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“WHAT A BABY” (Mr. Krabs, 2004)

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Summary

The study of symbiotic interactions is immensely valuable for understanding the development and evolution of all organisms on earth, the origin of eukaryotes and the formation of organelles. Chlamydiae are a group of obligate intracellular bacteria best known for their role as pathogens in humans and animals. While much of chlamydiae research is still largely focussed on the vertebrate-associated taxa, environmental chlamydiae have been valuable study models, harbouring a much broader host range and greater evolutionary and genomic versatility. This lends them higher metabolic independence from their hosts, more diverse transport capabilities, outer membrane structures, and effector proteins, that help in dealing with changing hosts and environments.

My focus in this thesis is on the impact chlamydial symbionts have on their hosts and environments in two very different host systems and experimental contexts from soil microcosms to freshwater systems.

First, I studied the impact of two different chlamydial strains on a common soil amoeba by establishing a controlled soil microcosm setup. Over an incubation period of 35 days I followed the effects of predation of symbiont carrying amoebae on three different levels: 1. the growth dynamics of host, symbionts and bacterial soil community; 2. changes in the active bacterial soil community structure; 3. the impact on soil nutrient cycles like respiration, growth and nitrogen mineralization rates. My results suggest the chlamydial symbionts have large impacts on the feeding behaviour of their amoeba hosts and by proxy on the soil microbial community and resulting ecosystem processes. I have also found evidence pointing to potential protective properties of the chlamydial symbionts, potentially increasing tolerance towards microbial antipredator defense systems, allowing for a broader spectrum of prey organisms to be consumed by the host.

Second, in a freshwater system, I characterized the first isolate of a chlamydial species associating with the freshwater cnidarian *Hydra oligactis*, using a combination of phylogenetic analysis, comparative genomics and imaging techniques. This showed that the symbionts' adaptations to its *Hydra* host consist mainly of genomic reorganization and streamlining, suggesting a relatively recent host shift and expanded our knowledge on capabilities, dispersion and transmission of cnidarian associated chlamydiae.

Together these studies highlight the evolutionary success of the chlamydial core genome and position bacterial endosymbionts not only as important components of their individual hosts, but also as regulators of broader microbial communities and processes.

Zusammenfassung

Die Untersuchung symbiotischer Interaktionen ist von hohem Wert für das Verständnis der Entwicklung und Evolution aller Organismen auf der Erde, des Ursprungs der Eukaryoten sowie der Entstehung von Organellen. Chlamydien sind eine Gruppe obligat intrazellulärer Bakterien, die vor allem für ihre Rolle als Pathogene beim Menschen und Tieren bekannt sind. Während sich ein Großteil der Chlamydienforschung nach wie vor hauptsächlich auf Wirbeltier-assoziierte Taxa konzentriert, haben sich Umwelt-Chlamydien als wertvolle Modellsysteme erwiesen, da sie ein deutlich breiteres Wirtsspektrum sowie eine größere evolutionäre und genomische Vielseitigkeit aufweisen. Dies verleiht ihnen höhere metabolische Unabhängigkeit von ihren Wirten, vielfältigere Transportkapazitäten und Außenmembranstrukturen sowie eine größere Diversität an Effektorproteinen, die ihnen helfen, mit wechselnden Wirten und Umweltbedingungen umzugehen. Der Schwerpunkt dieser Dissertation lag auf den Auswirkungen chlamydialer Symbionten auf ihre Wirte und deren Umwelt in zwei unterschiedlichen Wirtssystemen und experimentellen Kontexten, die von Bodenmikrokosmen bis hin zu Süßwassersystemen reichten.

Zunächst untersuchte ich den Einfluss zweier chlamydialer Stämme auf eine häufig vorkommende Bodenamöbe, indem ich ein kontrolliertes Bodenmikrokosmos-Setup etablierte. Über einen Inkubationszeitraum von 35 Tagen verfolgte ich die Effekte der Amöben-Prädation auf drei unterschiedlichen Ebenen: 1. die Wachstumsdynamik von Wirt, Symbionten und bakterieller Bodengemeinschaft; 2. Veränderungen in der aktiven Struktur der bakteriellen Bodengemeinschaft; 3. die Auswirkungen auf Nährstoffkreisläufe wie Respiration, Wachstum und Stickstoff Mineralisierungsraten. Meine Ergebnisse deuten darauf hin, dass chlamydiale Symbionten einen starken Einfluss auf das Ernährungsverhalten ihrer Wirte haben und indirekt auch auf die mikrobielle Bodengemeinschaft sowie daraus resultierende Ökosystemprozesse. Darüber hinaus fand ich Hinweise auf potenziell schützende Eigenschaften der Symbionten, die es dem Wirt ermöglichen, ein breiteres Spektrum an Beuteorganismen zu konsumieren.

Weiters charakterisierte ich in einem Süßwassersystem das erste Isolat einer Chlamydienart, die mit dem Süßwasser-Nesseltier *Hydra oligactis* assoziiert ist, mit einer Kombination aus phylogenetischen Analysen, vergleichender Genomik und bildgebenden Verfahren. Dabei zeigte sich, dass die Anpassungen des Symbionten an den *Hydra*-Wirt hauptsächlich aus genomischer Reorganisation und Optimierung bestehen, was auf einen relativ kürzlich erfolgten Wirtswechsel hindeutet. Diese Ergebnisse erweitern unser Wissen über die Fähigkeiten, Verbreitung und Übertragungswege Cnidarien assoziierter Chlamydien.

Diese Ergebnisse unterstreichen den evolutionären Erfolg des chlamydialen Genoms und positionieren bakterielle Endosymbionten nicht nur als wichtige Bestandteile ihrer jeweiligen Wirte, sondern auch als Regulatoren breiterer mikrobieller Gemeinschaften und ökologischer Prozesse.

