'
Distance	2	4.19	2.09	6.80	0.09***	2	3.67	1.84	5.31	0.07***
Origin x Treatment	1	0.28	0.28	0.90	0.01	1	0.34	0.34	0.98	0.01
Origin x Distance	2	0.54	0.27	0.87	0.01	2	0.63	0.32	0.92	0.01
Treatment x Distance	2	0.79	0.39	1.28	0.02	2	0.83	0.42	1.20	0.02
Origin x Treatment x Distance	2	1.00	0.50	1.63	0.02*	2	1.08	0.54	1.57	0.02**
Residuals	133	40.98	0.31		0.84	133	46.02	0.35		0.86
Total	144	48.53			1.00	144	53.40			1.00
August										
Origin	1	0.31	0.31	0.97	0.01'	1	0.34	0.34	0.94	0.01
Treatment	1	0.48	0.48	1.47	0.02	1	0.42	0.42	1.15	0.01
Distance	2	2.09	1.04	3.22	0.07***	2	2.44	1.22	3.36	0.08***
Origin x Treatment	1	0.43	0.43	1.32	0.01	1	0.42	0.42	1.15	0.01
Origin x Distance	2	0.52	0.26	0.81	0.02	2	0.64	0.32	0.88	0.02
Treatment x Distance	2	0.53	0.27	0.82	0.02	2	0.62	0.31	0.86	0.02
Origin x Treatment x Distance	2	0.74	0.37	1.14	0.03	2	0.77	0.38	1.06	0.02
Residuals	74	23.95	0.32		0.82	74	26.89	0.36		0.83
Total	85	29.04			1.00	85	32.53			1.00
Sylt July										
Origin	1	0.43	0.43	1.27	0.01	1	0.47	0.47	1.36	0.02'
Treatment	1	0.75	0.75	2.24	0.03**	1	0.78	0.78	2.24	0.03***
Distance	2	0.79	0.39	1.18	0.03	2	0.78	0.39	1.13	0.03
Origin x Treatment	1	0.53	0.53	1.58	0.02'	1	0.50	0.50	1.44	0.02'
Origin x Distance	2	0.41	0.20	0.61	0.01	2	0.46	0.23	0.66	0.02
Treatment x Distance	2	0.65	0.32	0.97	0.02	2	0.60	0.30	0.86	0.02
Origin x Treatment x Distance	2	0.90	0.45	1.35	0.03'	2	0.91	0.45	1.31	0.03'
Residuals	72	24.04	0.33		0.84	72	25.05	0.35		0.85
Total	83	28.49			1.00	83	29.55			1.00
Sylt August										
Origin	1	0.35	0.35	0.95	0.02	1	0.38	0.38	1.02	0.02
Treatment	1	0.38	0.38	1.06	0.02	1	0.38	0.38	1.00	0.02
Distance	2	0.84	0.42	1.16	0.04	2	0.86	0.43	1.14	0.04
Origin x Treatment	1	0.36	0.36	0.99	0.02	1	0.39	0.39	1.05	0.02
Origin x Distance	2	0.58	0.29	0.80	0.03	2	0.60	0.30	0.80	0.03
Treatment x Distance	2	1.02	0.51	1.41	0.05**	2	1.04	0.52	1.38	0.05**
Origin x Treatment x Distance	2	0.52	0.26	0.72	0.02	2	0.56	0.28	0.74	0.03
Residuals	47	16.99	0.36		0.81	47	17.70	0.38		0.81
Total	58	21.03			1.00	58	21.91			1.00

[1]significance levels: ' < 0.1, * ≤ 0.05, ** < 0.01, *** < 0.001

***Table II-2**. Permanova (adonis) showing the effects of oyster origin, antibiotic treatment and distance on hemolymph communities at different time points during the experiment. Left: including all OTUs, right: excluding the seawater OTUs. Samples from Sylt and Texel on comparable sampling points in June, July and August are analyzed together. Two additional sampling points on Sylt, one in July and one in August are analyzed separately.*

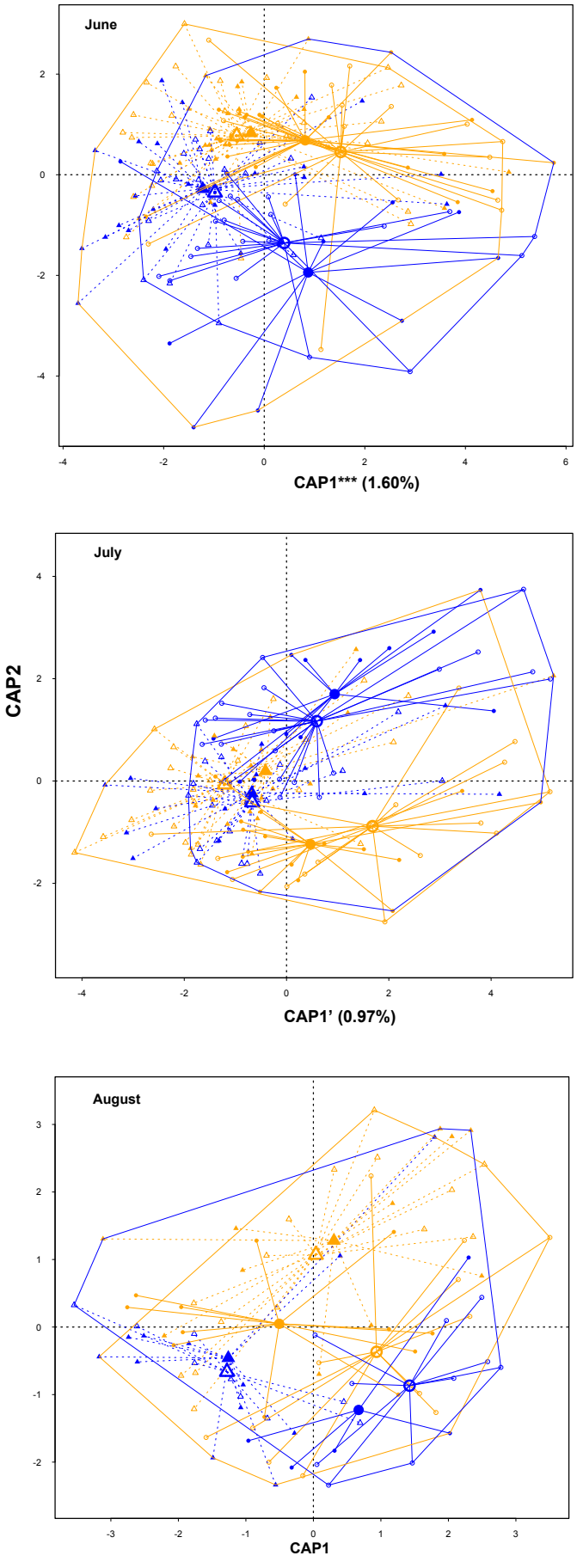


Figure II-3. *CAP of beta diversity of hemolymph communities on Sylt (orange) and Texel (blue) showing the effects of oyster origin and antibiotic treatment and accounting for distance throughout the summer. Hull indicates site, circle = control, triangle = antiobiotic treated.*

Distance-decay

Analysis of the distance-decay relationship (Figure II-4) revealed a small, but significant overall effect of distance (linear model: $F_{1, 32696} = 457.8$, $p < 10^{-6}$, adj. $R^2 = 0.013$, slope: -0.024). The slope was significantly higher when small (up to 1 m, within the spots) and intermediate (between spots, up to 186 m) spatial scales were considered separately (within spot: $F_{1, 1590} = 19.61$, $p < 10^{-5}$, adj. $R^2 = 0.012$, slope = -0.102, between spots: $F_{1, 17264} = 112.5$, $p < 10^{-6}$, adj. $R^2 = 0.012$ slope = -0.113). Exclusion of the seawater OTUs reduced the average community similarity at all scales (Figure II-S5), but it affected neither the overall ($F_{1, 32696} = 606.7$, $p < 10^{-6}$, adj. $R^2 = 0.002$, slope = -0.027) nor the small-scale slope ($F_{1, 1590} = 14$, $p < 10^{-4}$, adj. $R^2 = 0.001$, slope = -0.082). However, it decreased the distance-decay slope between the spots within a site Figure II-5, indicating that microenvironmental site conditions predominantly influence transient parts of the hemolymph microbiome ($F_{1, 17264} = 11.39$, $p < 10^{-5}$, adj. $R^2 = 0.001$ slope: -0.034).

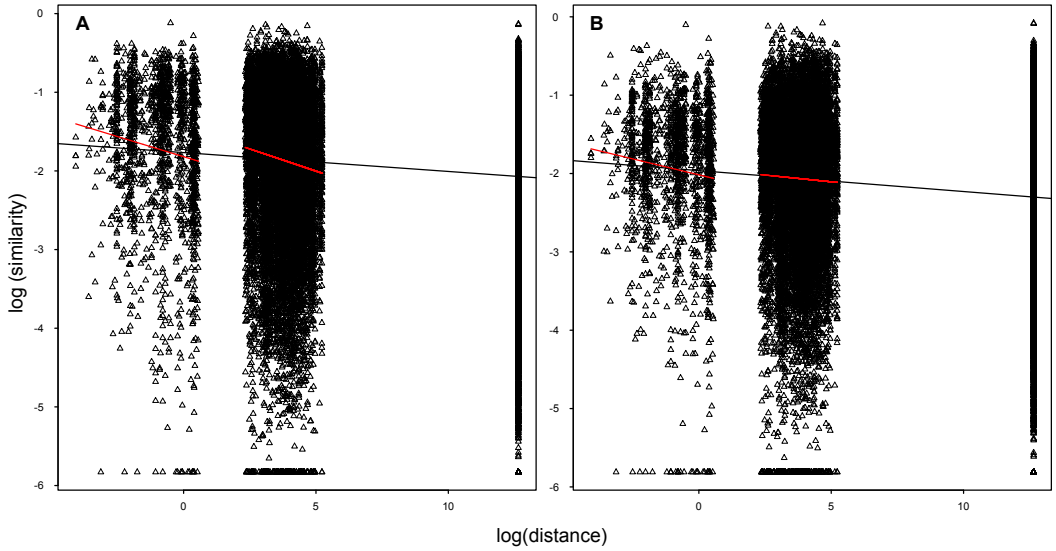


Figure II-4*. Distance-decay relationship including A) all OTUs, B) resident (excluding seawater) OTUs. Lines are linear models fitted for the given spatial scale.*

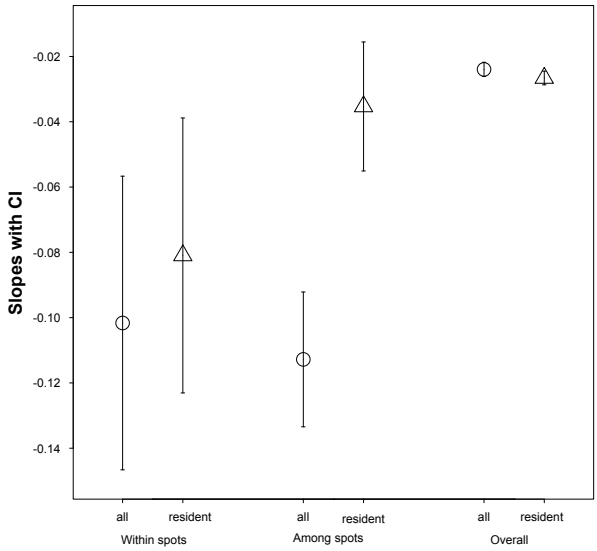


***Figure II-5**. Effect of transient OTUs on slope of distance-decay relationship on small, medium and overall spatial scale.*

Vibrio activity (Figure II-6, Table II-S1)

Vibrio activity at the Sylt site was higher in translocated oysters from Texel, but this difference disappeared over time. Interestingly, although the pre-deployment antibiotic treated communities had very low vibrio counts, all Texel oysters at the end of June had vibrio counts comparable to each other and much higher than the local ones, suggesting that the increased vibrio activity may be at least partially due to direct interactions of local strains with non-adapted oysters, rather than to interactions within the microbiome as a whole.

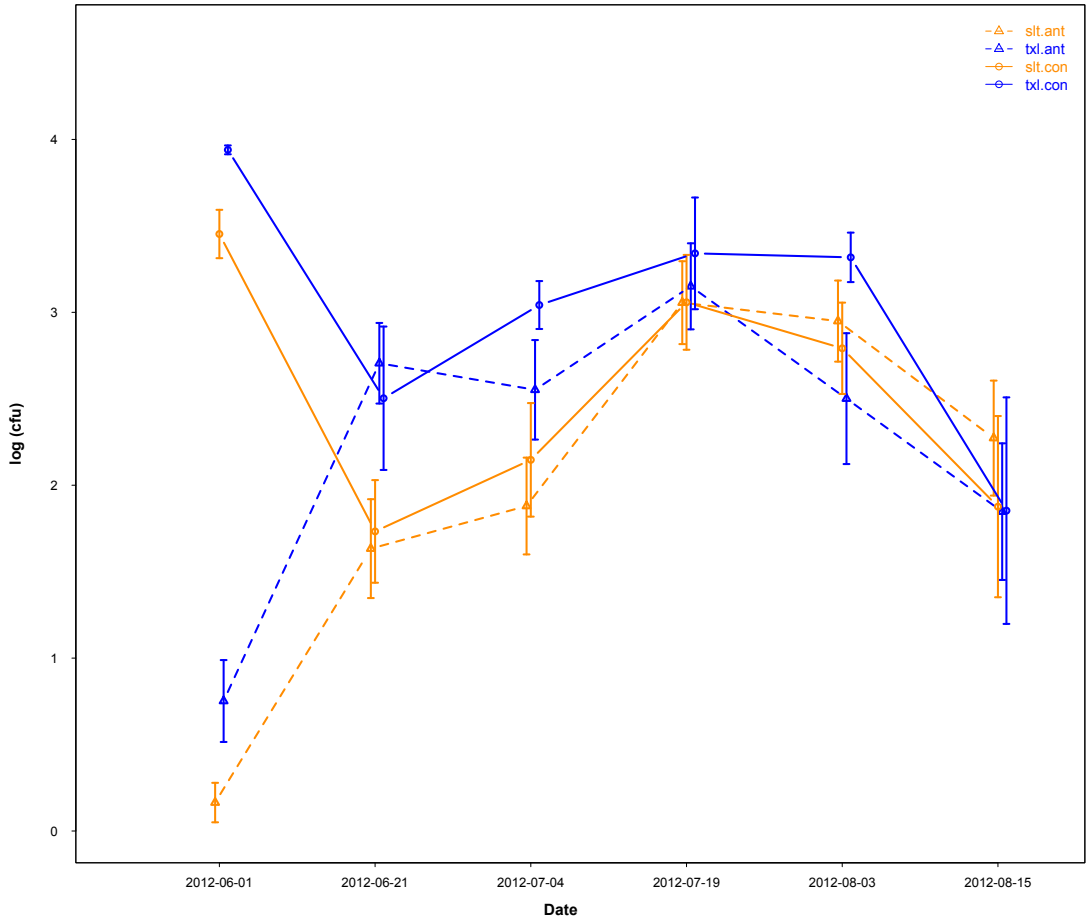


***Figure II-6**. Vibrio activity according to oyster origin and antibiotic treatment over the course of the experiment on Sylt. The dotted line shows temperature measurements.*

Discussion

Unveiling the microbial community dynamics is essential for the assessment of community assembly and stability and thus for understanding how the microbiota contribute to the fitness of their host. Disturbances, and the determinants of the community composition in general, act over different spatial and temporal scales; taking these into consideration provides a tool to disentangle the relative contributions of multiple factors in the complex natural environment, especially because the direct assessment of environmental parameters in highly variable habitats such as the intertidal can be challenging for theoretical and practical reasons. By examining the short- and long -term dynamics of the hemolymph microbiota over multiple spatial scales, we observed a quick response to major perturbations. While the large changes in abiotic conditions (for example, transfer to the laboratory conditions) caused prompt adjustments in the

composition of transient bacteria, the long-term persistence of the translocation and antibiotic treatment effects indicates stronger influence of biotic interactions - with the host and within the microbiome - on the resident part of the community.

Environmental effects on resident and transient hemolymph microbiota

The absence of typical seawater OTUs from laboratory communities implies that they represent a transient part of oyster microbiota, irrespective of how common they are in the field (Vasconcelos and Lee, 1972; Rong *et al.*, 2014). Unlike the putative resident bacteria (Vasconcelos and Lee, 1972; Pruzzo *et al.*, 2005), they are expected to strongly depend on the immediate environmental fluctuations, such as the immersion in the intertidal habitat (Chapter I). No effect of the exclusion of transient microbiota on the variability explained by antibiotic treatment or oyster origin, and the strong effect of the transient microbiota on the distance-decay slope over microenvironmentally variable scales indicate that this indeed could be the case here. The distance-decay relationship arises through spatially-correlated environmental conditions or through dispersal limitation (Martiny *et al.*, 2011; Costello *et al.*, 2012). Over small distances (< 1m), the abiotic conditions experienced by oysters are very similar and the transient part of the microbiome likely reflects this high environmental similarity. On the other hand, the microenvironmental variation at the distance of tens of meters is high in the intertidal, and the strong effect of transient microbiota on the distance-decay slope over these scales suggests, that this could be related to more similar immersion times or effects of tidal currents at smaller distances.

Other transient bacteria belonged to the Tenericutes and an unindentified bacterium close to Spirochaetes that were abundant in the field and rare in the laboratory conditions. These bacteria have been previously found in Pacific oysters in Tasmania (Fernandez-Piquer *et al.*, 2012) and could thus represent cosmopolitan oyster symbionts. Both Spirochaetes and Tenericutes have been found in various oyster tissues (Chapter I), (Prieur *et al.*, 1990; Green and Barnes, 2010; Husmann *et al.*, 2010; King *et al.*, 2012; Trabal *et al.*, 2012; Trabal Fernandez *et al.*, 2013; Wegner *et al.*, 2013) and could provide some benefits to the host (Prieur *et al.*, 1990; Tanaka *et al.*, 2004; Fraune and Zimmer, 2008). However, Spirochaetes and Tenericutes are usually very rare in the hemolymph (Chapter I, Chapter III), and the high abundance of Tenericutes was mainly linked to stress or even mortality (Chapter III). It is therefore not clear if Tenericutes are common hemolymph inhabitants in the wild or their increased abundance was due to a secondary infection of the injection site on the adductor muscle caused by repeated hemolymph sampling (Ayling *et al.*, 2011).