Chapter I

Introduction

Introduction

The earliest scientific work I was able to find, describing microorganisms living in symbiosis was by a biologist called Simon Schwendener. When he realized lichens were two organisms living as one composite organism, he proposed a “dual hypothesis” and chose to describe their relationship in terms of a fungal master and algal slave (Schwendener, 1868).

Shortly after, several people, unhappy with such a charged description, suggested using a term that is more appreciative of the diversity of interactions that could be had. This included the formulation of Albert Bernhard’s term “*Symbiotismus*” (Sapp, 2010) and later famous, but not first-time use of the term ‘symbiosis’, for ‘unlike organisms living together’ by Anton de Bary (de Bary, 1879).

Although de Bary’s definition seems to be the most widely accepted, the idea that most interactions start out parasitic and remain so at the core, was persistent as it fit with ideas of germ theory and a general societal attitude towards life at the time. As part of my introduction to this thesis I wanted to first have a better understanding of this debate and answer a few questions that came up for me, which is then followed by a more classical introduction of endosymbiosis and the model system of this thesis, the chlamydiae.

So to define how symbiosis emerges I needed to know when unlike organisms actually started living together.

The first symbiosis might have only taken as much time to develop as it took for two ‘unlike’ organisms to exist in the first place, although ‘unlike’ in itself is not straightforward to define. But first things first: earth has been busy producing life almost from the start. While life may have existed already on (very)proto-earth, it only really kicked off after the moon-forming impact around 4.5 billion years ago - which is the last impact known to have completely sterilized the planet (Betts 2018). Theories about how and where life came about are multiple. It seems likely however, that life and evolution for quite a while happened within microbial mats - these are layered communities of organisms that differentiated as they occupied emerging niches (Nisbet and Fowler, 1999). Remnants of such communities date back over 3.7 billion years, with evidence in the form of stromatolites preserving mixed microbial assemblages (Nutman et al., 2016). With very close proximity over long periods of time this allowed for division of labour and cooperative mechanisms between these organisms, so a good start for early ‘symbiogenic’ mechanisms (Pereira et al., 2012). A later key symbiogenic event was the formation of eukaryotes - these are organisms with a complex cellular organization like protists, fungi, animals and plants. Estimated around 2 to 1.2 billion years ago, a popular hypothesis suggests that symbiosis between an archaeon and an alphaproteobacterium formed

an organism that eventually contained organelles in the form of a DNA storing nucleus (archaeon-derived) and energy producing mitochondria (bacterium-derived) (Betts et al., 2018; Mills et al., 2022). Descendants of this eukaryote later incorporated a cyanobacterium, 1.7 to 1.1 billion years ago, that would evolve into modern-day chloroplasts, giving rise to plants and green algae (Betts et al., 2018).

So why does De Bary's definition involve only two 'unlike', independently evolved organisms interacting closely over a prolonged period of time. These have to be unlike because .. well, it's all about providing different skills. So for example algae provide food, while fungi provide shelter, or legumes provide sugar while rhizobacteria provide nitrogen, or humans provide eyeballs while chlamydiae provide nasty infections. Other close exchanges, like your partner washing your laundry for hugs, we like to call an intraspecies cooperation, because in principle you could wash your own laundry. A bacterial example of this are filamentous-growing cyanobacteria. They carry out oxygen-producing photosynthesis, while also snatching nitrogen from the atmosphere with an enzyme called nitrogenase (Esteves-Ferreira et al., 2017). This enzyme stops working in the presence of oxygen, so they make a specialised cell called a heterocyst, providing an anaerobic environment for the nitrogenase, which produces necessary nitrogen compounds for all the other cells (laundry) and gets sugar (hugs) in return (Muro-Pastor and Maldener, 2019).

Without knowing the origin of most symbioses, can we interpret them as fundamentally defined by domination and exploitation? Assumptions about hierarchy, competition, and control have often inspired attempts to derive fundamental models of society from early biological systems (Sapp, 2010). As such, theories of symbiosis and evolution, like Schwendener's "master-slave" model as well as later competition-centered interpretations of Darwinian evolution, have been criticized for reflecting contemporary sociopolitical ideas rather than biological necessity (Gontier, 2016). Symbiologists especially, have emphasized cooperation and synergistic interactions at the heart of evolutionary trends toward novelty and greater complexity (Gontier, 2016; Pereira et al., 2012). The persistence of this debate more than anything highlights some unresolved questions: whether symbiotic relationships necessarily originate in parasitism, and how we define the "beneficial" and "exploitative" in symbiotic interactions, when the same organisms will have different interactions under the influence of different environments.

"The difficulty lies, not so much in developing new ideas, as in escaping from the old ones"
(John Maynard Keynes, 1936)

So why is this all so important? For one, life would look quite different without symbioses, which act like a driver for the complexity of biological processes. Symbiosis brings about emergent properties

neither organism achieves alone, making life, stability and access to resources easier for at least one of the partners involved. The ways it does so are incredibly varied, depend on ecological context and are for many a mix of mutualistic, parasitic and (messy) commensal outcomes, sometimes all at the same time. Environments do not develop independently either, they are constructed and shaped by organisms and their interactions, just think of coral reefs, termites, or lichens. This reminder that living things are never as simple and isolated as they may seem makes symbioses challenging to pin down, but also so intriguing to study.

Endosymbiosis, chlamydiae, his wife and her lover

The cook

Endosymbiotic interactions refer to symbioses where one organism lives inside another. For multicellular hosts this is typically a specialised tissue or organ composed of so-called ‘bacteriocytes’, while for single-celled organisms endosymbionts are located directly inside each cell (Bennett et al., 2024). It is common in endosymbiosis to refer to the larger organism as the ‘host’ and the inhabiting organism as the ‘symbiont’. Symbionts are also seen as the ones providing new phenotypes to the host, often allowing access to nutrients and habitats, much like a toolkit, while the host is usually itself the habitat, providing shelter and energy (Bennett et al., 2024). The majority of single-cell endosymbionts are located in the host cytosol either directly, or housed in specialised host-derived inclusions. Less commonly they are also found in cellular organelles such as the nucleus (Schulz, 2015), chloroplasts (Schmid, 2003), mitochondria (Sassera et al., 2006), or directly associated with endoplasmic reticulum (Vogt, 1992). The development of such symbioses is not trivial and depends to a large degree on the quality of communication between the partners. Symbionts usually require the recruitment of host ribosomes and endocytic and secretory machinery to establish their intracellular niche (Canton and Kima, 2012). Additionally the symbiont transmission modes, host range of the symbiont and presence and uptake of other symbionts can have large impacts on the stability of the association.

“The complicated interplay between virulence and transmissibility of parasites leaves room for many revolutionary paths to be followed, with many endpoints” (Anderson and May, 1982).

Trajectories of symbiotic relationships are diverse, but often exhibit commonalities. Symbiont genome evolution is to a large part influenced by symbiont transmission mode and has been described as (loosely) following either ‘open’, ‘closed’ or ‘mixed’ evolutionary modes (Perreau and Moran, 2022).

Frequent host switches (i.e. open symbiosis) and mainly horizontal transmission routes have generally been considered to lead to uncoupling of host and symbiont fitness and allow symbionts the preservation of greater genetic repertoires (Drew et al., 2021; Perreau and Moran, 2022). As such, symbionts can switch hosts with relative ease. They show high replication rates and frequent horizontal gene transfer (HGT) events with other microbes, allowing high pathogenicity both to hosts and other competing microbes. An evolution experiment involving a chlamydial symbiont of *Acanthamoeba* has shown a shift to such a pattern. After exclusive horizontal propagation to naïve hosts for 560 symbiont generations, the symbiont had shown increased expression of parasitism genes and genes related to infectivity such as the type III secretion system (T3SS) and stability and infectiousness of extracellular stages (Herrera et al., 2020).

On the other hand, long term host-association with mainly vertical transmission routes, is generally considered as selecting for benign or even mutualistic traits on both sides as fitness of the two organisms becomes more intertwined over time (Drew et al., 2021; Turner et al., 1998). Here, processes like Muller's ratchet in host restricted clonal lineages with multiple bottlenecks leads to the accumulation of deleterious mutations and genetic drift, ultimately leading to genome reduction and growing dependence on compensation by host genes (McCutcheon and Moran, 2012; Perreau and Moran, 2022; Sørensen et al., 2025). The current lower limit of such continuous genome reduction can be found in the 112 kb strong genome of the leafhopper symbiont *Nasua deltocephalinicola* (Moran and Bennett, 2014).

The so-called 'mixed' mode of symbiosis shows features of both open and closed symbioses. These are to a large part host-restricted, but still have opportunities to exchange genetic material with other co-infecting endosymbionts, or occasionally transfer to novel hosts (Perreau and Moran, 2022). Famous examples include the widespread reproductive parasite of arthropods *Wolbachia* (Vavre et al., 1999), species of whitefly infecting *Rickettsia* and *Hamiltonella* (Chiel et al., 2009), aphid infecting species of *Buchnera* (Russell et al., 2003) and divergent insect infecting species of *Sodalis* (Medina Munoz et al., 2020; Renoz et al., 2024). Symbionts in this context typically retained sufficient mechanisms to evade host immune systems without imposing highly virulent traits like excessive replication. Most described cases in mixed symbiosis are concentrated in arthropod hosts (Perreau and Moran, 2022). Many of these symbionts carry pathogen defense mechanisms that stem from and are transmitted through horizontal gene transfer (HGT) from diverse lineages. In open symbioses, such systems are readily exchanged as well, however often accompanied by significant pathogenic traits by the symbiont. Thus, mixed symbiosis plays a crucial role in providing antimicrobial defense systems to hosts, without elevated cost.

Microbial symbionts can impact the lifestyles of their hosts. They can influence their hosts' access to different habitats, change their physiology, behaviour, resilience and level of pathogenicity to other organisms. For instance, several studies in the parthenogenetic pea aphid *Acyrtosiphon pisum* show symbionts of the genera *Buchnera*, *Rickettsia* and *Hamiltonella* influenced phenotypes of body colour, resistance to parasitoid infection and thermotolerance through alleles encoded in the symbiont genomes (Dunbar et al., 2007; Moran and Yun, 2015; Oliver et al., 2009; Tsuchida et al., 2010). Host-symbiont relationships evolve under the constraints of symbiont transmission, nutritional benefits and necessities, available gene pools and susceptibility to horizontal- and endosymbiont gene transfer.

The thief

Chlamydiae are a group of bacteria with a unique biphasic and obligate intracellular lifestyle, belonging to the phylum Chlamydiota. The first description of chlamydiae in 1907 by the German physician Ludwig Halberstädter and Czech parasitologist Stanislaus von Prowazek was based on conjunctival swabs during an expedition on syphilis in Java (Halberstaedter and Prowazek, 1907). In their publication they describe stainings of swabbed eye smears that showed strongly stained, large masses inside cells, which they correctly identified as the infectious agent responsible for the eye disease trachoma. This disease, potentially leading to blindness, likely afflicted humans already thousands of years back and remains a concern today (Nunes and Gomes, 2014). They went on to call these organisms “Chlamydozoa”, after their cloaked morphology inside the host-cell derived inclusion (Halberstaedter and Prowazek, 1907).