Although the dynamics of the bacteria such as *Vibrio* or *Arcobacter* (Chapter I, Chapter III) is also affected by the environment, and could be seasonally driven (compare this thesis, (Wegner *et al.*, 2013 32; Wendling *et al.*, 2014), their persistence in the hemolymph under variety of conditions suggests that they could be indeed considered as part of the resident hemolymph microbiota.

Spatiotemporal dynamics of oyster microbiota in the field

High small scale variability and little differentiation over large spatiotemporal scales - between the sites or between the sampling points - corresponds with the finding that the measured variation in community composition diminishes as the observation period increases (Shade *et al.*, 2013). Comparing the composite communities and/or decreasing the taxonomic resolution would result in much lower estimates of bacterial turnover within individuals and higher perceived seasonal stability observed in subtidal sessile marine invertebrates (Erwin *et al.*, 2012; Björk *et al.*, 2013; Pita *et al.*, 2013; Hardoim and Costa, 2014). However, higher turnover could also be attributed to extreme small-scale environmental fluctuations in the intertidal, as the the hemolymph microbiota respond quickly to disturbances by shifts in relative abundances (Chapter I, Chapter III). This could result in a cycling dynamics and high temporal variability (Shade *et al.*, 2014), not necessarily because the bacteria disappeared, but because rare taxa might fall below the detection limit (Caporaso *et al.*, 2012). This hypothesis is supported by the constant turnover rate observed here, suggesting that the bacterial populations disappear, but also reappear by different taxa dominating the community at different time points (Gobet *et al.*, 2012). On the other hand, while the high between-oyster variability at individual sampling points also likely reflects the microenvironmental variability, other factors, such as oyster condition (Chapter I) or genotype (Wegner *et al.*, 2013) could have significantly contributed to the observed differences.

Conversely, the large-scale spatiotemporal stability could be due to relatively stable mean abiotic conditions (for example, temperature, Figure II-S6) throughout the sampling period, which were unlikely to significantly alter the overall community composition (Nguyen and Landfald, 2015). Both coastal sediment and seawater communities (Campbell *et al.*, 2011; Gilbert *et al.*, 2012; Gobet *et al.*, 2012) are seasonally stable and, in addition, both study sites belong to the same water mass. The hemolymph microbiota are thus likely to reflect this seasonal stability, as they are strongly affected by abiotic environment (Chapter III) and environmental microbial communities (Chapter I, here).

Variation of oyster microbiota related to origin and antibiotic treatment

The effects of antibiotic treatment and oyster origin lasted for several weeks, indicating that the turnover of resident microbiota was gradual, as opposed to highly dynamic transient bacteria. Although antibiotics can have long-lasting negative effects on diversity in some cases (Stein *et al.*, 2013), they can increase the diversity in others, probably because the disturbed communities are more susceptible to invasions (Shea *et al.*, 2004; Robinson *et al.*, 2010). The open circulatory systems of oysters with its tight connection to the environment probably prevented antibiotics to induce permanent changes and shifts towards alternative stable states (Stein *et al.*, 2013). Interestingly, the micriobal diversity in non-treated translocated oysters initially did not differ from their local counterparts, but increased towards the end of the experiment at both sites (Figure II-2). Although the effect was only marginally significant, it is tempting to speculate that this trend might have been linked to colonization by novel microbiota from the environment complementing the resident microbiota while diversity of resident microbiota stayed constant in local oysters. The delay might suggest that the resident bacteria provided resistance against colonization in the beginning, before the better-adapted bacteria from the environment gained advantage to form a new resident microbiome. Marine microbial communities are characterized by periodically recurring bacterial populations (Gilbert *et al.*, 2012; Gobet *et al.*, 2012) and although such predictable environmental community shifts could affect local oyster and its microbiota to a lesser degree, they might represent a more significant disturbance for translocated communities.

Genetic differentiation between oysters from Texel and Sylt (Moehler *et al.*, 2011) could have contributed to the observed differences in β-diversity since oyster microbiota can assemble according to genotype (Wegner *et al.*, 2013). However, the gradually decreasing difference between the translocated and local oysters in both sites showed that the divergence in community composition was in the long run mainly affected by site (Linnenbrink *et al.*, 2013; Burgsdorf *et al.*, 2014). Previous studies have shown that *Vibrio* spp. gradually colonized oysters throughout a season (Wendling *et al.*, 2014) and such dynamics could also be observed for the large parts of the microbial community here. While antibiotics strongly affected *Vibrio* activity, they did not completely eradicate the indigenous populations (Chapter I). Therefore, the higher loads in all translocated oysters might represent the reaction of endogenous vibrios to their counterparts at the new site (Froelich and Oliver, 2013). However, resistance against vibrio pathogens is partially genetically determined (Rosa *et al.*, 2012) and we could already show that Sylt oysters rapidly adapted to their local vibrio populations and were able to efficiently control vibrio population size in their hemolypmh (Wendling and Wegner, 2015). While this suggests

that there are host genetic factors affecting parts of the microbial community, the open contact of hemolymph to the environment might dampen genetoype-specific community assembly for the whole community that could only be observed in other tissues that are more shielded from the environment (Wegner *et al.*, 2013). Overall, the persistence of translocation and antibiotic treatment effects suggests that such unpredictable and strong disturbances could affect the oyster fitness (Wendling and Wegner, 2013).

Conclusion

Although 16s amplicon studies provide helpful insight and are important first step in understanding microbial communities, they lack resolution power. Taxonomic composition is only partially consistent with ecology (Koeppel and Wu, 2012) and allows only for distinction between broad habitat types (Schmidt *et al.*, 2014). 16s rRNA defined OTUs may consist of variety of ecotypes, and, in case of host-associated bacteria, they can significantly differ in crucial traits such as virulence (Koeppel and Wu, 2013; Wendling and Wegner, 2015). Moreover, closely related bacteria exhibit adaptation at very small spatial scales (Belotte *et al.*, 2003). Therefore, although the communities at both sites and throughout the summer appear similar through the lense of 16s rDNA based taxonomy, they can actually consist of ecologically different bacteria with important consequences for hemolymph microbiota dynamics and their oyster hosts. Despite the strong environmental influence, factors such as oyster genotype, physiology and health condition are also likely to affect the composition and structure of hemolymph microbiome (Chapter III), (Wegner *et al.*, 2013), as suggested by a large portion of α-diversity variability attributed to individual differences.

This is the first study of seasonal dynamics of the complete oyster hemolymph microbiome at various spatial scales. High small-scale spatiotemporal variability probably reflects the acclimation potential of the hemolymph microbiome, and the quick changes in transient microbiota following major shits in abiotic environment suggest that the microbiome is strongly shaped by immediate environmental conditions. However, the changes in the resident community show that strong pulse disturbances can have long-lasting effects even on a highly dynamic microbiome and thus potentially on the host fitness.

CHAPTER II SUPPLEMENTS

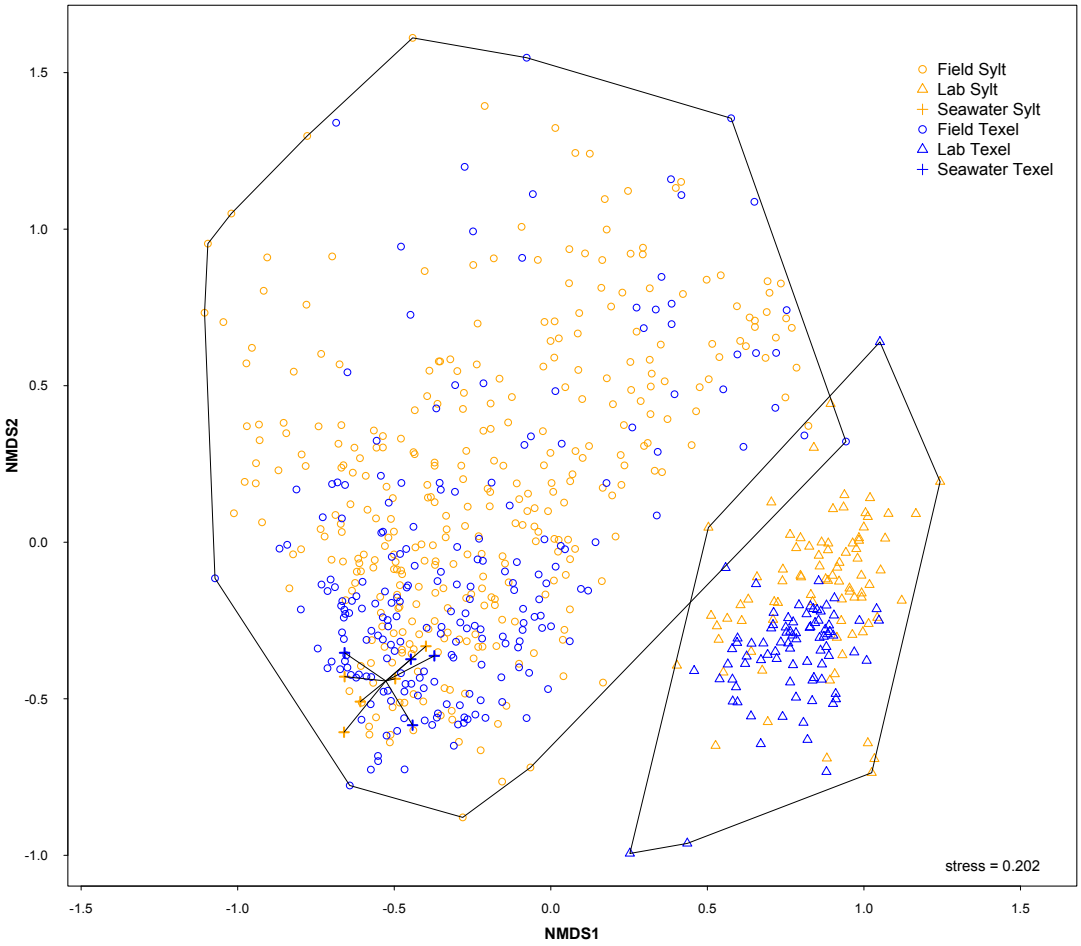


Figure II-S1. *Non-metric multidimensional scaling including complete dataset and visualizing the difference between the seawater and hemolymph samples and between the pre-deployment and field communities, as well as similarity of hemolymph and seawater communities on Sylt and Texel. Color denotes site, hull lab and field communities.*

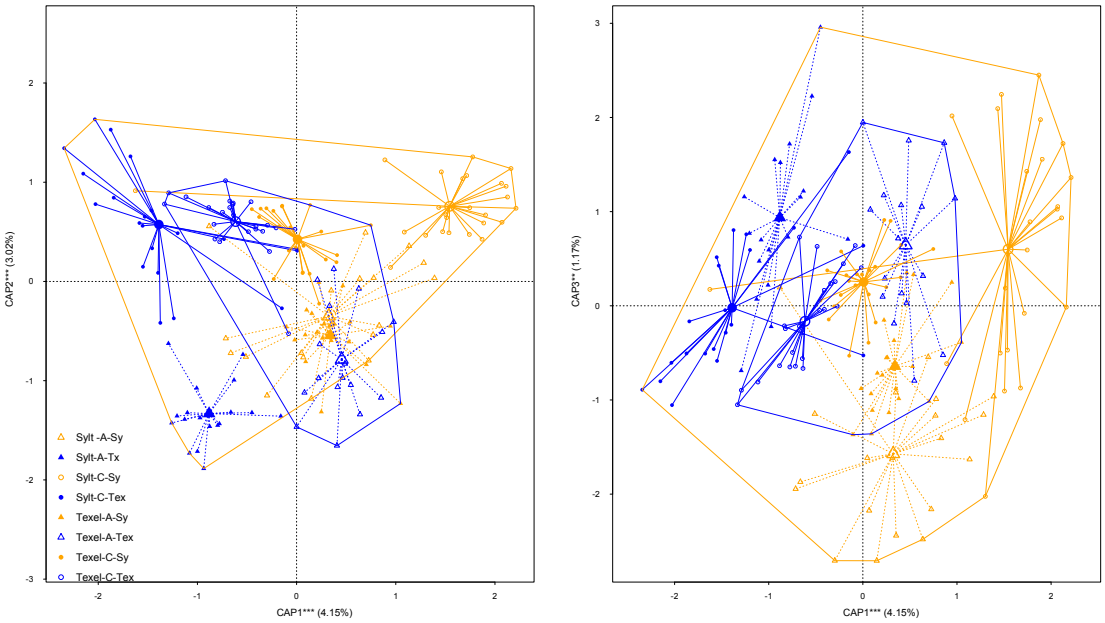


Figure II-S2. *CAP of pre-deployment hemolymph communities with marked significant axes and the amount of variability explained.*

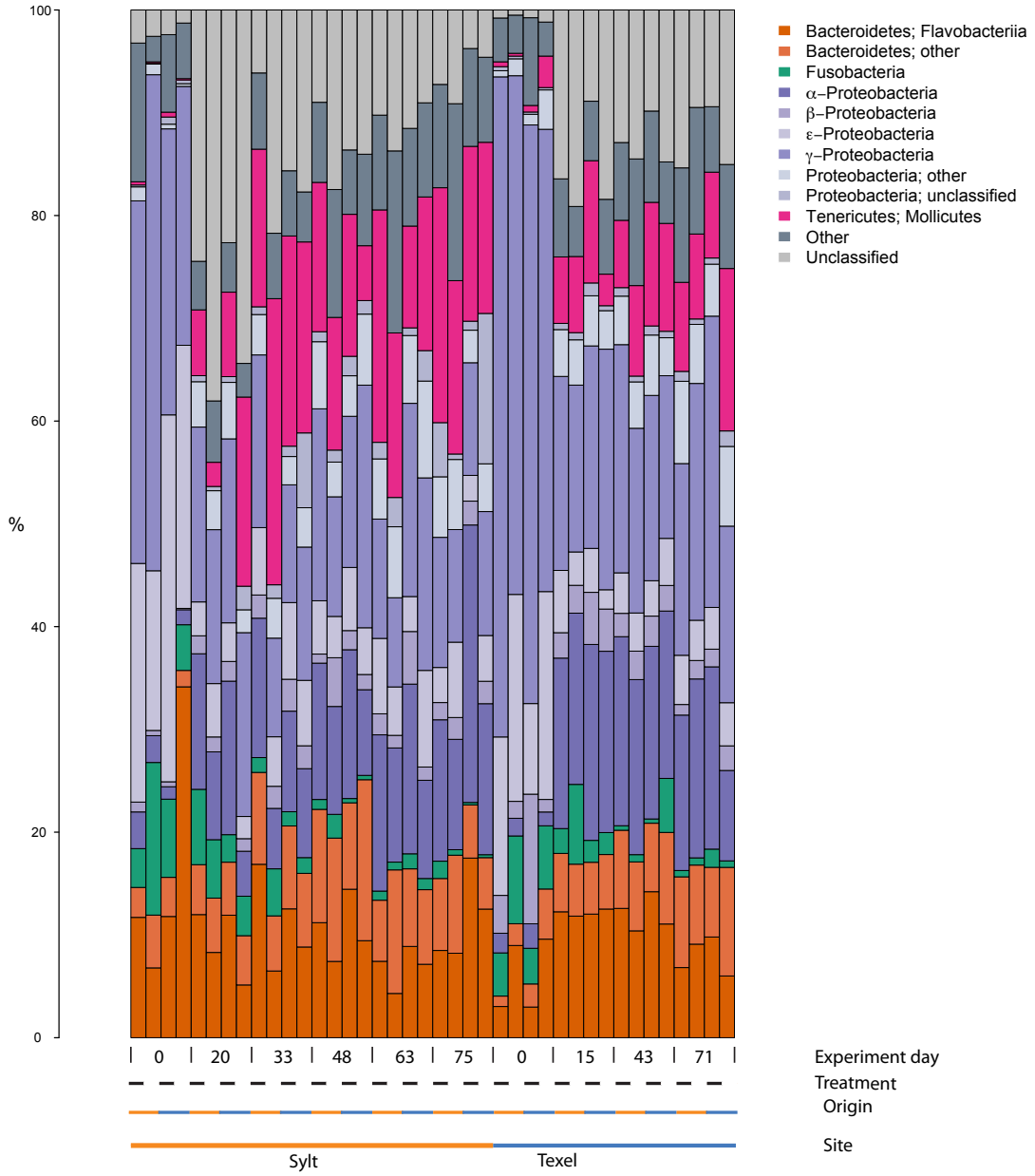


***Figure II-S3**. Class level composition of hemolymph microbiota on Sylt and Texel at different sampling points grouped according to oyster origin, antibiotic treatment and site.*