In the following decades, samples showing similar morphology from pneumonia, urethritis and lymphogranuloma venereum cases were found and the whole group was categorized as the lymphogranuloma-trachoma-psittacosis group (Nunes and Gomes, 2014). Yet, still misconsidered as viruses as they passed through bacterial filters and were unculturable on common artificial media. Finally, in 1945 they were shown as separate from ‘true viruses’ due to their susceptibility to antibiotic sulfonamides and suggested to be placed between bacteria and viruses with the genus name *Chlamydia* (Jones et al., 1945).

With developments in the electron microscopy field, more observations on the morphology of chlamydiae were made, revealing two morphotypes appearing in two different phases of the infection progression. These were described to be composed of a large cell over 0.5 µm in diameter, termed the reticulate body or RB and a small form of about 0.3 µm with an electron dense nucleoid, termed the elementary body or EB (Bedson and Bland, 1932). In 1966 James Moulder in his comprehensive review on the biology of this group finally recognised chlamydiae as gram-negative bacteria mainly based on the presence of DNA, RNA, ribosomes and cellular division (Moulder, 1966). By that point

they were already believed to be energy parasites, depending on host metabolites to generate ATP, but Moulder also suggested they pirate ATP directly from the host (Moulder, 1962, 1966).

“It is generally agreed that there are properties that are highly characteristic of these life forms and some even unique to them” (James Moulder 1966)

These findings and suggestions have sparked much research, revealing the communication between chlamydiae and their hosts. Later discoveries like nucleotide transport proteins showed chlamydiae were indeed capable of scavenging ATP from their hosts (Tjaden et al., 1999). The sequencing of the first *Chlamydia trachomatis* genome in 1998 has led to a series of discoveries on chlamydial metabolic processes and virulence factors (Stephens et al., 1998) and was followed by many studies on a diverse range of chlamydial genomes, revealing genome structure, phylogeny and evolution (Dharamshi et al., 2023; Lagkouravdos et al., 2014; Luu et al., 2023).

Today, despite the expanding set of genes, metabolic pathways and capabilities that are found in the ever growing diversity of chlamydial lineages, at the core, chlamydiae still share basic hallmark features. Features common to all chlamydiae include the reliance on a host organism for energy parasitism by scavenging ATP, nucleotides and glucose (Collingro et al., 2020; Horn, 2008), manipulation of host cells through secretion of effectors and virulence factors using the highly conserved type III secretion system (T3SS) (Collingro et al., 2011; Pillonel et al., 2018)) and control of the biphasic developmental cycle via the EUO gene, a regulator repressing late genes and enabling the alternation between the infectious EB and replicating RB stages (Collingro et al., 2020).

Chlamydiae are immensely successful at infecting a large and very diverse number of eukaryotic hosts, from humans and livestock, to fish, arthropods and diverse unicellular eukaryotes such as protists (Collingro et al., 2020; Taylor-Brown et al., 2015) (Figure 1). This success lies partly in their ancient association with eukaryotic hosts that dates back about 1-2 billion years (Dharamshi et al., 2023).

Environmental lineages of chlamydiae, i.e. those not falling into the vertebrate-infecting Chlamydiaceae, have proven to be a resourceful model for understanding the evolutionary and molecular mechanisms behind this symbiosis. They are environmentally widespread, phylogenetically diverse and often form symbiotic associations with free-living protists, that present challenging model systems to study in the lab, but can in some cases be substituted with easy-to-cultivate model protist hosts (Collingro et al., 2020; Corsaro and Venditti, 2006; Horn, 2008).

Some environmental chlamydiae show association with a range of human and animal diseases, like the early on described *Simkania negevensis* Z, discovered as a cell culture contaminant, was later

associated with pneumonia in humans (Kahane et al., 1993; Lieberman et al., 1997), or *Waddlia chondrophila*, which was isolated from an aborted bovine fetus (Rurangirwa et al., 1999).

However, further descriptions of environmental chlamydiae showed the story is not that simple and though there is association with humans and animals, primary hosts are often environmentally widespread protists (Collingro et al., 2020). This wide range of associations informed later hypotheses of protist hosts acting as ‘training grounds’ for the acquisition and development of virulence traits (Molmeret et al., 2005). As such protists would provide a place for the exchange of genes with other co-infecting microbes, conferring a multitude of functions.

In the case of environmental chlamydiae, a large number of genes were predicted to be of horizontally transferred origin, rates of which were estimated to be similar to free-living relatives in the Planctomycetotoa-Verrucomicrobiota-Chlamydiota superphylum (Dharamshi et al., 2023).

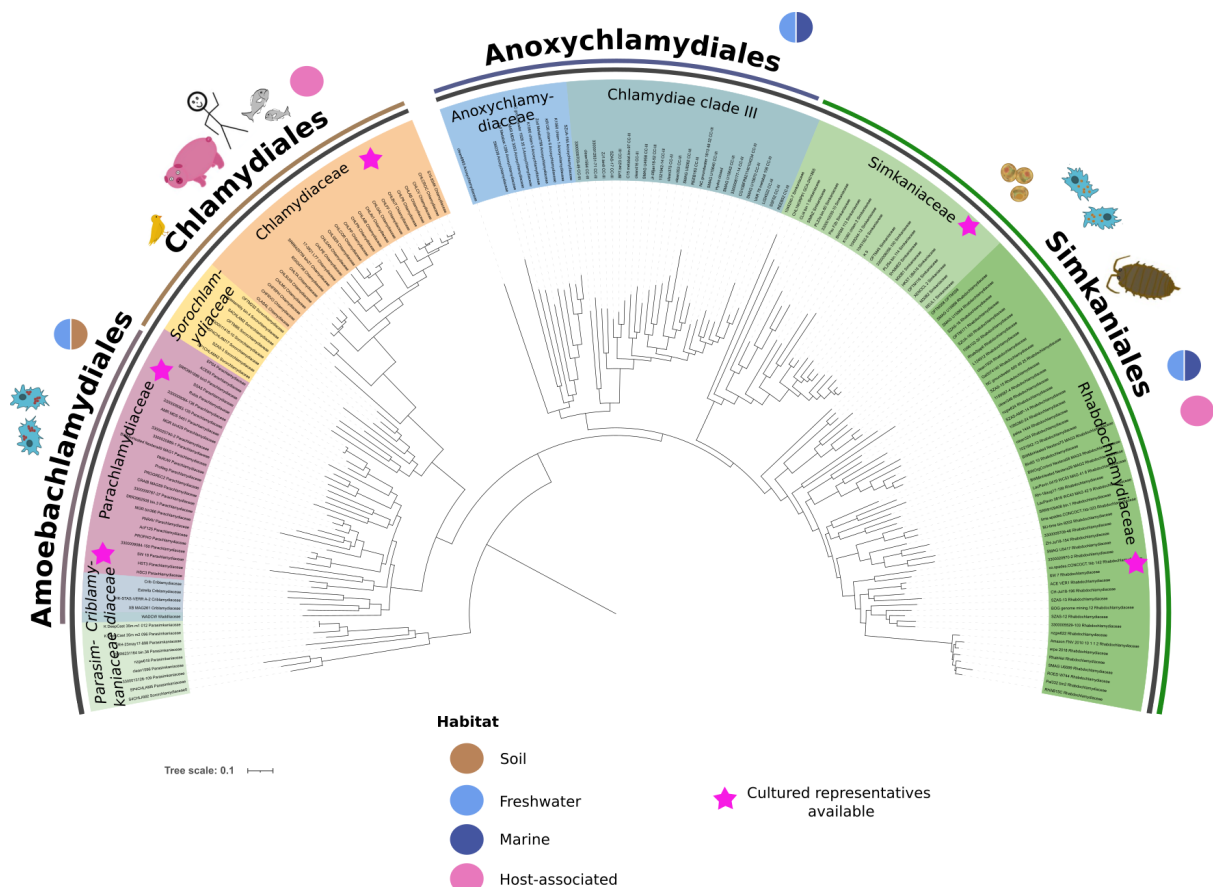


Figure 1. Phylogenetic tree showing the relationship of species in the phylum Chlamydiota. A maximum likelihood phylogenetic tree based on 43 concatenated conserved marker proteins in 227 chlamydial genomes is shown. Scale bar represents 0.1 amino acid substitutions per site. Circles represent common habitat types they are found in together with a selection of common host organisms for each respective group.

Although chlamydiae-host interactions always constitute a demand on host energy resources, the communication and interaction between host and symbiont appears more complex and balanced in areas not immediately visible. Several studies have shown a protective function against other amoeba-infecting bacteria and viruses (Arthofer et al., 2022; Ishida et al., 2014; König et al., 2019). Sponge-associated chlamydiae additionally show an enrichment in genes for degrading diverse compounds found in sponges and on a whole the phylum harbours a widespread genetic potential for secondary metabolite production (Dharamshi et al., 2022), possibly explaining some of the observed protective effects.

Environmental chlamydiae impose an energy demand on their hosts and come at a high cost, but are often very successful at maintaining their intracellular niche. From our current standpoint, environmental chlamydiae mostly follow the genomic trajectory of an open symbiosis, with frequent host switches and a tendency to pick up virulent traits via HGT (Dharamshi et al., 2023; Köstlbacher et al., 2021), lending them higher independence and a broader host range than the vertebrate pathogenic chlamydiae. They can appear in unusually high abundances especially in highly diverse and competitive environments like soil or wastewater (Collingro et al., 2020), which indicates they play a potentially important ecological factor for the hosts in these environments.

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Thesis aims

This thesis is centered around the environmental chlamydiae and aims to show their importance as model systems for both diverse host-microbe interactions and as environmentally important members of microbial communities.

Key aims are as follows:

Protist symbionts - overlooked players in the microbial soil food web (Manuscript I)

Authors:

Angelika Schwarzhans, Christian Ranits, Andreas Richter, Matthias Horn, Astrid Collingro

- Develop a eukaryote-less soil microcosm setup with a near-natural microbial community to investigate protist predation under the influence of chlamydial parasites with varying degrees of host parasitism.
- Compare host and symbiont growth dynamics under laboratory and in complex soil microcosms.
- Assess changes in microbial community composition driven by predation using fast-response rRNA-based analyses across the different host-microbe systems.
- Identify potential groups especially affected by predation from symbiont-carrying hosts compared to aposymbiotic hosts.
- Reveal effects on ecosystem-level processes, including microbial biomass, growth and respiration as well as carbon use efficiency and nitrogen mineralization, using stable isotope techniques.

Environmental chlamydiae at the animal-protist interface: A Hydra-associated chlamydial symbiont (Manuscript II)

Authors:

Angelika Schwarzhans, Justine Boutry, Jácint Tökölyi, Norbert Cyran, Martin Kunert, Matthias Horn, Astrid Collingro

In revision at:

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- Employ genome sequencing, fluorescence microscopy and electron microscopy to characterize the first isolate of a chlamydial family so far represented by metagenomic data only.
- Describe the stability of the *Hydra*-symbiont association.
- Investigate symbiont transmissibility and potential environmental sources.
- Analyse genomic content and rearrangements that indicate adaptations to *Hydra* as a host.
- Investigate *Hydra* as a host for other environmental chlamydiae by screening natural *Hydra* populations and identifying conserved genes involved in host communication among the chlamydiae.