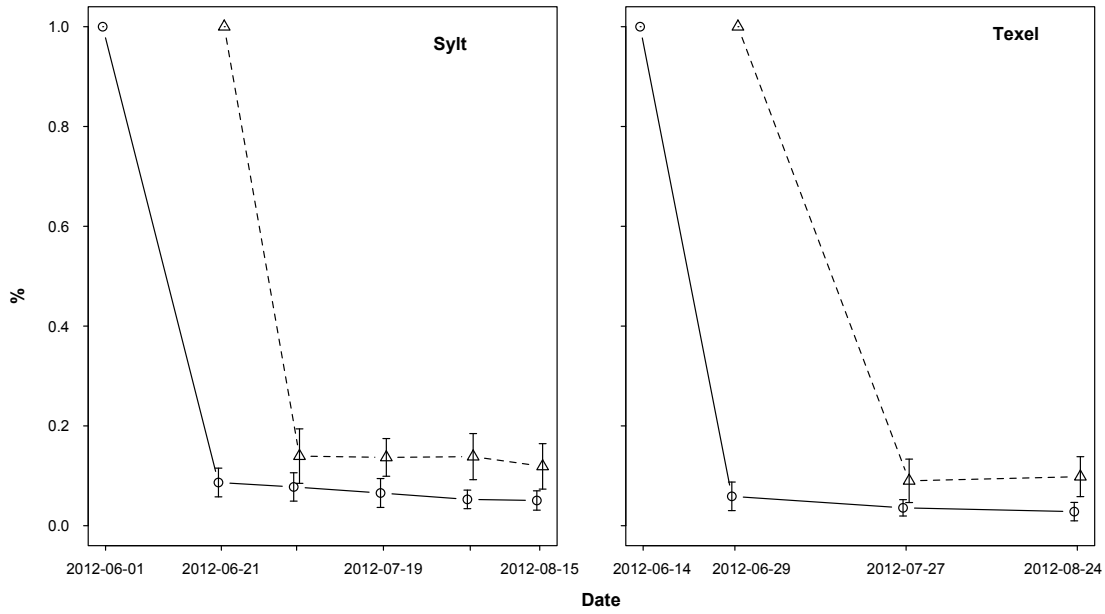


***Figure II-S4**. Bacterial turnover calculated as percentage of OTUs shared with the initial community*

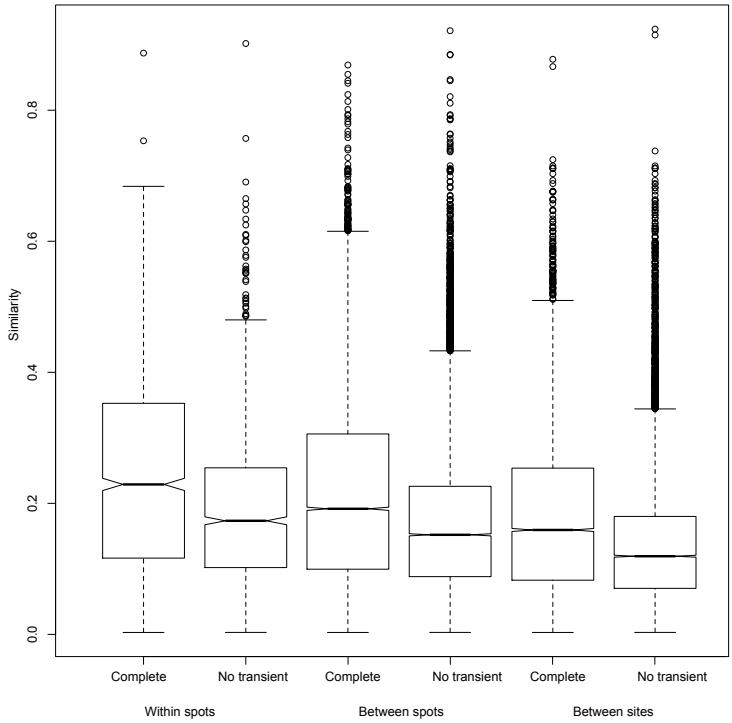


***Figure II-S5**. Average Bray-Curtis similarities at small, medium and large spatial scales based on all and only resident OTUs.*

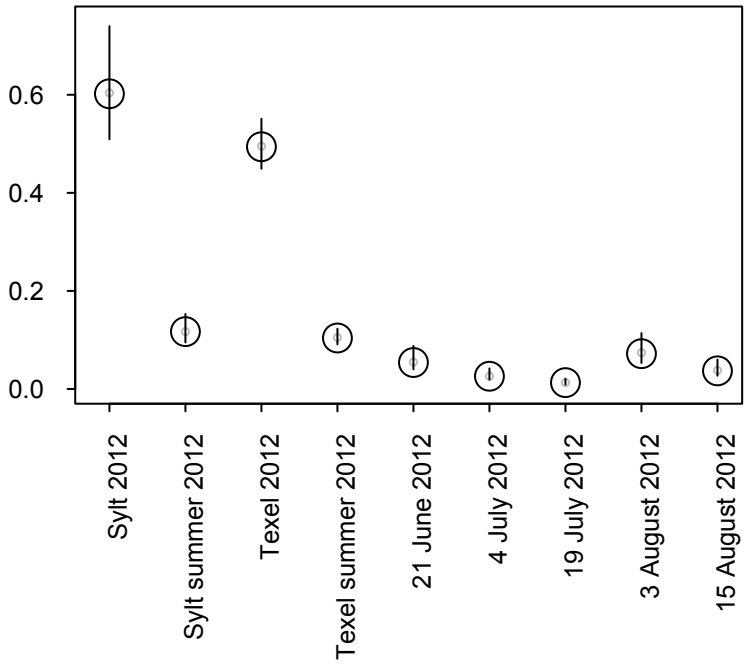


Figure II-S6. *Coefficient of variation of all-year temperature measurements, as well as during the summer only for Texel and Sylt. Last five points are coefficients of variations of measurements that I took during sampling at various spots. I sampled usually early morning before it got really warm.*

	Estimate	*2.5% CI*	*97.5% CI*	*Z*	*p*
Sylt-Texel	-0.28	-0.48	-0.08	-2.78	0.01
Antibiotic-Control	-0.45	-0.65	-0.25	-4.42	0.00
Linear trend	0.72	0.22	1.22	2.82	0.00
Quadratic trend	-1.17	-1.65	-0.68	-4.72	0.00
Cubic trend	-0.65	-1.14	-0.16	-2.61	0.01
samdate^4	0.20	-0.28	0.69	0.81	0.42
samdate^5	0.68	0.22	1.15	2.87	0.00
Sylt-Texel : Antibiotic-Treated	-0.08	-0.27	0.12	-0.75	0.45
Sylt-Texel : Linear trend	0.94	0.44	1.44	3.69	0.00
Sylt-Texel : Quadratic trend	-0.26	-0.75	0.22	-1.07	0.29
Sylt-Texel : Cubic trend	0.13	-0.37	0.62	0.50	0.62
Sylt-Texel : Quartic trend	0.09	-0.40	0.57	0.36	0.72
Sylt-Texel : Quintic trend	0.01	-0.46	0.47	0.03	0.98
Antibiotic-Control : Linear trend	1.29	0.79	1.79	5.06	0.00
Antibiotic-Control : Quadratic trend	-1.05	-1.54	-0.56	-4.24	0.00
Antibiotic-Control : Cubic trend	0.85	0.36	1.34	3.40	0.00
Antibiotic-Control : Quartic trend	-0.52	-1.01	-0.04	-2.11	0.03
Antibiotic-Control : Quintic trend	0.13	-0.34	0.59	0.54	0.59
Sylt-Texel : Antibiotic-Control : Linear trend	0.49	-0.01	0.99	1.93	0.05
Sylt-Texel : Antibiotic-Control : Quadratic trend	-0.36	-0.84	0.13	-1.45	0.15
Sylt-Texel : Antibiotic-Control : Cubic trend	0.19	-0.30	0.68	0.77	0.44
Sylt-Texel : Antibiotic-Control : Quartic trend	-0.27	-0.75	0.22	-1.07	0.28
Sylt-Texel : Antibiotic-Control : Quintic trend	0.05	-0.42	0.51	0.19	0.85

Table II-S1. *Negative binomial generalized model of Vibrionaceae CFU counts on Sylt (including pre-deployment samples). Loglk = -3582.45.*

CHAPTER III

Hemolymph microbiome of Pacific oysters in response to temperature, temperature stress and infection

Abstract

Microbiota provide their hosts with a range of beneficial services, including defense from external pathogens. However, host-associated microbial communities themselves can act as a source of opportunistic pathogens depending on the environment. Marine poikilotherms and their microbiota are strongly influenced by temperature, but experimental studies exploring how temperature affects the interactions between both parties are rare. In order to assess the effects of temperature, temperature stress and infection on diversity, composition and dynamics of the hemolymph microbiota of Pacific oysters (*Crassostrea gigas*), we conducted an experiment in a fully-crossed, three-factorial design, in which the temperature acclimated oysters (8°C or 22°C) were exposed to temperature stress and to experimental challenge with a virulent *Vibrio* spp. strain. We monitored oyster survival and repeatedly collected hemolymph of dead and alive animals in order to determine the microbiome composition by 16s rRNA gene amplicon pyrosequencing. We found that the microbial dynamics and composition of communities in healthy animals (including infection survivors) were significantly affected by temperature and temperature stress, but not by infection. The response was mediated by changes in the incidence and abundance of OTUs and accompanied by little change at higher taxonomic levels, indicating dynamic stability of the hemolymph microbiome. Dead and moribund oysters, on the contrary, displayed signs of community structure disruption, characterized by very low diversity and proliferation of few OTUs. We can therefore link short-term responses of host-associated microbial communities to abiotic and biotic factors and assess the potential feedback between microbiota dynamics and host survival during disease.

Introduction

Over the last couple of decades, it has become clear that microbiota are of vital importance for survival, homeostasis and development of animals (McFall-Ngai *et al.*, 2013). Tight relationships between hosts and their symbionts even inspired a hologenome theory of evolution (Rosenberg *et al.*, 2007), proposing a holobiont - a host together with the associated microorganisms - as the unit of selection. One prominent service that microbiota provide for their hosts, is protection from pathogens (Kamada *et al.*, 2013). However, in compromised hosts or under (un)favorable environmental conditions, the symbionts themselves can act as opportunistic pathogens (Olafsen *et al.*, 1993b; Garnier *et al.*, 2007; Cerf-Bensussan and Gaboriau-Routhiau, 2010) understand how the environmental factors and stress affect the composition and function of microbiota and the outcome of host-microbe interactions.

Tissues of healthy marine invertebrates usually harbor species-rich microbial communities (Prieur *et al.*, 1990; Gomez-Gil *et al.*, 1998; Romero *et al.*, 2002a; Gomez-Gil *et al.*, 2010; King *et al.*, 2012b; Trabal *et al.*, 2012; Wegner *et al.*, 2013). This also applies to the hemolymph (Olafsen *et al.*, 1993b; Garnier *et al.*, 2007; Wendling *et al.*, 2014) - the functional analogue of blood in vertebrates (Bachere *et al.*, 2004). The presence of viable bacteria in the hemolymph of healthy oysters can influence the outcome of pathogen infections by either stimulating immunity or by competitive exclusion (Schmitt *et al.*, 2012). Isolation of antimicrobial compounds of bacterial origin from oyster hemolymph has provided support for the latter hypothesis (Defer *et al.*, 2013). Yet oyster hemolymph microbiota have rarely been studied and so far only by means of culture-dependent methods (Olafsen *et al.*, 1993b; Garnier *et al.*, 2007; Wendling *et al.*, 2014). However, neither cultivation nor molecular fingerprinting methods provide realistic estimates of community diversity and composition (Pedrós-Alió, 2006; Bent and Forney, 2008). Next generation sequencing (NGS), although by no means free of biases (Fierer and Lennon, 2011; Sergeant *et al.*, 2012; Cai *et al.*, 2013) enables detailed characterization of microbial community composition and dynamics, including rare phylotypes (Huse *et al.*, 2008) that can act as a seed bank and mediate community response to environmental change (Caporaso *et al.*, 2012; Pedros-Alio, 2012; Sjostedt *et al.*, 2012). Recently, amplicon sequencing has been used to characterize oyster gut (King *et al.*, 2012b; Trabal Fernandez *et al.*, 2013) and gill microbiomes (Wegner *et al.*, 2013) , resulting in higher estimates of α–diversity and challenging the long-held view that the oyster microbiota were dominated mainly by pseudomonads and vibrios (Prieur *et al.*, 1990; Olafsen *et al.*, 1993b; Garnier *et al.*, 2007).

Temperature is an important factor in shaping microbial communities in marine abiotic and biotic habitats (e.g. (Fuhrman *et al.*, 2008)). Shifts from mutualist- to pathogen-dominated

communities due to global warming have already been reported (Ritchie, 2006), as well as an increase in occurrence of infectious diseases (Altizer *et al.*, 2013). At lower latitudes, warming usually implies crossing the upper limits of thermal tolerance and is almost certain to have adverse effects on the affected organisms (Lafferty *et al.*, 2004; Fan *et al.*, 2013). The outcome in temperate regions can be much less predictable, depending on thermal optima and ranges of hosts and pathogens (Thomas and Blanford, 2003). For example, temperatures over 20°C are necessary for oyster summer mortalities to occur (Samain *et al.*, 2007; Watermann *et al.*, 2008), but it is low temperatures (< 14°C) that promote development of brown ring disease in clams (Paillard *et al.*, 2004).

Many temperature-dependent disease outbreaks have been linked to various *Vibrio* species (Lacoste *et al.*, 2001; Lee *et al.*, 2001; Garnier *et al.*, 2007; Cervino *et al.*, 2008; Elston *et al.*, 2008) that are commonly isolated from healthy marine animals (Prieur *et al.*, 1990; Olafsen *et al.*, 1993a; Gomez-Gil *et al.*, 1998; Iida *et al.*, 2000; Vega Thurber *et al.*, 2009; Trabal *et al.*, 2012; Zhao *et al.*, 2012). The effects of temperature on *Vibrio* spp. virulence have also been demonstrated experimentally (Kushmaro *et al.*, 1998; Wendling and Wegner, 2013), making this group a suitable candidate for experimentally exploring temperature-dependent host-pathogen interactions in the marine environment.

So far, most of the research on microbial dynamics in marine poikilotherms has been observational and focused on seasonal changes (Beleneva and Zhukova, 2009; Preheim *et al.*, 2011; Zurel *et al.*, 2011; Erwin and López-Legentil…, 2012; Carlos *et al.*, 2013; Mahalaxmi *et al.*, 2013). However, seasonality does not equal temperature (Farcy *et al.*, 2007; Gilbert *et al.*, 2012) and experimental studies addressing the temperature-dependent short-term microbial dynamics, which may be highly relevant to development of disease, are mostly confined to corals and sponges (Vega Thurber *et al.*, 2009; Simister *et al.*, 2012; Fan *et al.*, 2013).

Although higher temperatures usually promote microbial growth including pathogens, acclimated eurythermic hosts may be well prepared to deal with them. *In vitro* experiments on oyster hemocytes revealed little change in immunocompetence over wide range of environmentally relevant temperatures (Ashton-Alcox and Ford, 1998; Gagnaire *et al.*, 2006). Temperature stress, on the other hand, severely compromised host defenses (Malham *et al.*, 2009) illustrating the need to examine temperature effects in broader context of animal condition and history. Mortalities observed in wild populations of marine poikilotherms are often due to complex interplay of multiple stressors, such as in the well documented case of oyster summer mortalities, e.g. (Samain, 2011; Wendling and Wegner, 2013) or *Vibrio harveyi*

infection in abalones (Travers *et al.*, 2008). Controlled experiments are therefore the only way to assess the importance and relative contributions of individual factors.

In order to examine how infection, temperature and temperature stress affect oyster survival and the composition and dynamics of hemolymph microbiota, we experimentally challenged Pacific oysters with a virulent *Vibrio* spp. strain and exposed them to different temperature treatments. We thus present experimental data describing the short-term microbial dynamics in response to abiotic and biotic stress in Pacific oysters. With the combination of the above experimental factors and a high temporal resolution of microbial community dynamics, we can now try to link changes in microbial communities to different stressors and host survival.

Material and methods

Biological Material

All oysters were collected in a Wadden Sea tidal flat in Königshafen, Germany (55° 1' 44" N, 8° 26' 3" E) and subsequently kept in flow-through aquaria in climate chambers set to either 22°C (warm-acclimated) or 8°C (cold-acclimated). To avoid temperature shock during the transfer to the lab, the warm-acclimated oysters were collected in late august 2010, while the cold-acclimated oysters of matching size were collected ten days prior to the experiment (beginning of November 2010). Warm-acclimated oysters were fed three times a week using shellfish diet instant algae mix (Varicon Aqua, UK).

For infection, we used the isolate *Vibrio* sp. D29w affiliated to *Vibrio orientalis/tubiashii* clade based on MLST sequencing (Thieltges *et al.*, 2013), which was obtained from hemolymph of the local oyster population. This isolate was shown to induce intermediate levels of mortality upon injection in adults at ambient temperatures (Thieltges *et al.*, 2013), but was highly virulent for larvae at elevated temperatures (Wendling *et al.*, 2014). We cultured bacteria overnight in 8 ml of soya-peptone medium at 25°C and shaking at 240 rpm. Bacterial cells were then collected by centrifugation for 10 min at 5000 rpm, resuspended in fresh soya-peptone medium and adjusted to the concentration of 2 x 10^8 cells/ml.

Experimental design and hemolymph sampling

The experiment was designed to examine the effects of temperature, temperature stress and infection in a fully crossed 3-factorial design for duration of 7 days. A total of 48 oysters (24 animals from each acclimation group) were kept singly in aerated 2.5 l glass jars with no flow-

through, after being randomly assigned to experimental treatments. The oysters were either left at their acclimation temperature or stressed by non-abrupt transfer (together with seawater, allowing for gradual temp. equalization) to the opposite temperature, resulting in four temperature treatments: cold-acclimated (CC), warm-acclimated (WW), cold-stressed (WC) and warm-stressed (CW). Half of the oysters in each of the four groups were injected either with *Vibrio* sp. D29w strain or with the pure soya-peptone medium. Injections of 500 µl (~10^8 bacteria) were applied into the adductor muscle with 23x1/4 gauge (dm 0.60 x 30 mm) needle introduced via a notch drilled on a ventral shell side. During the experiment, the oysters were kept individually in fully-aerated 2.5 jars filled with the filtered seawater.

In order to examine the composition and dynamics of hemolymph microbiota, 200 µl of hemolymph were drawn from the adductor muscle just before the experiment and on the third, fifth and seventh day of the experiment. Samples were immediately stored on ice and transferred to -80°C as soon as possible. The oysters were checked for survival during hemolymph collection. In order to have a proxy for the abundance of vibrios, four µl of hemolymph were streaked on vibrio-selective thiosulfate-citrate-bile-salts-sucrose (TCBS) agar immediately after sampling, the plates were grown at 25°C, and colony forming units (CFUs) were counted after 24 h cultivation.