Chapter II

Protist symbionts - overlooked players in the
microbial soil food web

Protist symbionts - overlooked players in the microbial soil food web

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Abstract

Protists in soil contribute to primary production and play key roles in decomposition pathways as major consumers of bacteria and other microbes. *Acanthamoeba* species are part of a dominant group of soil protists that served as model organisms, unravelling the significant effects of protist predation on soil microbial communities and ecosystem processes, while also frequently serving as hosts to intracellular bacterial symbionts such as members of the phylum Chlamydiota. While chlamydiae are traditionally studied as intracellular pathogens or symbionts in simplified laboratory systems, their role in modulating ecological interactions and ecosystem functioning in soil remains unexplored. We hypothesised that by affecting their hosts' fitness, chlamydiae indirectly shape microbial community structure and key ecosystem processes. To test this, we investigated the impact of two chlamydial symbionts with different levels of parasitism towards the same *Acanthamoeba* host in a controlled soil microcosm setup over a period of 35 days. Digital PCR and amplicon sequencing based on reverse transcribed 16S and 18S ribosomal RNA showed that the impact chlamydial symbionts have on their host and the microbial community depends on the fitness-cost imposed on the amoeba host. The altered host abundance and predation pattern resulted in shifts of the bacterial community even after a significant decrease of the amoeba host population. At the same time, host effects on microbial growth, respiration and gross nitrogen mineralization rates were amplified with only the milder of the two symbionts. Our findings demonstrate that intracellular bacterial symbionts can profoundly influence soil ecosystem dynamics by altering the behavior and survival of microbial predators. These results position bacterial endosymbionts not only as important components of the protist interactome, but also as regulators of broader microbial communities and soil processes.

Introduction

Protists make up the bulk of eukaryotic diversity. They are keystone organisms in many environments, but play especially crucial roles in soil ecosystems as primary consumers of bacterial biomass, driving the microbial loop by releasing nutrients bound in microbial cells and transferring it to higher trophic levels when serving as a food source themselves (Geisen and Bonkowski 2018).

Chlamydiae are a group of obligate intracellular bacteria best known for their role as pathogens in humans and animals. Environmental lineages of chlamydiae are widespread and phylogenetically diverse, often forming symbiotic associations with free-living protists (Lagkouvardos, Weinmaier, et al. 2014; Astrid Collingro et al. 2020). In laboratory settings, chlamydiae have proven to be a resourceful model for studying host-symbiont interactions, particularly due to their obligate intracellular lifestyle and long-standing evolutionary relationship with eukaryotic hosts during which

they have evolved a unique biphasic developmental cycle. This developmental cycle consists of an alternating replicative intracellular stage (reticulate body, RB) and a non-replicative infectious stage (elementary body, EB) which is able to survive extracellular conditions (Taylor-Brown et al. 2015; Greub and Raoult 2004; AbdelRahman and Belland 2005). Protists are natural hosts of chlamydiae and often serve as isolation tools, with the genus *Acanthamoeba* being the most commonly used due to its ease of cultivation and frequent natural association with intracellular microbes (Rayamajhee et al. 2021; Corsaro and Venditti 2006). The typically low abundance of chlamydiae in many microbiomes has led to their categorization as part of the "rare biosphere" (Astrid Collingro et al. 2020), yet their frequent detection in soil and rhizosphere samples suggests ecological significance (Astrid Collingro et al. 2020; Dharamshi et al. 2023).

Despite extensive laboratory research, the ecological role of environmental chlamydiae in natural ecosystems remains poorly characterized. One described example of ecological context shaping the relationship between chlamydiae and amoeba hosts, is the *Acanthamoeba* symbiont *Protochlamydia amoebophila*. In a pure host-microbe culture, the presence of the chlamydial symbiont causes a reduction of its host's growth-rate. However under more complex conditions involving the amoeba pathogen *Legionella pneumophila*, the symbiont protects its host from infection with the pathogen, ultimately enhancing host fitness (König et al. 2019), an effect also found for the chlamydial species *Neochlamydia* S13 (Ishida et al. 2014). Another example is the *Acanthamoeba*-infecting chlamydial strain *Parachlamydia acanthamoebae*, which in a recent study has been shown repressing the replication of two giant viruses from divergent lineages of the Nucleocytoviricota (Arthofer et al. 2022). Both the proto- and parachlamydial symbionts were used as model organisms in the current study.

Chlamydiae are ideally suited as a model system to study endosymbiotic interactions under more ecologically relevant, complex conditions: They have known functional impacts on their hosts, are integral parts of microbial food web components through their protist hosts, and are unable to replicate outside a host cell, rendering them unlikely to have an ecological impact on their own outside the context of their hosts.

Acanthamoeba is a globally distributed and ecologically versatile genus of free-living amoebae, capable of thriving in diverse environments including freshwater, marine, and especially soil habitats (Siddiqui and Khan 2012). In soil, *Acanthamoeba* functions as a key microbial predator, feeding mainly on bacteria and fungi through phagocytosis. This grazing behavior plays a pivotal role in shaping microbial community composition and function. Several studies have shown that through predation *Acanthamoebae* can increase the relative abundance of plant beneficial, predation-resistant bacterial taxa such as *Pseudomonas*, *Sphingomonas*, and *Azospirillum*, while suppressing plant-pathogenic bacteria (Martins et al. 2022; Rønn et al. 2002; Rosenberg et al. 2009; Guo et al. 2021). Beyond reshaping microbial communities, *Acanthamoeba* and other protists are known to influence

key soil ecosystem processes, particularly nutrient cycling. By consuming microbial biomass, protists contribute to the mineralization of nitrogen and other nutrients, releasing them in plant-available forms and stimulating microbial respiration (Sinclair et al. 1981; Bonkowski and Clarholm 2012; Clarholm 1985). This protist-driven turnover enhances plant growth and ecosystem productivity. However, the influence on the outcomes of these processes likely depend on the internal state - i.e. symbiont carrying or aposymbiotic - of the predator itself.