DNA extraction and amplicon sequencing

DNA was extracted from 174 whole hemolymph samples (Table III-1) with the Illustra TriplePrep kit (GE Healthcare Life Sciences, Hamburg, Germany) according to the manufacturer's protocol. DNA concentration and purity were checked with a Nanodrop ND-1000 spectrometer (peqlab, Erlangen, Germany) and all samples were adjusted to equal DNA concentration (5 ng/µl).

Ribosomal 16S rDNA V1-V2 region was amplified with a barcoded universal bacterial 27F (CTATGCGCCTTGCCAGCCCGCTCAG-MID-TCAGAGTTTGATCMTGGCTCAG)-338R (CGTATCGCCTCCCTCGCGCCATCAG-MID-TGCTGCCTCCCGTAGGAGT) primer pair. Forward primers were marked with barcodes MID02 and MID03 and the reverse primers with MID01-MID98 (excluding MID09 and MID12). Each individual PCR reaction including negative controls was coded by a unique combination of forward and reverse MIDs (Binladen et al, 2007; Wegner, 2009). Twenty µl PCR reactions contained 1.5 mM $MgCl_2$, 0.2 mM of each nucleotide, 0.2 µm of each primer, 0.5 unit of Taq polymerase (GoTaq Flexi DNAp, Promega, Mannheim, Germany) and 20 ng of DNA template. DNA was amplified using the following

protocol: 1 min initial denaturation at 94°C, 30 cycles of 40 s at 94°C, 30 s at 55°C, 30 s at 72°C and final extension 2 min at 72°C.

Treatment[1]	*Sampling point (day)*				*Oysters per treatment*	*Samples per treatment*
	0	*3*	*5*	*7*		
CC_0	6/6	6/6	6/6	6/6	6	24/24
CC_I	6/6	6/6[2]	6/6[2,3]	4/4[3]	6	22/22[2,3]
CW_0	6/6	6/6	6/6	6/6	6	24/24
CW_I	6/6	6/6[3]	1/1	1/1	6	14/14[3]
WC_0	6/6	5/6	5/6	6/6	6	22/24
WC_I	6/6	6/6	5/6[2]	6/6[3]	6	23/24[2,3]
WW_0	6/6	6/6[3]	3/5	4/5	6	19/22[3]
WW_I	6/6	6/6	4/4[2]	4/4[3]	6	20/20[2,3]
Total	48/48	47/48[2,3]	36/40[2,3]	37/38[3]	48	168/174[2,3]

[1] 1st letter denotes acclimation temp., 2nd experimental temp., followed by infection status

[2] Moribund oysters included. Total moribund samples: 5/6

[3] Dead oysters included. Total dead samples: 14/14

***Table III-1**. Number of oysters and hemolymph samples (analyzed/collected) per treatment and sampling point.*

Quality of PCR products was checked on the QIAxcel system using a QIAxcel DNA Screening kit (Qiagen, Hilden, Germany). Equal amounts (10 µl) of each product were then pooled together and cleaned by ethanol precipitation. Pooled samples were finally adjusted to 150 ng/µl and sequenced at Roche GS-FLX 454 platform at the Institute of clinical molecular biology at the Christian-Albrechts-University Kiel, Germany.

Processing of raw sequence data

Raw reads were demultiplexed using modified Python scripts from the cogent package (Knight *et al.*, 2007). In short, original binary file (sff) was split into multiple sff files corresponding to individual samples allowing for only perfect matches to both barcodes and primers and translated into sfftxt-files using Mothur (Schloss *et al.*, 2009). Quality control included denoising and chimera removal and was performed using the AmpliconNoise V1.23 pipeline (Quince *et al.*, 2011). Only flowgrams with minimum length of 200 bp before the first noise signal were kept for further analysis. Initial cutoff value for removing sequencing noise was 0.01 and cluster size 60. No ambiguities were allowed and maximum homopolymer length was set to 7. PCR noise removal cutoff value was 0.08 with the cluster size of 30. Chimeras were identified with Perseus and sequences with probability higher than 50% of being chimeras were excluded from further analysis. A custom perl script was then used in order to trim primer sequences and create an input fasta file for further analysis.

Raw demultiplexed sequence data are available at European Nucleotide Archive (ENA) under the study accession number PRJEB5702 (sample accession numbers ERR457899 - ERR458074).

We used the QIIME pipeline (Caporaso *et al.*, 2010) to create OTU (operational taxonomic unit) tables and perform rarefaction, taxonomical composition assessment and phylogenetic diversity analyses. OTUs were picked with uclust (Edgar, 2010) at a 97% similarity threshold. Taxonomy was assigned with RDP classifier (Wang *et al.*, 2007) to the genus level, with 60% confidence using the 110 Greengenes taxonomy 12_10 (McDonald *et al.*, 2012) as a reference database. The sequences assigned to the genus *Vibrio* were then compared to 16S rDNA sequences obtained from cultured strains isolated from hemolymph of local oysters (courtesy of C. Wendling) in order to identify OTU corresponding to the injected strain. We defined contaminants as OTUs with more than 5 reads in negative controls and removed them from further analysis. This threshold was chosen because negative control samples represented pools of several individual reactions and most sequences were represented with a single read. Sequences were aligned with mafft (Katoh *et al.*, 2002) and a phylogenetic tree was built using fasttree (Price *et al.*, 2010). We calculated rarefaction curves for Shannon, evenness, species richness and PD (phylogenetic diversity, (Faith, 1992)) in order to assess the effects of sample sizes on these α-diversity metrics.

Statistical analyses

All other statistical analyses were performed in R (R Core Team, 2013). Host survival analysis was conducted using the survival package (Therneau, 2013). For bacterial communities, we analyzed relative species abundance, α and β-diversity with the Vegan package (Oksanen *et al.*, 2013). Differences in α–diversity patterns between treatments were analyzed using non-parametric tests and linear mixed models (nlme package, (Pinheiro *et al.*, 2013)). In order to assess β-diversity, we calculated Bray-Curtis and weighted UniFrac distances (Hamady *et al.*, 2010) between the samples and analyzed them by non-metric multidimensional scaling (NMDS) and the *Adonis* implementation of Permanova (nonparametric permutational multivariate analysis of variance (Anderson, 2001)). Results are reported for Bray-Curtis distances if not explicitly stated otherwise. We also determined how abundant or dominant (≥1% of sample) community members contributed to explaining the variation between the treatments. In order to statistically examine taxonomical composition and identify phylotypes

associated with individual treatments and conditions, we applied indicator species approach implemented in indicspecies package (Cáceres and Legendre, 2009).

Results

Oyster survival upon infection

Experimental challenge with *Vibrio* sp. D29w resulted in 54% mortality of infected oysters, as opposed to a single death event in the control treatment ($\chi^2(1) = 14.52$, $p<0.001$, odds = 25.33 (3.10, 1195.27)). Survival analysis of infected oysters revealed significant differences between warm-stressed animals and all the other groups (Peto & Peto modification of the Gehan-Wilcoxon test, $\chi^2(3) = 9$, $p = 0.029$). Not only did the warm-stressed oysters experience the highest mortality, but they also died earlier, within the first three days of the experiment (Figure III-1).

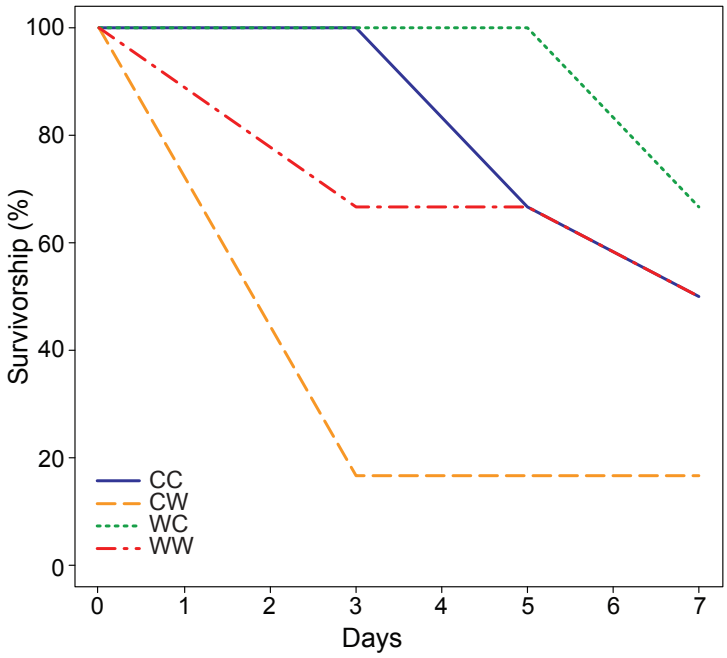


Figure III-1. *Survival curves for infected oysters. CC = cold acclimated, CW = warm stressed, WC = cold-stressed, WW = warm-acclimated.*

Hemolymph microbiome: general characteristics

Results of sequence data processing are shown in Table III-S1. Singletons (OTUs represented with a single read in the dataset) and the samples with fewer than 100 reads were excluded from

further analyses. It is noteworthy that 4 out of 6 low-coverage libraries came from two uninfected oysters (Table III-1), suggesting that low number of reads in these cases may reflect true absence of bacteria.

Phyla Proteobacteria and Bacteroidetes encompassed the bulk of OTU diversity and abundance (Figure III-2a, Figure III-2b, Figure III-S1). Relative OTU abundances fitted well to Fisher's log-series distribution, both in the dataset as a whole and in the individual samples (Kolmogorov-Smirnoff test, D = 0.000 - 0.196, p>0.881, details not shown), indicating that few OTUs accounted for the majority of reads. The amount of variability in community composition explained by treatments was higher when only abundant OTUs (≥1%), and not the complete dataset, were considered (Figure III-S2, Figure III-S3b).

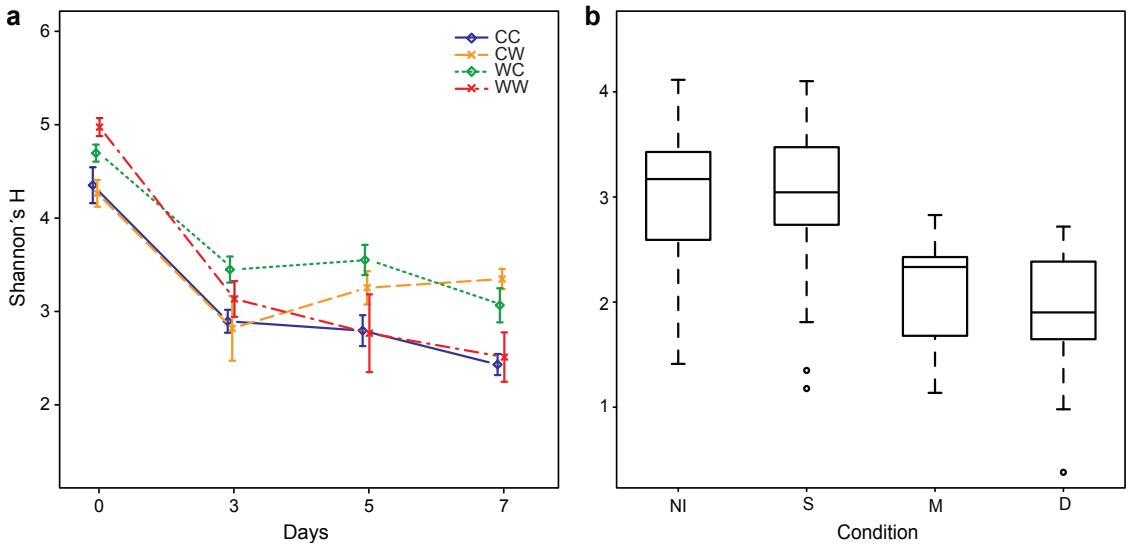


Figure III-2. *α-diversity, expressed as Shannon's H in response to: temperature treatments over time (a), oyster condition (b). Infection is not shown for clarity. Error bars represent standard error of mean. CC = cold acclimated, CW = warm stressed, WC = cold-stressed, WW = warm-acclimated, NI = non-infected, S = survivors, M = moribund, D = dead.*

Rarefaction curves of α-diversity metrics show that Shannon's H and evenness - the indices we based our conclusions upon - were relatively stable even at the sampling depths of <100 reads. Furthermore, the relative differences between the treatments were fairly constant over a large range of sampling depths (Figure III-S4). To assure negligible effects of sampling effort on α- and β-diversity analyses, we generated 10 random subsets of 100 reads per sample and calculated correlations between the α-diversity metrics (Spearman) and NMDS ordinations (Procrustes) based on subsets and the original dataset. High correlation values indicated that

our results were not influenced by differences in sampling depths of individual libraries (Table III-S2, Table III-S3). The main analysis was thus based on the complete dataset (excluding singletons) in order to keep the estimates of α-diversity realistic and to avoid information loss.

The transfer from the flow-through system to the experimental conditions was followed by significant drop in OTU diversity of alive oysters (39% OTUs were found only on day 0, Figure III-3a) contributing to a clear distinction between pre-experimental and experimental communities (Figure III-S3a). Loss of rare OTUs (mean relative abundance of OTUs present only on day 0 was 0.29%, quartiles: 0.14%, 0.36%) and increase in dominance were reflected in substantially lower Shannon's H (median: day 0 = 4.545, days 3-7 = 3.059, $W = 4931$, $p < 0.001$, $r = -0.718$) and evenness (median: day 0 = 0.899, days 3-7 = 0.675, $W = 4836$, $p < 0.001$, $r = -0.720$), regardless of treatment (Figure III-3a, Figure III-S5). Since pre-experimental communities could not be meaningfully assigned to the tested groups, they were omitted from the analyses concerning the effects of experimental treatments and oyster condition.

Hemolymph microbiome: effects of infection

In order to describe effects of infection on hemolymph microbiota, we grouped the infected oysters into three categories: surviving (S, alive at the next sampling point), moribund (M, dead at the following sampling point) and dead (D). We refer to control and surviving oysters together as healthy (H).

Experimental challenge barely affected α– (Table III-2) and β-diversity (Table III-3). Microbiomes from healthy oysters (H) formed largely overlapping clusters (Figure III-4b), reflecting a comparatively small but significant effect of infection on β-diversity (Table III-3). In contrast, moribund (M) and dead (D) oysters formed separate groups from healthy oysters in the NMDS plot (Figure III-4a). M and D communities were characterized by proliferation of one or very few OTUs and therefore displayed very low α-diversity (Figure III-3b). At 8°C, the microbiomes of dead oysters (D) closely resembled those of moribund animals (M, Figure III-2c), whereas we observed more variation in community composition and an increase in anaerobic bacteria soon after death at 22°C.

In contrast to our expectation, the microbiomes of moribund oysters were dominated by the genus *Arcobacter* and not, as expected, by *Vibrio* spp. (Figure III-2c, Supp III-Ind). Overall, the infected oysters harbored more strains from other genera with described pathogenic species, such as *Photobacterium* and *Shewanella* (Supp III-Ind).

	Factors	*DF*	*F*[1]	*Significant contrasts*	*Estimate*	*SE*	*2.5% CI*	*97.5% CI*
Main effects	Acclimation temp.	1, 43	1.776					
	Experimental temp.	1, 43	2.547					
	Time	2, 62	0.735					
	Health status	2, 62	13.569***	Moribund & dead VS healthy	0.298	0.06	0.177	0.418
	Infection	1, 43	0.735					
Interaction terms	A. temp. x E. temp.	1, 43	14.280***	Stressed VS acclimated	-0.421	0.111	-0.646	-0.196
	A. temp. x Time	2, 62	1.407					
	E. temp. x Time	2, 62	4.954*	E. temp. on (day 3 VS days 5-7)	0.148	0.053	0.042	0.253
	A. temp. x E. temp. x Time	2, 62	3.361*	(Stress. VS acc.) on day 3 VS on days 5-7	-0.178	0.071	-0.321	-0.035
Random intercept	Oyster				0.113		0.005	2.671

[1]significance levels: * ≤ 0.05, ** < 0.01, *** < 0.001

***Table III-2**. Linear mixed model for treatment and condition effects on Shannon's H, day 0 samples excluded (AIC = 275.599, BIC = 320.717, logLik (df = 17) = -120.800).*

The indicator species analysis revealed that higher *Vibrio* sp. D29w abundances were associated with infection (IndVal = 0.769, p = 0.017), but not directly with mortality or disease (Supp III-Ind). Overall, the OTUs assigned to *Vibrionaceae* resp. genus *Vibrio* (including the injected strain) were common (present in 98.85%, resp. 92.26% of the samples), but not very abundant (5.1%, resp. 1.6% mean relative abundance). Presence of vibrios (other *Vibrionales* occasionally grow on TCBS agar, hence the taxonomically non-specific term) was lower (87.36%) when calculated from CFU counts. The discrepancy was most likely due to the low abundance coupled with the low volume of hemolymph plated. Unlike relative read abundances, higher CFU counts were related to disease and death (GLMM: Z =-2.61, p = 0.009, (moribund & dead) vs. healthy parameter estimate: -0.249 ± 0.095, CI = (-0.436, -0.062), Table III-S4).