Importantly, many fresh *Acanthamoeba* isolates were found to naturally contain bacterial endosymbionts, including chlamydiae, suggesting that endosymbiosis is the rule rather than the exception for these protists (Lagkouvardos, Shen, et al. 2014; Husnik et al. 2021). Yet, a major gap persists: although we know that chlamydiae can influence the fitness and behavior of their amoeba hosts under laboratory conditions, and we know that amoebae impact nutrient dynamics and microbial communities in soil, to our knowledge no study has bridged these two domains so far. Therefore we aimed to give a first understanding of how symbiont-induced changes in amoebal behavior translate into altered ecosystem functions. We made use of the common soil amoeba *Acanthamoeba terricola* (formerly *Acanthamoeba castellanii*) and its chlamydial symbionts *Protochlamydia amoebophila* and *Parachlamydia acanthamoebae* to investigate how bacterial endosymbionts influence microbial predation and ecosystem processes.

Specifically we address:

- (I) The impact of chlamydiae on host growth known from the lab to the complex habitat of soil and in the presence of a complex microbial community.
- (II) Protist grazing and feeding preferences. Does this grazing behaviour change with the presence of intracellular symbionts?
- (III) Release of mineralized nitrogen, microbial respiration and growth rates. Does the presence of intracellular symbionts have an increasing, or decreasing effect on these ecosystem-wide processes?

Results

To investigate the ecological impact of chlamydial symbionts on their amoeba host and in turn a soil bacterial community, we established a simplified soil microcosm system. This system consisted of sterilised soil that was re-inoculated with a filtered (1.2 μm pore size) microbial community stemming from the same soil, excluding most eukaryotes (Figure 1b). These “microcosms” were amended with four different treatments.

The soil community control (**SC**) has no additions and represents a host-free treatment consisting only of the re-introduced filtered microbial community. The second treatment contains only the aposymbiotic amoeba *Acanthamoeba terricola* NEFF (formerly known as *A. castellanii* NEFF; Figure 1 a(i)), a bacterivorous soil amoeba (**Apo**). The third treatment is that same amoeba strain carrying the chlamydial symbiont *Protochlamydia amoebophila* UWE25 (Figure 1 a(ii), Supp. Figure 1) (**Mild**), termed the “Mild” treatment, for the symbionts’ comparatively small influence on the host growth under laboratory conditions. The fourth treatment includes the same host carrying the chlamydial symbiont *Parachlamydia acanthamoebae* UV7 (Figure 1 a(iii)) (**Strong**), termed “Strong” treatment for its relatively strong reduction of host population size through its lytic effect under laboratory conditions. Initially, all microcosms were pre-incubated at ambient temperatures for 13 days with the bacterial soil community only. The different treatments were then set up and further incubated for up to 35 days to monitor microbial community structure and ecosystem processes (Figure 1b). In total 140 microcosm vessels were set up in this way, inoculated with the respective treatments on day 0 of the experiment and then sampled destructively. This meant per sampling time point 28 such microcosms were sampled, 7 per treatment were always used for DNA and RNA extractions (community analyses), only 5 of those 7 replicates were used for isotope incubations (ecosystem measurements).