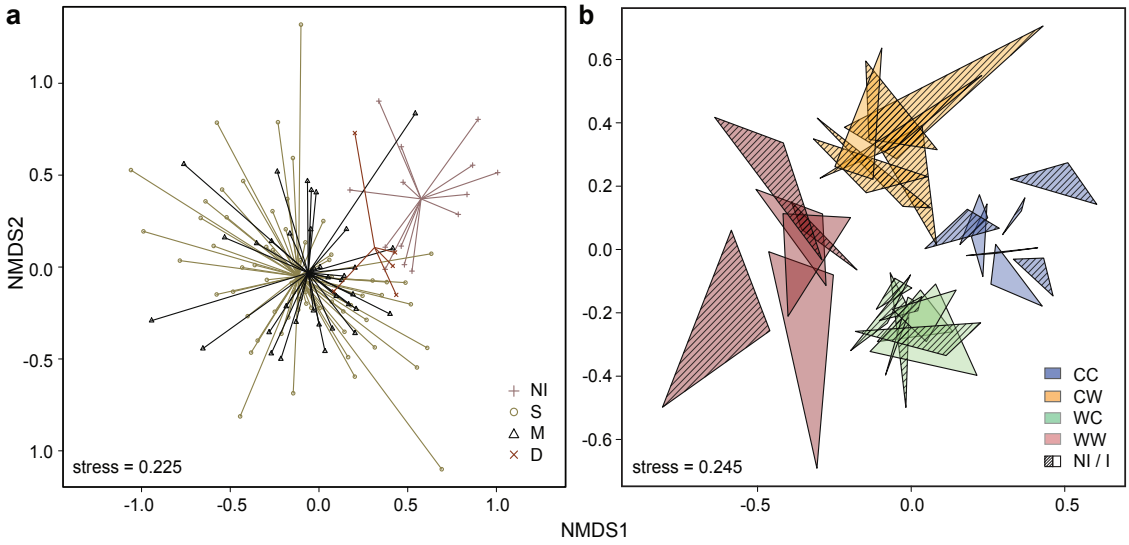


Figure III-3. *NMDS plots of Bray-Curtis distances between the microbial communities (day 0 excluded): according to oyster condition (a), showing temporal stability and effects of treatments (b). Triangle vertices represent samples, each triangle represents an oyster. CC = cold acclimated, CW = warm stressed, WC = cold-stressed, WW = warm-acclimated, NI = non-infected, I = infected, S = survivors, M = moribund, D = dead.*

		df	*SS*	*MS*	*pseudoF*	*R2*[1,2]
Main effects	Acclimation temp.	1	3.308	3.308	12.152	0.076***
	Experimental temp.	1	4.062	4.062	14.923	0.093***
	Time	2	1.435	0.717	2.636	0.033***
	Infection	1	0.853	0.853	3.133	0.020***
	Health status	2	1.769	0.885	3.25	0.041***
Interaction terms	A. temp. x E. temp.	1	1.154	1.154	4.239	0.026***
	A. temp. x Time	2	0.729	0.365	1.34	0.017**
	E. temp. x Time	2	1.034	0.517	1.899	0.024***
	A. temp. x E. temp. x Time	2	0.672	0.336	1.235	0.015**
	Residuals	105	28.579	0.272		0.656
	Total	119	43.594			1

[1]Significance values based on 999 permutations
[2]significance levels: * ≤ 0.05, ** < 0.01, *** < 0.001

Table III-3. *Adonis (Permanova) results for experimental communities (day 0 excluded) based on Bray-Curtis distances.*

Hemolymph microbiome: effects of temperature and temperature stress

Prior to the experiment, Shannon's H (median: 8°C = 4.228, 22°C = 4.711, W = 117, p<0.001, r = -0.525) and PD indices (median: 8°C = 19.697, 22°C = 28.216, W = 81, p<0.001, r = -0.652) were higher in warm-acclimated communities, with no difference in evenness between the groups (median: 8°C = 0.653, 22°C = 0.717, W = 214, p<0.128). During the experiment, only the

phylogenetic component (PD) of α-diversity remained positively correlated with higher ambient temperature (Figure III-S5, Table III-S, Table III-S6), indicating the presence of more rare, divergent phylotypes in warmer environment.

Both directions of temperature stress prevented a steady decrease of diversity and evenness that we observed in the acclimated communities (Figure III-3a, Table III-2, Figure III-S5). The effect became more pronounced towards the end of the experiment, indicating its persistence and reflecting the gradual and lagged microbiome response to stress. We expected that the OTUs present or dominating the acclimated communities were already established in hemolymph prior to the experiment, thus getting a head start; and that, in contrast, the temperature stress would facilitate colonization by new species and promote growth of rare or dormant OTUs. However, we found little support for this hypothesis, as the proportion of the OTUs that were dominant in experimental communities and already present at the beginning did not significantly differ between the warm-acclimated and stressed oysters, although it was higher for cold-acclimated microbiomes (Table III-S7).

Acclimation and experimental temperature, as well as their interaction, considerably affected the composition of hemolymph microbiota throughout the experiment (Table III-3). Ordination by NMDS clearly separated the communities by temperature treatments (Figure III-4b). Ordination and Permanova based on weighted UniFrac showed that little of phylogenetic β-diversity was attributable to the treatments (Figure III-S2, Figure III-S3), reflecting similarity of the microbiome composition at above-OTU taxonomic levels. However, we found some indication for increase in potentially pathogenic genera in stressed oysters and at 22°C in general (Supp III-Ind).

In order to estimate how experimental treatments affected microbial dynamics, we used the area of polygons connecting all samples from a single oyster in the NMDS plot as a proxy for temporal stability: the smaller the area, the more stable the community. ANOVA confirmed the visual impression (Figure III-4b) that microbiomes in the cold environment were more stable compared to microbiomes from the warm environment ($F_{1,26}$ = 17.86, $p<0.001$). Stress and infection, on the other hand had no significant effect on temporal microbiome dynamics ($F_{1,26}$ = 0.157, p = 0.695, $F_{1,26}$ = 0.89, p = 0.367).

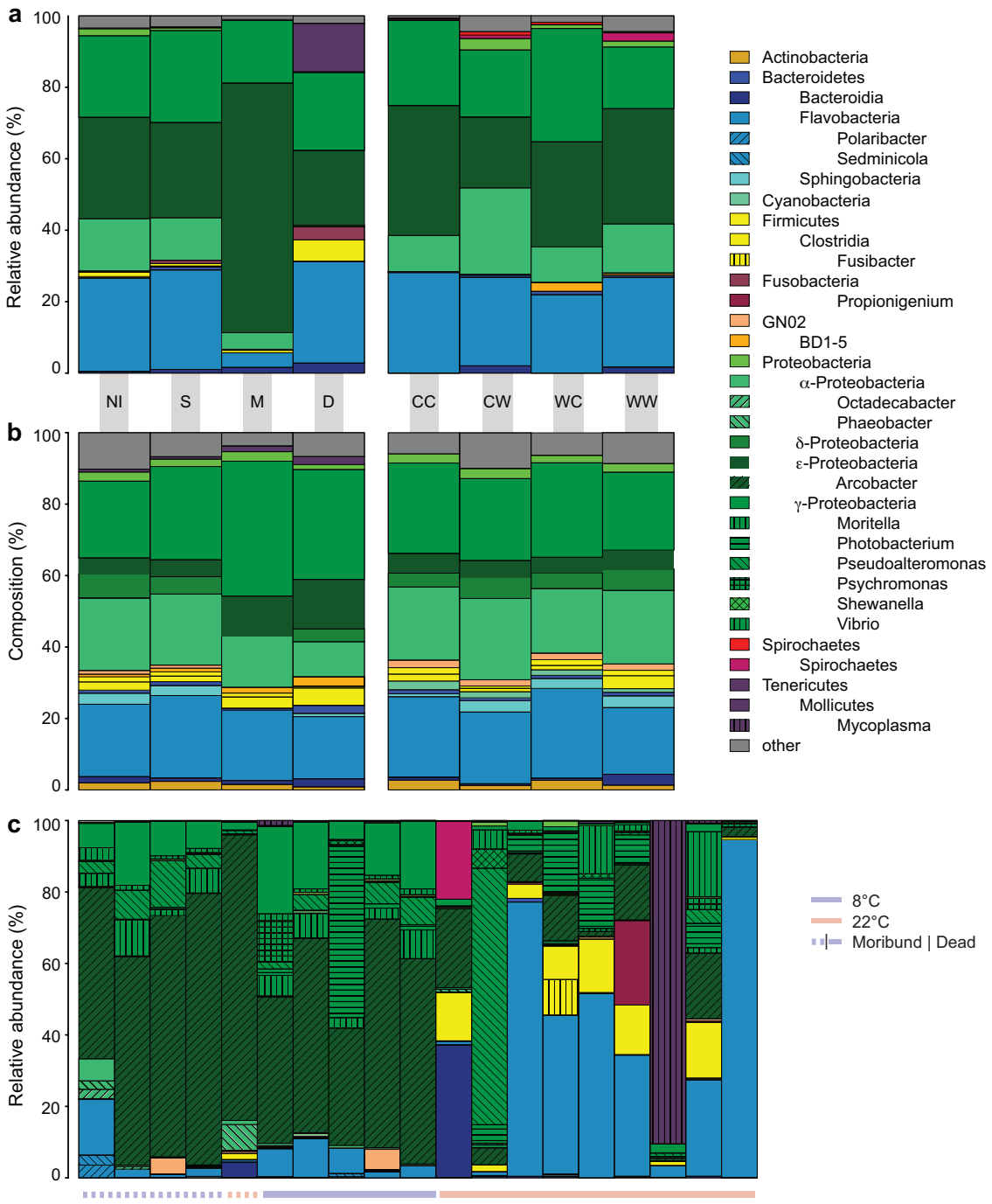


Figure III-4. *Relative abundances (a, c) and taxonomical composition (b) of hemolymph microbiota in response to oyster condition and temperature treatments at the class level (a,b) and in individual moribund and dead oysters at the genus level (c). Dashed line denotes moribund, full line the dead oysters. Blue denotes experimental temperature of 8°C, red 22°C.*

Discussion

Host-pathogen interactions play an important role in population dynamics and evolution of organisms. Although microbes inherently differ in their pathogenic potential, a disease usually arises from a complex interplay of multiple factors. Here, we show that temperature represents a notable determinant of microbial dynamics in oyster hemolymph, and propose that the lower temporal stability of microbiomes at higher temperatures may have contributed to the higher mortality of the heat-stressed hosts upon infection. We also show that a decrease in diversity and proliferation of opportunistic pathogens precede death, thus representing a good indicator of declining health.

Infection, microbiota and oyster health

Higher mortality at 22°C can be partially attributed to faster growth of the injected strain (Figure III-S6) and/or to temperature-dependent increase in expression of virulence factors (Kimes *et al.*, 2012). The effect of absolute temperature, however, cannot account for the difference in mortality between stressed and acclimated oysters. (Wang *et al.*, 2012) reported synergic effects of heat stress and infection on scallop survival and attributed it to energetic stress. A similar mechanism may have played a role for the oyster mortality, because only heat-, but not cold stress has been shown to increase energy consumption in Pacific oysters (Bougrier *et al.*, 1995; Malham *et al.*, 2009).

Injection of the virulent *Vibrio* sp. strain clearly caused mortality, but was also associated with an increased number of cultivable vibrios in the oyster hemolymph. In contrast, we could not directly link incidence or abundance of the injected strain in the sequenced libraries to disease (Supp III-Ind). For once, we cannot discriminate between sequences from active and inactive cells that would not cause disease (Williams *et al.*, 2009). Moreover, *Vibrio* spp. can have significant influence on the host health, despite the low relative abundance (Vega Thurber *et al.*, 2009). Still, low abundance of the injected strain suggests that other vibrios could have contributed to the mortalities, as the exogenous bacteria can be cleared quickly from the hemolymph (Parisi *et al.*, 2008), while stimulating growth of inactive residents (Froelich and Oliver, 2013).

The abundance of *Arcobacter* spp. in moribund oysters also suggests a pronounced role of the indirect effects for the mortalities (Hauton *et al.*, 1997). *Arcobacter* spp. is often found in association with marine organisms - ranging from bottle-nosed dolphins (Lima *et al.*, 2012), marine seaweeds (Hollants *et al.*, 2011), crabs (Givens *et al.*, 2013), mussels (Collado *et al.*,

2009), abalones (Tanaka *et al.*, 2004) and oysters (Romero *et al.*, 2002b). The ε-Proteobacteria are usually rare in coastal seawater and sediments (Campbell *et al.*, 2011; Gobet *et al.*, 2012), and they were rare in recent studies of oyster stomach (King *et al.*, 2012a), gut (Trabal Fernandez *et al.*, 2013) and gill microbiota (Wegner *et al.*, 2013). Thus the high abundance of *Arcobacter* spp. here could mean that these strains represent hemolymph-specific symbionts, which were not investigated in previous studies of oyster microbiota. *Arcobacter* spp. are often microaerophilic (Vandamme and Deley, 1991) and growth in the oysters could be facilitated by periodical valve closing (Sow *et al.*, 2011) or high variation in respiratory time activity (Bougrier *et al.*, 1998) - both of which may promote microaerophilic conditions. Dominance of *Arcobacter* spp. in moribund oysters, on the other hand, might have been a consequence of increased hypoxia due to disease-induced reduction of filtration activity (Mchenery and Birkbeck, 1986). Nevertheless, the high abundance of *Arcobacter* spp. strains in moribund oysters, starved abalones (Tanaka *et al.*, 2004) and necrotic sponges (Fan *et al.*, 2013) may suggest their potential as opportunistic pathogens when occurring in high enough densities (Olson *et al.*, 2014). Dominance of *Arcobacter* spp. in unhealthy animals resulted in low microbial diversity, in contrast to the diverse microbiomes of infection survivors, which were barely distinguishable from controls (Figure III-3b, Figure III-4b). This might reflect the crossing of a resilience threshold that a healthy community has against disturbance (Lozupone *et al.*, 2012). We cannot directly relate microbiome stability to disease resistance, but low diversity has repeatedly been found to coincide with impaired health in various animals (Garnier *et al.*, 2007; Chang *et al.*, 2008; Green and Barnes, 2010).

Microbial community dynamics in relation to infection and temperature stress

Warm temperatures can lead to higher heterogeneity in microbiome composition (Erwin *et al.*, 2012; Boutin *et al.*, 2013) and stress can favor shift towards more pathogen-dominated communities (Boutin *et al.*, 2013). Thus the lower community stability at 22°C (Figure III-4b) and temperature- or stress-related increase in potentially pathogenic bacteria (Supp III-Ind) could have contributed to the observed mortality pattern. However, microbiomes often remain stable and pathogen-free even in stressful conditions (Erwin *et al.*, 2012; Pita *et al.*, 2013; Wegner *et al.*, 2013), indicating their potential role in host acclimation (Rosenberg *et al.*, 2007). Phylogenetic similarity of the microbiomes suggests a common origin from the local seawater microbiota (Lozupone *et al.*, 2007). The drop in diversity following the transfer to the non-flow-through conditions was associated with a loss of rare and probably transient OTUs, underlining the importance of immigration for the assembly of the hemolymph microbiome. However, the

persistence of relatively rare, presumably resident bacteria - such as *Vibrio* spp. (Pruzzo *et al.*, 2005) - contrasts the loss of transient OTUs (Romero *et al.*, 2002b) and indicates that the hemolymph microbiome is not a simple result of filter-feeding lifestyle. While the individual stability indicates the importance of host genotype for the community assembly (Wegner *et al.*, 2013), the fine-scale differences between the treatments also illustrate acclimation potential of the hemolmyph microbiome. Multiple competing ecotypes usually coexist in bacterial populations (Cohan and Koeppel, 2008) and even the isolates with virtually identical 16s rRNA sequences are sometimes adapted to very different conditions (Cohan and Koeppel, 2008; Hall *et al.*, 2010), We cannot determine bacterial activity (Campbell *et al.*, 2011) nor function (Salles *et al.*, 2012) based on 16s rDNA sequences; moreover, our coverage is insufficient to capture very rare bacteria and thus estimate the full potential for community acclimation (Sjostedt *et al.*, 2012). Nevertheless, relatively high persistence of bacterial residents and the adjustments in fine-scale community composition following the environmental change could represent the microbiome's way to buffer the impact of environmental stress. Shifts in composition of the endogenous OTUs can thus prevent growth of external pathogens (Sjostedt *et al.*, 2012) (Froelich and Oliver, 2013) and contribute to the maintenance of homeostasis.

The microbiome of warm-acclimated oysters might have been influenced by their extended time to acclimate to laboratory conditions. This bias would mainly influence day 0 samples, where we cannot discriminate between effects of temperature or acclimation. Since we can assume that cold acclimated oysters recovered from handling stress at the start of the experiment (Thompson *et al.*, 2012), we mainly focus on those results where oysters could uneqivocally be assigned to experimental treatments (day 3 -7) for our interpretation to avoid any such bias. During those stages of the experiment, the quick shift of communities in response to experimental conditions, the strong effect fo experimental temperature (Suplementary Figure III-2 and 3) and the similarity of microbiomes from surviving oysters in response to infection indicate that oyster history did not largely affect our results throughout the experiment.

Our detailed insight into short-term microbial dynamics of Pacific oyster hemolymph microbiota in response to environmental conditions and infection shows that temperature is indeed a master switch determining community structure and dynamics of oyster associated microbiota. Microbial communities responded quickly to environmental change, but remained relatively stable within individuals for the duration of the experiment. Community disturbance by heat stress, coupled with host stress and faster bacterial growth at 22°C may have acted in concert to cause high mortality rates. Stress can also amplify the direct immediate effects by giving rise to secondary opportunistic pathogens (e.g. *Arcobacter*). Heat stress alone was not

sufficient to cause mortality, showing that direct or indirect effects of pathogenic bacteria are necessary to induce mortality. To disentangle direct from indirect effects mediated by resident microbial communities further studies are needed increasing the temporal resolution during the early phases of an infection to cover more bacterial communities associated to moribund oysters. The robustness of microbial communities against infection and plasticity in response to temperature suggest that the hemolymph microbiome can indeed play a vital role for host defense in changing environments.

CHAPTER III SUPPLEMENTS

	Raw	After denoising and chimera removal	w/o contaminants	w/o singletons	w/o low coverage samples (<100 reads)
No. sequences	256 748	165441	136607	133617	133285
No. bp	90 975 641				
No. libraries		176	174	174	168
No. 97% OTUs		5634	5616	2623	2622
Sequences/library					
min		29	26	24	100
max		2832	2164	2156	2156
median ± abs. dev		901.50 ±448	758.50±379	732.50±380.0	766.5±353.5
mean ± st.dev.		878.67±534.48	785.09±475.59	767.91±474.79	793.36±463.34

***Table III-S1**. Results of raw sequence data processing.*

Rarefied	Complete
Shannon`s H	0.979 ± 0.0025***
Evenness	0.956 ± 0.0030***
PD	0.842 ± 0.0093***

***Table III-S2**. Spearman correlation coefficients between alpha-diversity metrics of the complete and rarified (size = 100) datasets. Mean coefficient values and their standard deviation are shown.*

	Complete[1]
Abundant	0.79***
Rare	0.825***
Rarefied	0.85***

[1]significance levels: * ≤ 0.05, ** < 0.01, *** < 0.001

***Table III-S3.** Procrustes correlations between NMDS ordinations based on the complete dataset and: datasets including only abundant (≥1%) or rare (<1%) OTUs and the average of 10 rarified datasets.*

		Contrasts	*Z-value*[1]	*Estimate*	*SE*	*2.5% CI*	*97.5% CI*
Main effects	Acclimation temp.	Cold VS warm	-0.67				
	Experimental temp.	Cold VS warm	-7.31***	-0.766	0.1047	-0.971	-0.560
	Time	Day 3 VS days 5-7	-4.39***	-0.245	0.0557	-0.354	-0.136
		Day 5 VS 7	0.03				
	Health status	Moribund & dead VS healthy	-2.61**	-0.249	0.0954	-0.436	-0.062
		Dead VS moribund	-0.63				
	Infection	Control VS infected	3.53***	0.402	0.1138	0.179	0.625
	Day 0 CFUs		0.74				
Interaction terms	A. temp. x E. temp.		0.28	0.029	0.1033		
	E. temp. x Time	E. temp. on (day 3 VS days 5-7)	-3.32***	-0.192	0.0579	0.-305	-0.079
		E. temp. on (day 5 VS day 7)	0.48				
Random intercept	Oyster			0.174	0.4169		

[1]significance levels: * ≤ 0.05, ** < 0.01, *** < 0.001

Table III-S4*. Generalized linear mixed model (negative binomial family) for treatment and condition effects on CFU count data (day 0 as covariate). AIC = 1246.6, BIC = 1286.29, logLik (df = 14) = -609.291. Negative binomial dispersion parameter: 1.537±0.2498.*

		df	*F*[1]	*Significant contrasts*	*Estimate*	*SE*	*2.5% CI*	*97.5% CI*
Main effects	**Acclimation temp.**	1, 43	6.720*	Cold VS warm	3.178	1.226	0.706	5.65
	Experimental temp.	1, 43	9.735**	Cold VS warm	2.732	0.876	0.966	4.498
	Time	2, 62	4.545*	Day 3 VS days 5-7	1.463	0.497	0.469	2.457
	Health status	2, 62	9.608***	Moribund & dead VS healthy	2.542	2.542	1.288	3.796
	Infection	1, 43	0.828					
Interaction terms	A. temp. x E. temp.	1, 43	2.225					
	A. temp. x Time	2, 62	6.181**	A. temp. on (day 3 VS days 5-7)	-2.397	0.702	-3.8	-0.993
	E. temp. x Time	2, 62	5.109**	E. temp. on (day 3 VS days 5-7)	1.273	0.512	0.249	2.297
				E. temp. on (day 5 VS day 7)	1.918	0.93	0.058	3.778
	A. temp. x E. temp. x Time	2, 62	0.676					
Random intercept	Oyster				2.128		0.803	5.643

[1]significance levels: * ≤ 0.05, ** < 0.01, *** < 0.001

Table III-S5. *Linear mixed model for treatment and condition effects on PD (phylogenetic diversity). AIC = 761.903, BIC = 807.020, logLik (df = 17) = -363.951.*

		df	F[1]	Significant contrasts	Estimate	SE	2.5% CI	97.5% CI
Main effects	Acclimation temp.	1, 43	0.016					
	Experimental temp.	1, 43	1.275					
	Time	2, 62	0.597					
	Health status	2, 62	9.221***	Moribund & dead VS healthy	0.049	0.012	0.027	0.072
	Infection	1, 43	1.411					
Interaction terms	**A. temp. x E. temp.**	1, 43	13.016***	Stressed VS acclimated	-0.077	0.021	-0.117	-0.037
	A. temp. x Time	2, 62	0.173					
	E. temp. x Time	2, 62	3.501*	E. temp. on (day 3 VS days 5-7)	0.027	0.011	0.007	0.047
	A. temp. x E. temp. x Time	2, 62	2.245					
Random intercept	Oyster				0		0	7.00E+66

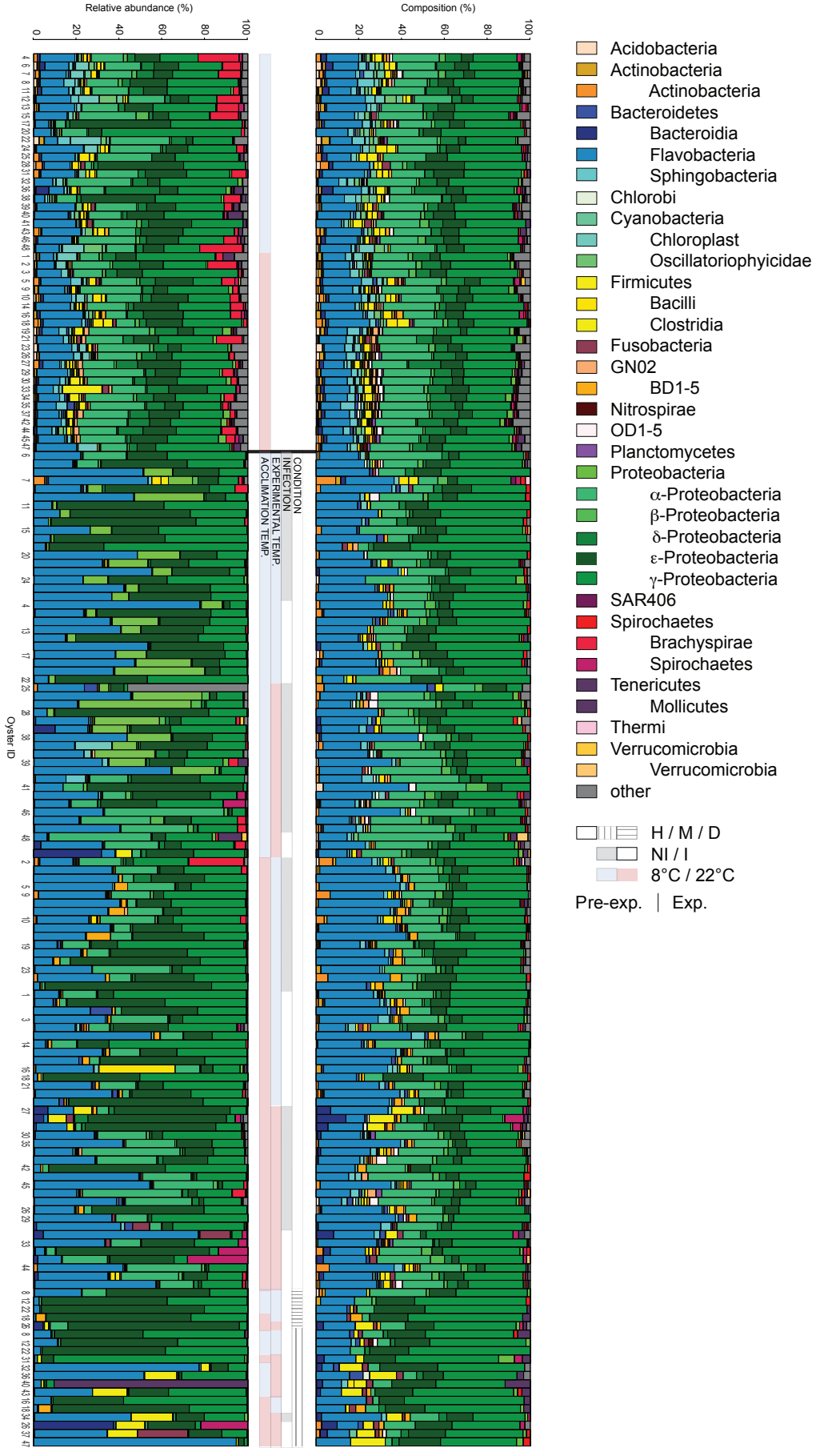
[1]significance levels: * ≤ 0.05, ** < 0.01, *** < 0.001

***Table III-S6**. Linear mixed model for treatment and condition effects on evenness. AIC = -165.083, BIC = -117.696, logLik (df = 17) = 99.542.*

	%
CC**	**78.66**
CW	58.64
WC	62.79
WW	64.61

***Table III-S7**. Proportion of abundant (≥1%) OTUs in days 3-7 communities that were present on day 0, listed by temperature treatments. Cold-acclimated communities (CC) had significantly higher percentageof persistent OTUs than the rest (F3,36 = 6.003, p = 0.002). CC = cold acclimated, CW = warm stressed, WC = cold-stressed, WW = warm-acclimated.*

***Figure III-S1**. OTU composition (number of OTUs per taxon) and relative abundances at the class level in individual samples. Horizontal bars between the upper and the lower barplot describe oyster condition and experimental treatments (for additional information on oysters and treatments see Supplementary File 1). Only acclimation temperature is given for the pre-experimental communities (left of the vertical bar). Experimental communities are first grouped according to condition, then to reatments. Within a treatment, healthy oysters are grouped by their ID in the chronological order (the label is placed at the beginning of a sample group). H = healthy, M = moribund, D = dead, NI = non-infected, I = infected.*


Relative abundance (%)
Composition (%)
0
20
40
60
80
100
Oyster ID
CONDITION
INFECTION
EXPERIMENTAL TEMP.
ACCLIMATION TEMP.
Acidobacteria
Actinobacteria
Actinobacteria
Bacteroidetes
Bacteroidia
Flavobacteria
Sphingobacteria
Chlorobi
Cyanobacteria
Chloroplast
Oscillatoriophyicidae
Firmicutes
Bacilli
Clostridia
Fusobacteria
GN02
BD1-5
Nitrospirae
OD1-5
Planctomycetes
Proteobacteria
α-Proteobacteria
β-Proteobacteria
δ-Proteobacteria
ε-Proteobacteria
γ-Proteobacteria
SAR406
Spirochaetes
Brachyspirae
Spirochaetes
Tenericutes
Mollicutes
Thermi
Verrucomicrobia
Verrucomicrobia
other
H / M / D
NI / I
8°C / 22°C
Pre-exp. | Exp.

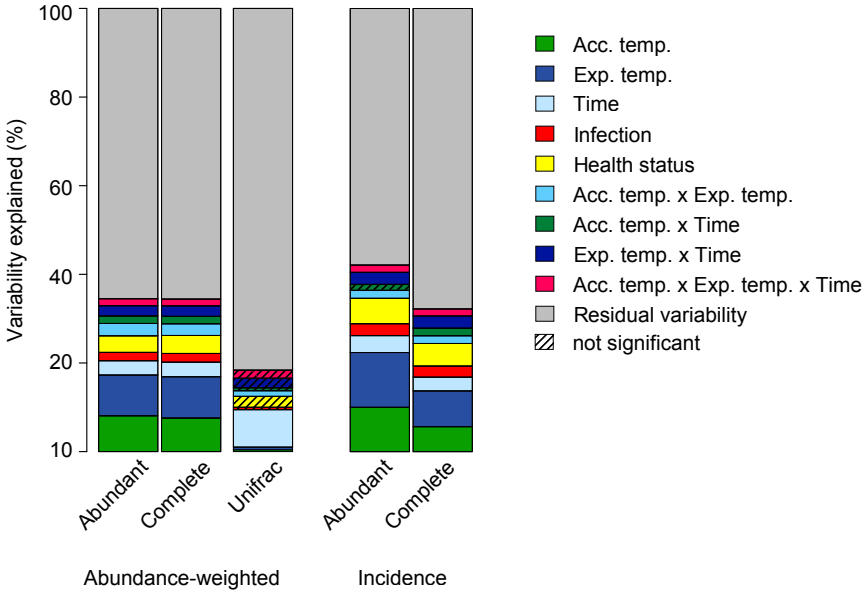


Figure III-S2. *Graphical representation of Adonis (Permanova) results for abundance-weighted (left) and incidence data (right). Analyses are based on complete (Bray-Curtis & weighted Unifrac distances) and abundant (≥1%) portions (Bray-Curtis only) of experimental communities (day 0 excluded).*

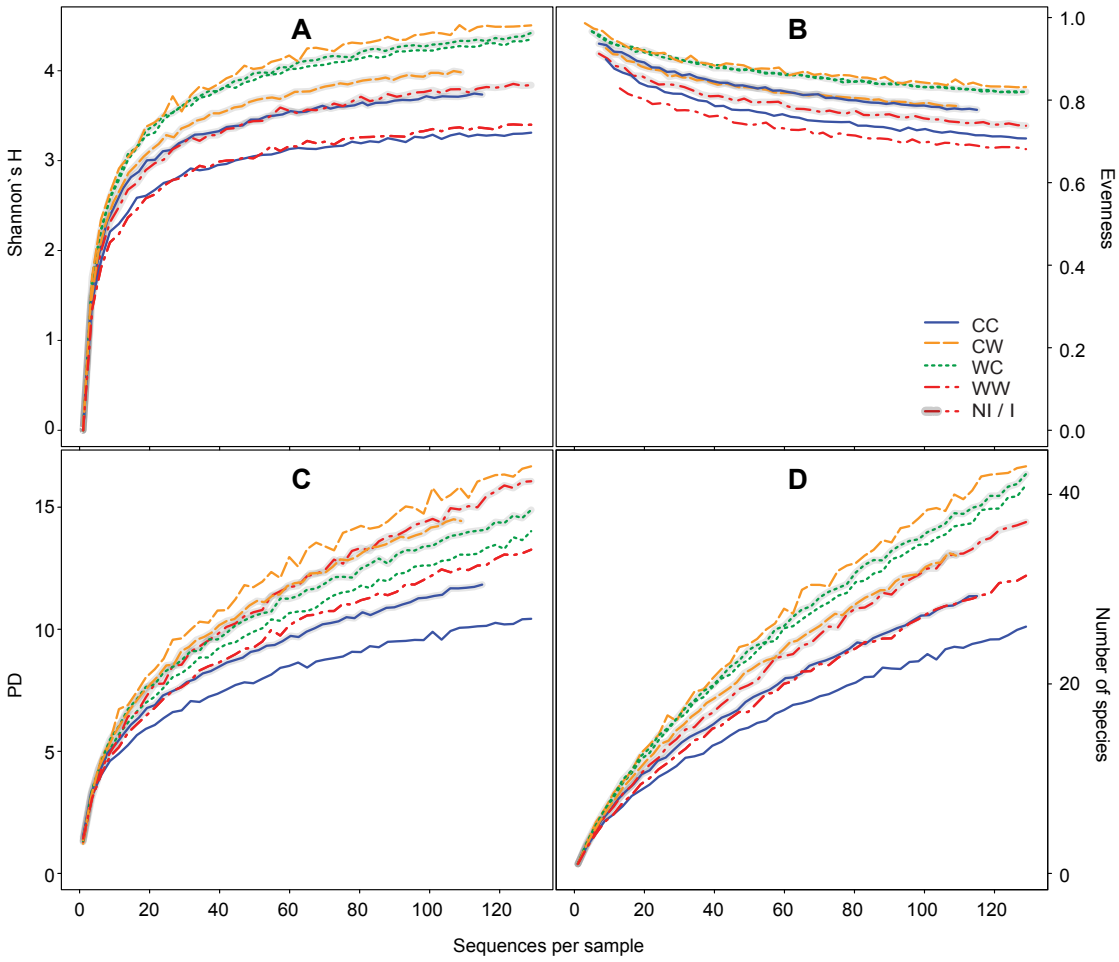


Figure III-S3. *Mean rarefaction curves of α-diversity indices of experimental communities (day 0 excluded) according to treatments: (A) Shannon's H, (B) evenness, (C) phylogenetic diversity, (D) number of species, CC = cold acclimated, CW = warm stressed, WC = cold-stressed, WW = warm-acclimated, NI = non-infected, I = infected.*

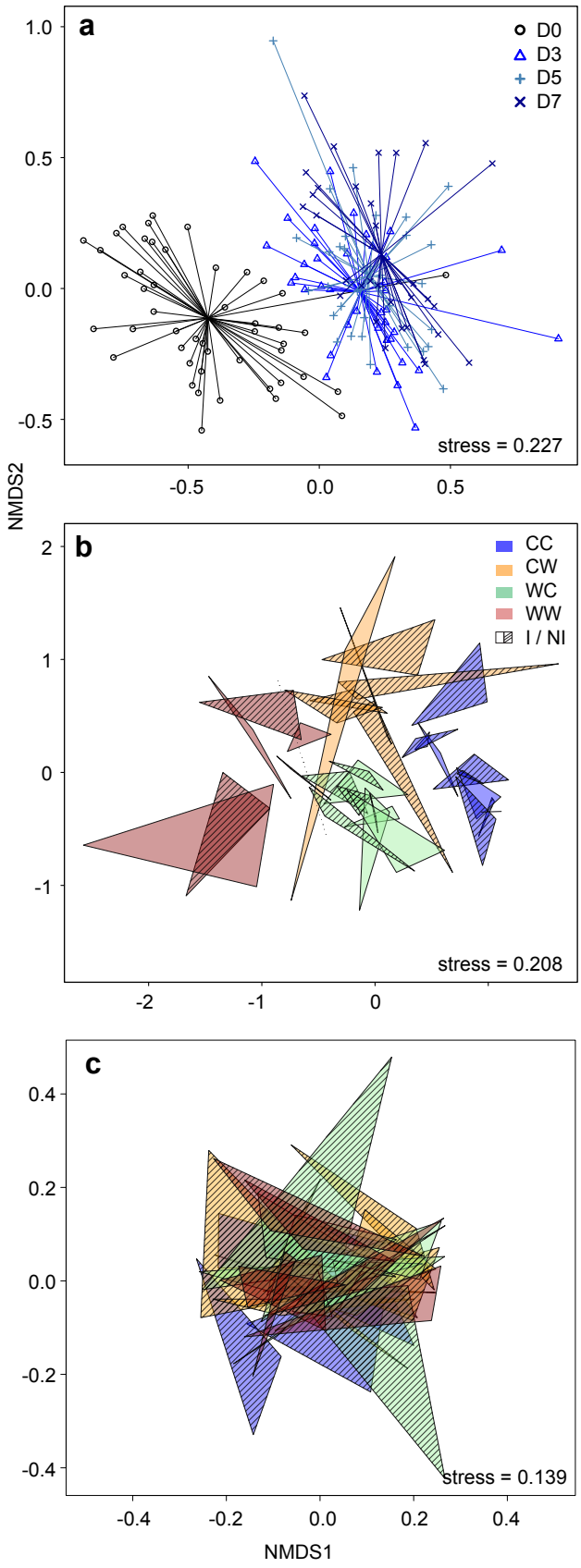


Figure III-S4. *NMDS plots showing: (a) the difference between pre-experimental and experimental communities (only alive oysters); (b, c) the temporal stability and response to the treatments based on the (b) Bray-Curtis distances between the abundance-truncated (>1%) dataset, and on the (c) weighted UniFrac in the full dataset (day 0 excluded). CC = cold acclimated, CW = warm-stressed, WC = cold-stressed, WW = warm- acclimated, I = infected, NI = non-infected, D = Day.*

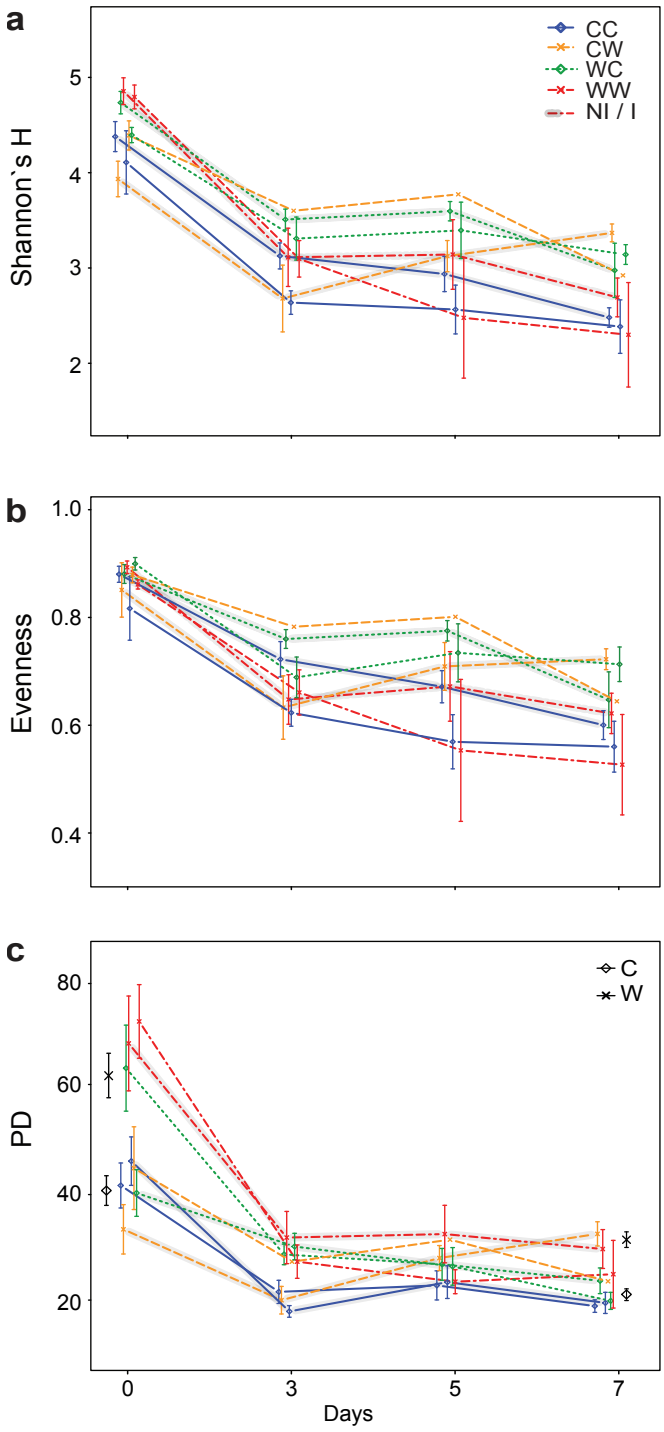


Figure III-S5. *α-diversity indices - (a) Shannon's H, (b) evenness and (c) PD - in response to treatments. In (c), means of PD at the beginning and the end of the experiment are shown for communities at ambient (current) temperature (C = 8°C, W = 22°C). CC = cold-acclimated, CW = warm stressed, WC = cold-stressed, WW = warm-acclimated, NI = non-infected, I = infected.*

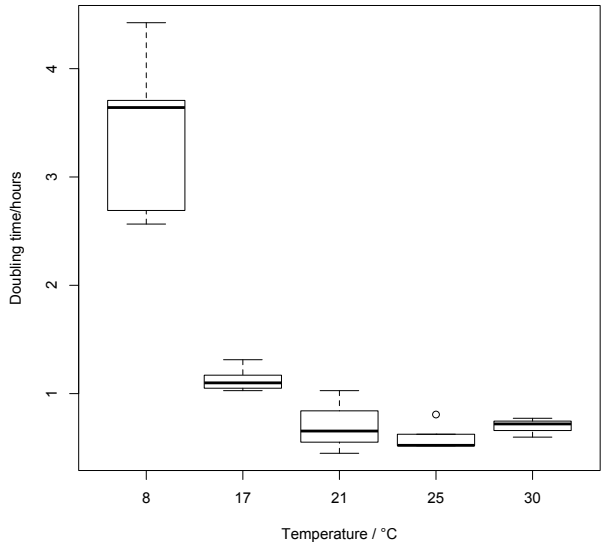


Figure III-S6. *Effect of temperature on doubling time of Vibrio sp. D29w (3-5 replicates at each temperature, OD550 measured in 15 min intervals).*

Sup III-Ind

Indicator species analysis

We used indicator species approach implemented in the R package indicspecies to quantify association of OTUs and genera with conditions and treatments of interest.

Taxon names are written as in output of RDP classifier using Greengenes taxonomy 12_10.

A separate analysis has been performed at OTU and genus level for abundance and presence/absence data.

P-values were adjusted for multiple comparisons with Benjamini & Hochberg correction.

Indicator value index is a product of two indices:

- specificity (A) is the probability that A site (oyster) belongs to the tested group given that the taxon was found

- sensitivity (B) is the probability of finding the taxon in sites (oysters) belonging to the tested group

We excluded indicators with low specificity ($A < 0.6$) and sensitivity ($B < 0.5$) from the main list of indicators ("Indicators" sheet) in order to remove taxons that were abundant in a single oyster or only few samples in a tested group.

Legend is on the next page (109) and list of indicators starts on page 110.

Tested groups		*Significant indicators found:*	
Day 0 communities			
T0	Time 0	+	
C	Cold-acclimated		
W	Warm- acclimated	+	
	Experimental communities		
A	Alive	+	
CC	Cold-acclimated	+	
CC_0	Non-infected		
CC_I	Infected		
CW	Warm-stressed	+	
CW_0	Non-infected	+	
CW_I	Infected	+	*only 1 oyster in the group
WC	Cold-stressed	+	
WC_0	Non-infected	+	
WC_I	Infected	+	
WW	Warm-acclimated	+	
WW_0	Non-infected		
WW_I	Infected		
Cx	Cold acclimation temperature	+	
Wx	Warm acclimation temperature	+	
xC	Cold experimental temperature	+	
xW	Warm experimental temperature	+	
I	Infected	+	
A_I	Alive infected	+	
H	Healthy	+	
S	Surviving		
M	Moribund		
DM	Dead and moribund	+	
D	Dead	+	

Abbreviations

uc	match to unclassified sequence
na	not assigned to the given taxonomical level
ab	abundance-weighted
pa	non-weighted (presence/absence)
gen	genus

Level	*Phylum*	*Class*	*Order*	*Family*	*Genus*	*OTU ID*	*Group*	*IndVal*	*A*	*B*	*Padj.*	*Data*
Genus	Bacteroidetes	Bacteroidia	Bacteroidales	uc	uc		CW_I	0.643	0.620	0.667	0.041	ab
OTU		Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Aquimarina	5385	CW_I	0.775	0.901	0.667	0.010	ab
OTU						5385	CW_I	0.740	0.901	0.667	0.008	pa
OTU					Flavobacteriumgelidilacus	3029	A	0.776	0.952	0.632	0.013	ab
Genus							A	0.755	0.902	0.632	0.016	ab
OTU					Mesonia	746	WC	0.655	0.803	0.533	0.019	ab
OTU						746	WC	0.512	0.803	0.533	0.027	pa
Genus							WC	0.624	0.730	0.533	0.012	ab
OTU					Polaribacter	129	A	0.708	0.966	0.519	0.010	ab
OTU						129	A	0.681	0.966	0.519	0.008	pa
OTU						275	xC	0.856	0.885	0.828	0.010	ab
OTU						275	A	0.756	0.982	0.689	0.008	pa
OTU						4144	xW	0.701	0.918	0.535	0.017	ab
Genus							A	0.937	0.931	0.943	0.005	ab
Genus							A	0.860	0.784	0.943	0.013	pa
OTU					Polaribacterirgensii	1067	xC	0.854	0.920	0.793	0.010	ab
OTU						1067	A	0.719	0.978	0.642	0.034	pa
Genus							xC	0.834	0.876	0.793	0.005	ab
Genus							A	0.725	0.808	0.651	0.033	pa
OTU					Sediminicola	1120	Wx	0.899	0.875	0.923	0.010	ab
Genus							Wx	0.885	0.849	0.923	0.005	ab
Genus					Tenacibaculum		H	0.680	0.865	0.535	0.007	ab
Genus							A	0.664	0.849	0.519	0.015	pa
OTU					uc	506	WW	0.640	0.750	0.545	0.019	ab
OTU						1066	A	0.892	0.937	0.849	0.010	ab
OTU							A	0.814	0.937	0.849	0.035	pa
OTU						1193	xW	0.692	0.763	0.628	0.010	ab
OTU							xW	0.655	0.763	0.628	0.008	pa
OTU						1551	WC_I	0.763	0.740	0.786	0.010	ab
OTU							WC_I	0.585	0.740	0.786	0.021	pa
OTU						1797	CC	0.725	0.775	0.679	0.025	ab
OTU						2360	Wx	0.753	0.921	0.615	0.010	ab
OTU							Wx	0.641	0.921	0.615	0.008	pa
OTU						2486	Wx	0.836	0.909	0.769	0.010	ab
OTU							Wx	0.705	0.909	0.769	0.008	pa
OTU						2487	WC	0.698	0.913	0.533	0.017	ab
OTU					na	1468	xW	0.934	0.938	0.930	0.010	ab
OTU							H	0.724	0.981	0.663	0.030	pa
OTU				uc	uc	3114	WC	0.715	0.903	0.567	0.019	ab
OTU							WC	0.527	0.903	0.567	0.021	pa
OTU						4365	A	0.804	0.926	0.698	0.010	ab
OTU							A	0.746	0.926	0.698	0.033	pa
OTU				na	na	3607	A	0.731	0.978	0.547	0.025	ab
OTU							A	0.681	0.978	0.547	0.020	pa
OTU		Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	uc	1488	CW_I	0.648	0.630	0.667	0.010	ab
OTU							CW_I	0.606	0.630	0.667	0.016	pa
Genus				Saprospiraceae	uc		T0	0.611	0.689	0.542	0.007	ab
OTU		na	na	na	na	4384	WC_I	0.710	0.706	0.714	0.010	ab
Genus	Cyanobacteria	Chloroplast	Stramenopiles	uc	uc		T0	0.893	0.869	0.917	0.005	ab
OTU	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	uc	4789	D	0.778	0.940	0.643	0.013	ab
OTU							D	0.541	0.940	0.643	0.023	pa
OTU		na	na	na	na	454	W	0.726	0.843	0.625	0.021	ab
OTU	Firmicutes	na	na	na	na		W	0.542	0.843	0.625	0.008	pa
OTU	GN02	BD1.5	uc	uc	uc	2576	WC	0.516	0.919	0.567	0.030	pa
OTU							WC_0	0.798	0.849	0.750	0.013	ab
OTU						2814	Wx	0.676	0.626	0.731	0.013	ab
OTU							Wx	0.668	0.626	0.731	0.008	pa
Genus							Wx	0.740	0.663	0.827	0.038	ab
OTU		IIB17	uc	uc	uc	3900	W	0.803	0.910	0.708	0.010	ab
OTU							W	0.696	0.910	0.708	0.008	pa
Genus	Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	uc		W	0.721	0.781	0.667	0.005	ab
Genus							W	0.690	0.713	0.667	0.009	pa
Genus	Proteobacteria	α-Proteobacteria	Rhizobiales	Hyphomicrobiaceae	uc		T0	0.718	0.727	0.708	0.005	ab
OTU			Rhodobacterales	Rhodobacteraceae	Loktanella	260	A	0.780	0.908	0.670	0.032	ab
OTU						2073	T0	0.657	0.769	0.563	0.010	ab

Level	*Phylum*	*Class*	*Order*	*Family*	*Genus*	*OTU ID*	*Group*	*IndVal*	*A*	*B*	*Padj.*	*Data*
OTU							T0	0.605	0.769	0.563	0.008	pa
OTU					Octadecabacter	1986	Cx	0.875	0.914	0.837	0.010	ab
OTU							A	0.731	0.968	0.670	0.042	pa
Genus							Cx	0.845	0.853	0.837	0.005	ab
OTU					Phaeobacter	3642	Cx	0.744	0.798	0.694	0.010	ab
OTU							Cx	0.684	0.798	0.694	0.008	pa
OTU						3918	CW_I	0.769	0.888	0.667	0.010	ab
OTU							CW_I	0.712	0.888	0.667	0.020	pa
OTU						4004	H	0.867	0.863	0.871	0.013	ab
OTU							A	0.816	0.875	0.849	0.031	pa
OTU						4118	A_I	0.726	0.851	0.619	0.028	ab
Genus							A	0.892	0.836	0.953	0.007	ab
Genus							A	0.862	0.780	0.953	0.021	pa
OTU					uc	527	CW	0.752	0.913	0.619	0.013	ab
OTU							CW	0.558	0.913	0.619	0.027	pa
Genus							CW	0.677	0.740	0.619	0.026	ab
OTU	Proteobacteria	α-Proteobacteria	Rhodobacterales	Rhodobacteraceae	na	355	CW_I	0.816	1.000	0.667	0.010	ab
OTU							CW_I	0.816	1.000	0.667	0.008	pa
OTU						807	H	0.882	0.904	0.861	0.013	ab
OTU						3205	WC	0.609	0.618	0.600	0.027	ab
Genus			Rhodospirillales	Rhodospirillaceae	uc		T0	0.738	0.688	0.792	0.005	ab
Genus			Rickettsiales	Rickettsiaceae	uc		xW	0.779	0.931	0.651	0.009	ab
Genus							xW	0.631	0.611	0.651	0.009	pa
OTU				uc	uc	484	xW	0.752	0.935	0.605	0.010	ab
OTU							xW	0.689	0.935	0.605	0.008	pa
Genus							xW	0.843	0.899	0.791	0.005	ab
Genus							xW	0.696	0.612	0.791	0.009	pa
OTU			na	na	na	4044	xW	0.794	0.905	0.698	0.017	ab
OTU							xW	0.730	0.905	0.698	0.008	pa
OTU		β-Proteobacteria	Methylophilales	Methylophilaceae	uc	14	xW	0.681	0.738	0.628	0.017	ab
OTU						1701	CW_0	0.692	0.616	0.778	0.010	ab
OTU							xW	0.614	0.857	0.558	0.008	pa
Genus							A	0.679	0.814	0.566	0.038	pa
Genus							xW	0.729	0.692	0.767	0.005	ab
Genus			Nitrosomonadales	Nitrosomonadaceae	uc		T0	0.632	0.768	0.521	0.009	ab
Genus							T0	0.561	0.605	0.521	0.016	pa
OTU		∂-Proteobacteria	Bdellovibrionales	Bacteriovoracaceae	Bacteriovorax	5344	WC_0	0.660	0.697	0.625	0.029	ab
Genus					uc		T0	0.615	0.606	0.625	0.018	ab
Genus			Desulfobacterales	Desulfobulbaceae	uc		T0	0.721	0.960	0.542	0.009	ab
Genus							T0	0.653	0.787	0.542	0.009	pa
Genus				Nitrospinaceae	Nitrospina		W	0.578	0.618	0.542	0.018	pa
OTU			Myxococcales	Nannocystaceae	uc	592	W	0.713	0.762	0.667	0.013	ab
OTU							W	0.619	0.762	0.667	0.016	pa
Genus							W	0.707	0.750	0.667	0.005	ab
Genus				uc	uc		T0	0.687	0.780	0.604	0.007	ab
Genus							T0	0.606	0.607	0.604	0.009	pa
Genus			Sva0853	JTB36	uc		W	0.687	0.809	0.583	0.007	ab
Genus							W	0.613	0.645	0.583	0.015	pa
OTU				uc	uc	658	W	0.755	0.978	0.583	0.010	ab
OTU							W	0.738	0.978	0.583	0.008	pa
Genus							W	0.756	0.981	0.583	0.005	ab
Genus							W	0.699	0.838	0.583	0.015	pa
OTU			na	na	na	148	CW	0.656	0.646	0.667	0.021	ab
OTU							CW	0.516	0.646	0.667	0.047	pa
OTU		ε-Proteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	692	D	0.794	0.981	0.643	0.010	ab
OTU							D	0.664	0.981	0.643	0.016	pa
OTU						1330	A	0.890	0.943	0.840	0.019	ab
OTU						4156	DM	0.810	0.734	0.895	0.013	ab
OTU						4435	A	0.893	0.949	0.840	0.043	ab
OTU						4917	CW_I	0.715	0.767	0.667	0.013	ab
OTU							CW_I	0.676	0.767	0.667	0.016	pa
Genus							A	0.919	0.852	0.991	0.005	ab
OTU				na	na	4369	W	0.758	0.656	0.875	0.010	ab
OTU		γ-Proteobacteria	Alteromonadales	Alteromonadaceae	Glaciecola	236	H	0.811	0.948	0.693	0.010	ab
OTU							H	0.758	0.948	0.693	0.008	pa
Genus							xC	0.783	0.671	0.914	0.007	ab

Level	*Phylum*	*Class*	*Order*	*Family*	*Genus*	*OTU ID*	*Group*	*IndVal*	*A*	*B*	*Padj.*	*Data*
Genus							H	0.753	0.774	0.733	0.028	pa
Genus				Colwelliaceae	Thalassomonas		A_I	0.588	0.604	0.571	0.041	ab
OTU					uc	1702	xC	0.789	0.737	0.845	0.010	ab
OTU							A	0.716	0.939	0.623	0.008	pa
OTU						4116	xC	0.861	0.796	0.931	0.010	ab
OTU							A	0.765	0.942	0.708	0.008	pa
Genus							A	0.861	0.883	0.840	0.007	ab
Genus				Shewanellaceae	Shewanella		I	0.632	0.699	0.571	0.038	ab
OTU					Shewanellahanedai	3720	I	0.626	0.685	0.571	0.042	ab
OTU							I	0.617	0.685	0.571	0.030	pa
Genus							I	0.617	0.666	0.571	0.034	pa
OTU				na	na	3855	W	0.671	0.832	0.542	0.021	ab
OTU							W	0.548	0.832	0.542	0.016	pa
Genus			Chromatiales	uc	uc		T0	0.836	0.839	0.833	0.005	ab
OTU			Legionellales	Francisellaceae	Francisella	2747	CW_I	0.851	0.724	1.000	0.010	ab
OTU							CW_I	0.871	0.724	1.000	0.008	pa
Genus					uc		CW	0.671	0.726	0.619	0.005	ab
Genus				Legionellaceae	uc		CW_I	0.854	0.730	1.000	0.007	ab
OTU					uc	133	CW	0.793	0.943	0.667	0.010	ab
OTU					uc		CW	0.633	0.943	0.667	0.013	pa
OTU					uc	2148	CW_I	0.710	0.756	0.667	0.013	ab
OTU					uc		CW_I	0.621	0.756	0.667	0.013	pa
Genus			Oceanospirillales	Halomonadaceae	Chromohalobacter		W	0.758	0.861	0.667	0.007	ab
OTU					Chromohalobacter	4581	W	0.785	0.925	0.667	0.013	ab
Genus				Oceanospirillaceae	Amphritea		H	0.730	0.869	0.614	0.005	ab
Genus							A	0.717	0.865	0.594	0.009	pa
OTU					Marinomonas	1444	WC	0.667	0.702	0.633	0.022	ab
OTU						4052	Wx	0.727	0.834	0.635	0.010	ab
OTU							Wx	0.634	0.834	0.635	0.008	pa
Genus							A	0.752	0.869	0.651	0.012	ab
Genus							A	0.719	0.794	0.651	0.028	pa
OTU	Proteobacteria	γ-Proteobacteria	Oceanospirillales	Oceanospirillaceae	Oleispira	1855	A	0.744	0.930	0.594	0.010	ab
OTU							A	0.703	0.930	0.594	0.013	pa
Genus							A	0.728	0.878	0.604	0.005	ab
Genus							A	0.707	0.829	0.604	0.015	pa
OTU					uc	595	A	0.736	0.973	0.557	0.013	ab
OTU							A	0.683	0.973	0.557	0.022	pa
OTU						1190	H	0.899	0.938	0.861	0.010	ab
OTU						1549	xW	0.777	0.865	0.698	0.019	ab
OTU							xW	0.630	0.865	0.698	0.008	pa
Genus							A	0.935	0.909	0.962	0.005	ab
OTU					na	3370	Wx	0.698	0.791	0.615	0.013	ab
OTU						4781	CW_0	0.665	0.663	0.667	0.017	ab
Genus			Salinisphaerales	Salinisphaeraceae	Salinisphaera		W	0.739	0.873	0.625	0.005	ab
OTU						226	W	0.709	0.929	0.542	0.010	ab
OTU							W	0.539	0.929	0.542	0.021	pa
OTU			Vibrionales	Vibrionaceae	Photobacterium	753	I	0.653	0.769	0.554	0.028	ab
OTU							I	0.608	0.769	0.554	0.026	pa
OTU					**Vibrio**	**4770**	**I**	**0.769**	**0.849**	**0.696**	**0.017**	**ab**
Genus			Xanthomonadales	uc	uc		T0	0.895	0.916	0.875	0.005	ab
Genus							T0	0.745	0.634	0.875	0.009	pa
Genus			uc	uc	uc		T0	0.831	0.737	0.938	0.005	ab
OTU						1638	W	0.576	0.613	0.542	0.021	ab
OTU							W	0.585	0.613	0.542	0.016	pa
OTU			na	na	na	5079	CW_I	0.816	1.000	0.667	0.010	ab
OTU							CW_I	0.816	1.000	0.667	0.008	pa
OTU						5191	W	0.730	0.799	0.667	0.013	ab
OTU							W	0.574	0.799	0.667	0.022	pa
OTU						5578	T0	0.688	0.811	0.583	0.010	ab
OTU							T0	0.575	0.811	0.583	0.008	pa
OTU		na	na	na	na	356	CW_I	0.786	0.617	1.000	0.010	ab
OTU							CW_I	0.844	0.617	1.000	0.008	pa
OTU						4758	CW_I	0.830	0.690	1.000	0.010	ab
OTU							CW_I	0.720	0.690	1.000	0.013	pa
OTU						5098	W	0.729	0.910	0.583	0.013	ab
OTU							W	0.657	0.910	0.583	0.008	pa

Level	*Phylum*	*Class*	*Order*	*Family*	*Genus*	*OTU ID*	*Group*	*IndVal*	*A*	*B*	*Padj.*	*Data*
OTU	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Persicirhabdus	1019	CW_I	0.816	1.000	0.667	0.013	ab
OTU							CW_I	0.816	1.000	0.667	0.013	pa
Genus							CW_I	0.790	0.937	0.667	0.009	ab
Genus							CW_I	0.724	0.787	0.667	0.013	pa

CONCLUSION

The evolution is driven by the pressure that abiotic and biotic environment exert on individual organisms. However, they do not face these challenges alone, as each individual is actually an ecosystem hosting numerous and distinct microbial communities. Both host and its associated microbiota are affected by the environment, and the fitness of an organism also depends upon the outcome of these complex interactions. Deciphering host-microbiota-environment interactions has thus important theoretical and practical implications for understanding the evolutionary and ecological consequences of the climate change.

Pacific oysters have experienced positive as well as negative consequences of the global warming: they invaded coastal habitats worldwide due to the rising temperatures, which at the same time increased the risk of oyster mass mortalities. The mortalities are result of a complex interplay of numerous factors, including abiotic stress and opportunistic pathogens. The oyster-associated microbial communities might be important for the outcome of such disease outbreaks, both because of their putative ability to protect the host and because they themselves may act as opportunistic pathogens. Translocations of Pacific oysters for commercial purposes represent yet another interesting aspect of microbiota-environment interactions, namely the interactions of co-adapted microbiota with the unknown biotic environment.

In this thesis, I set out to explore how abiotic and biotic conditions affect the Pacific oyster microbiota and how these changes might reflect upon the oyster fitness. I combined field and laboratory experiments over short (Chapter I, Chapter III) and long (Chapter II) timescales in order to get a comprehensive picture of oyster microbiota variability in response to the environment. I addressed the consequences of complex biotic and abiotic stress imposed by translocation (Chapter I, Chapter II) as well as of the controlled stress imposed by experimental infection and abrupt temperature change (Chapter III).

I found that the hemolymph, unlike the gut, gill and mantle tissues, hosted highly diverse microbiota including active potentially pathogenic bacteria, and that stable hemolymph communities prevented passage of these pathogens to solid tissues (Chapter I). Destabilization of hemolymph microbiota, likely through interactions with a new biotic environment, caused systemic potentially fatal infections in translocated oysters. In contrast, hemolymph microbiota were robust against infection with a sympatric *Vibrio* sp. (Chapter III), although the community destabilization through temperature stress increased the risk of disease development. While the composition of the structurally stable communities stayed the same after infection, it shifted rapidly in response to temperature change (Chapter III). However, the community structure

was still preserved, indicating that the hemolymph microbiota also take part in oyster acclimation. Such rapid shifts in composition in response to abiotic could also partially explain high community variability observed in the field, since the intertidal represents spatiotemporally extremely heterogeneous environment (Chapter II). Interestingly, despite the strong influence of external environment, the resident microbiota were replaced only gradually after the translocation, which may reflect a beneficial stability, but also cause unpredictable consequences, since the microbiota are not adapted to respond to local biotic challenges (Chapter I, Chapter II).

Differing role of microbiota in a new environment and under local disturbances

Investigating various disturbance scenarios revealed that the response of resident hemolymph microbiota varies accordingly: they exhibit resistance against colonization by external pathogens (Buffie and Pamer, 2013), while quickly responding to abiotic conditions by shifts in composition (Reshef *et al.*, 2006). This demonstrates that the two ways in which microbial communities can benefit their host are not mutually exclusive (Erwin *et al.*, 2012). While the above examples (Chapter III) mimic conditions that can occur in nature, the abrupt encounter with a completely foreign microbial environment (Chapter I) is basically impossible for sessile animals in nature and thus represents conditions to which the community would not be able to predictably respond. It is possible that the community's resistance to colonization, which is beneficial against intruders in the generally familiar microbial environment, prevents the integration of similar, but locally adapted bacteria into the community (Koeppel and Wu, 2013). Considering that the hemolymph microbiota are largely influenced by immigration (Chapter II), subsequent loss of diversity could result in the loss of function (Shade *et al.*, 2012). However, the proposed mechanism is highly speculative, as the response of stable microbial communities to entirely new microbial context is *terra incognita*.

Will the climate change affect the Pacific oyster holobiont?

High small-scale variability, be it temporal or spatial, and low large-scale variability of the hemolymph community composition at least partially reflect the similar pattern in environmental variation (Chapter II): the intertidal is a highly heterogeneous habitat and daily oscillations in abiotic conditions by far exceed long-term mean fluctuations. Prompt response of hemolymph microbiota to temperature change (Chapter III) and possibly to tidal cycle (Chapter

I, Chapter II) indicates the ability of the oyster holobiont to acclimate quickly to large shifts in abiotic environment (Reshef *et al.*, 2006). Oysters should thus be well prepared for future challenges imposed by global warming. However, warmer temperatures do decrease community stability (Chapter III), which can subsequently affect the resistance to colonization by external pathogens. This, in combination with the temperature-dependent increase in virulence (Kimes *et al.*, 2012), can result in more severe and more often occurring disease outbreaks. Moreover, other factors, such as ocean acidification, can further lower community stability and affect the oyster physiology (Clark *et al.*, 2013). For example, lower pH and hypoxia (Levican *et al.*, 2014) could promote the growth of oyster symbionts and opportunistic pathogens of the genus *Arcobacter* and thus add to the selection pressure imposed by temperature-driven vibriosis.

Limitations and perspectives

Here I would like to address some limitations of my thesis and recommendations for future research. Firstly, 16s rDNA sequencing reveals nothing about the bacterial activity and viability (Campbell *et al.*, 2011). Including 16s rRNA could partially solve the problem; however, even some less active and abundant bacteria can have disproportional effects on the host (Thurber *et al.*, 2009). Another problem is that 16s rRNA is a housekeeping gene and cannot distinguish between closely related but ecologically distinct bacteria (Preheim *et al.*, 2011; Koeppel and Wu, 2013). Meta-omic studies in controlled and natural conditions could help identify ecologically relevant genes and elucidate the mechanisms behind the community response to internal and external host environment (Fritz *et al.*, 2013). Nevertheless, the health condition seems to be well correlated with general community properties such as diversity and structure, which are well detected by DNA sequencing (Chapter I, Chapter III). Secondly, survival is a rough, all-or-nothing measure of host response. However, choosing the right set of parameters to measure is a challenging task when it comes to oysters: it is hard to judge their condition from the outside and any kind of sampling is invasive. In fact, with the exception of hemolymph, it requires sacrificing the animals. Although immune parameters would directly address the oyster defense, the interpretation is not always straightforward and their measurement requires relatively high sample amounts. Quantifying physiologically important parameters such as oxygen consumption or filtration rate may be a non-invasive solution in controlled experiments, and should become more feasible in highly replicated studies due to technological developments. Analyzing the differences in gene expression is another, more or less invasive, but promising option. However, identifying the relevant genes is not straightforward and will require

comprehensive transcriptomic studies. Thirdly, although a constant experimental setting is necessary to quantify the effects of particular factors, it does not reflect the conditions in the natural habitat of Pacific oysters - the harsh, extreme intertidal, and it is the variation that may significantly increase the risk of disease (Ben-Horin *et al.*, 2013; Paaijmans *et al.*, 2013). Simulating tides and temperature fluctuations in a controlled way would bridge the gap between the natural and experimental conditions. Despite the above limitations, this thesis represents, the most comprehensive account of diversity and dynamics of bivalve microbiota in response to different disturbances, and it shows that Pacific oyster microbiota can profoundly affect various aspects of the interactions of the oyster holobiont with the environment.

Predicting the impact of disturbances including those linked to global climate change requires thorough understanding of the role of microbiota in a holobiont's interactions with the environment. By combining laboratory and field experiments, and manipulation and observation across spatial and temporal scales, this thesis sets solid ground for the future studies of the Pacific oyster holobiont in the changing abiotic and biotic environment. It reveals a special role of hemolymph microbiota and identifies potentially interesting groups of symbionts to study environmentally-dependent host-microbe interactions such as *Arcobacter*. Furthermore, it offers an overview of oyster-associated microbial diversity and composition in time and space, estimates the effects of different factors and disturbances on community stability and dynamics and therefore provides a baseline for assessing the relative impact of other, so far uninvestigated factors such as acidification. Finally, it illustrates the need to study the consequences of multiple stressors acting in concert - this is a vitally important matter for the future research, as disturbances, especially in time of the global climate change, rarely come alone.

I propose that the hemolymph microbiota represent an important "organ" of the Pacific oyster holobiont for the interactions with abiotic and biotic environment. Although their composition is determined by the bacterial populations from the external environment, the hemolymph communities posses internal dynamics and stable structure that is a prerequisite for their proposed beneficial functions: protection against pathogens and acclimation. While the hemolymph microbiota maintain their structure over a wide range of conditions (Chapter II), unpredictable disturbances (Chapter I) or combined stress (Chapter III) can negatively affect the community stability and result in shifts towards pathogenic states.

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ACKNOWLEDGEMENTS

First of all I would like to thank my supervisor, Mathias Wegner, for his patience and positive attitude in difficult times.

Without Anouk Goedknegt and David Thieltges, it would not have been possible to conduct simultaneous experiments of Sylt and Texel. I would also like to thank Mirjana Markovic, Jennifer Welsh, Anne-Karin Schuster, Cátia Carreira, who also helped sampling on Texel.

Big thanks goes to Tobias Mayr and Kaibil Escobar Wolf, who saved me from having to clean hundreds of oysters by myself.

Efficient processing of the samples would be hard and also quite boring for a single person, and I really appreciate the assistance I got from Laura Burbaum, Silke Vollbrecht and Franziska Schade with drilling, disecting, injecting….

Thanks to John Baines, and his Evolutionary Genomics group - Katja Cloppenborg-Schmidt, Philipp Rausch, Meriem Belheouane and Marie Vallier, I learned many secrects about Miseq sequencing. I enjoyed the time spent in Kiel and discussions with Philipp, Meriem and Marie.

I would further like to thank Carolin Wendling, for providing *Vibrio* strains, and for interesting disscusions, scientific and non-scientific as well as for useful comments on parts of this manuscript.

Speaking German is hard, but writing is even harder, and therefore thanks to Jonas Geburzi who produced the Zusammenfassung for me.

Pleasant environment is important for getting the things done, and therefore thanks to my working group, to Mathias, Caro, Franziska, Lisa and also Christian, Tobi, Marieke, Feli, Sonja, Jonas und Annika for being a fun and inspiring company.

Daily drinking of good Italian coffee made by Dario, in combination with interesting conversations about everything, made my finishing days much brighter.

Leisure time is also an important part of doing an PhD. I enjoyed time spent with Nina W., cooking and doing yoga, as well as parties and pleasant evenings in the garden with Nina E., Moritz, Finn, Svenja…

I would like to thank my family, and especially my parents for supporting me and not asking to often about the state of my studies. I really appreciate that.

I have not seen much of my friends from home during my PhD studies, but when I have, it was really worth it, and it definitely lifted my spirits.

And last but not the least, I would like to thank Ondrej for supporting me and cooking for me in these last months. Without his help, it would be much harder to focus on my work.

MiSeq libraries were run by Sven Kuenzel at Max Planck Institute for Evolutionary Biology in Plön.

All experimental and laboratory work was performed at Wadden Sea Station Sylt of Helmholtz Centre for Polar and Marine Research, Alfred Wegener Institute, at NIOZ Texel, and Institute for Experimental Medicine, Christian-Albrechts-University of Kiel.

This work was supported by DFG (Deutsche Forschungsgemeinschaft) Emmy Noether Programme (We4641/1-3), International Max Planck Research School for Evolutionary Biology and Excellence Cluster 306 "Inflammation at Interfaces" (Chapter I & II).

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AFFIDAVIT

I hereby confirm that the content and design my thesis entitled:

"Pacific oyster holobiont in the changing environment: a microbial perspective"

are the result of my own work apart from my supervisor's guidance and co-operation with my co-autors. I prepared this thesis following the Rules of Good Scientific Practice of the German Research Foundation.

Furthermore, I confirm that this thesis has not been submitted as a part of another examination process neither in identical nor in similar form

Parts of this thesis have been published or submitted to scientific journals:

- Chapter III has been published with K.M. Wegner in ISME Journal: Lokmer, A., and Wegner, K.M. (2015) Hemolymph microbiome of Pacific oysters in response to temperature, temperature stress and infection. *ISME J* 9: 670-682.
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