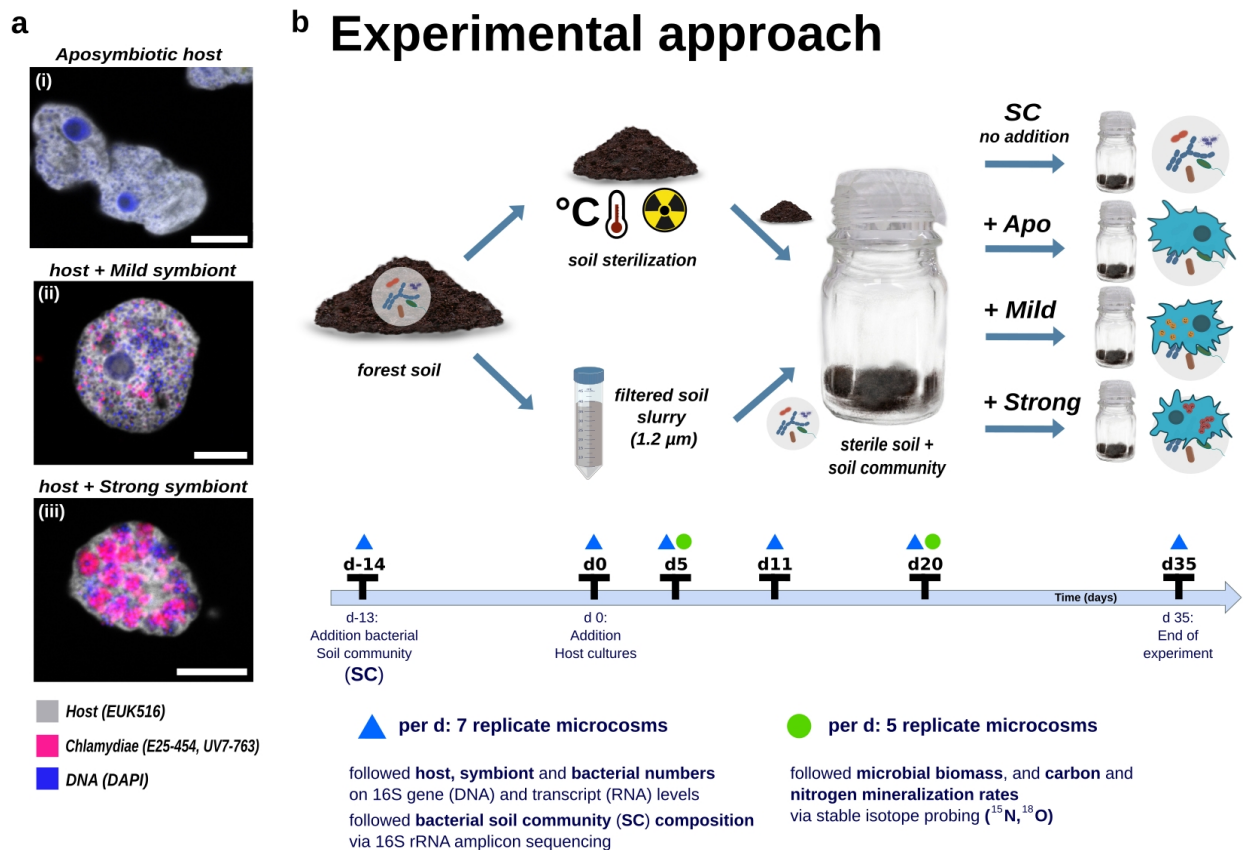


Fig. 1. Experimental set-up to study the impact of bacterial symbionts of amoeba on microbial community structure and ecosystem processes in soil microcosms. **a)** Confocal microscopy images of fluorescence *in situ* hybridizations of the organisms used in this study: **(i)** Aposymbiotic host *Acanthamoeba terricola* NEFF co-cultivated with either **(ii)** the “Mild” parasite *Protochlamydia amoebophila* UWE25 or **(iii)** the “Strong” parasite *Parachlamydia acanthamoebae* UV7. Host is depicted in grey (probe EUK516), symbionts in magenta (specific probes E25-454 and UV7-763, respectively), DNA in blue (stained with 4',6'-diamidino-2-phenylindole (DAPI)). Scale bars indicate 10 µm. **b)** Overview of the experimental approach. Sterilized soil was amended with a bacterial soil community 13 days (d-13) prior to addition of three different soil protist amendments (Apo, Mild or Strong). Per time point seven independent microcosm flasks were set up for each treatment. On each of those time points microcosms were sampled destructively and DNA and RNA were extracted (blue triangles). Extracts were used to follow host, symbiont and bacterial community numbers and to analyse bacterial community composition. On days 5 and 20 (green circles) 5 replicate microcosms were sampled to follow N and C mineralization and DNA synthesis.

Symbiont parasitism shapes host survival in soil

Under axenic conditions, i.e. during cultivation in nutrient-rich standard media without the addition of bacteria as foodsource, the aposymbiotic *Acanthamoeba* host displays a typical logarithmic growth curve. When freshly infected with the mild parasite (*P. amoebophila*), the amoeba host exhibits a generally slower initial growth followed by a gradual decline in abundance after about two weeks of cultivation. Infection with the strong parasite (*P. acanthamoebae*) leads to a cessation of host growth within two days. This is then followed by a later resurgence of host growth 21 days post infection,

when host numbers start to rapidly increase again with no loss of the parasite, reminiscent of Lotka-Volterra predator-prey relationships (Fig. S1).

To assess how symbiont parasitism affects *Acanthamoeba* survival and activity in soil, we quantified both 18S rRNA and 16S rRNA gene and transcript abundance of host and symbiont using droplet digital PCR (ddPCR) with *Acanthamoeba*- and chlamydiae-specific primers (Figure 2a-d). During the soil microcosm incubation the aposymbiotic *Acanthamoeba* hosts community did not change significantly in abundance over a period of 35 days (Figure 2ac). In contrast, symbiont-carrying hosts showed significant declines in both 18S rRNA transcriptional activity and gene abundance in a manner reflective of the symbiont parasitism. Hosts carrying the mild symbiont showed a significant reduction in rRNA transcripts (GLM, $p=0.031$) and a non-significant decrease of rRNA gene copy number (Figure 2 ac). The strong symbiont had a more drastic impact on host activity and numbers, with average rRNA transcripts significantly decreasing by more than two orders of magnitude (GLM, $p<0.0001$) and rRNA gene copy number decreasing below the limit of calculation for ddPCR (GLM, $p<0.0001$) (Figure 2 ac).

At the same time, we noticed the rRNA transcript abundance remained remarkably high compared to rRNA gene copy abundance in the Strong treatment on days 20 and 35 (Figure 2 ac). This could be indicative of a remaining host population, only detectable on rRNA level, would mirror the resurgence of the host population we observed under axenic conditions after 21 days (Fig. S1).

The chlamydial rRNA gene copy and rRNA transcript numbers remained mostly stable and similar for both symbiont strains (Figure 2 bd). Although rRNA gene copy numbers of the mild symbiont decreased by trend leading to lower genome copy abundance by day 35 (GLM, $p=0.002$) (Figure 2d). Differences in symbiont transmission modes became evident as the drop in host abundance in the Strong treatment is not reflected in the symbiont abundance. To visualize this we compared the ratios of symbiont-to-host rRNA transcript copies. In the mild symbiont, which is primarily vertically transmitted (during cell division (Herrera et al. 2023)), symbiont abundance closely mirrors that of the host, resulting in a stable ratio over time (Figure 2e). Whereas in the case of the strong symbiont, which primarily relies on horizontal transmission (through cell exit and infection of new cells), we see a progressive increase in symbiont abundance compared to host abundance over time (Wilcoxon test; $p=0.0005$) (Figure 2e), consistent with an accumulation of EBs outside of amoeba and reflective of its more parasitic lifestyle .

Together, our observations show that chlamydial effects on their host in a complex environment broadly mirrors laboratory findings. In addition we show that rRNA levels of the host can remain more stable and do not follow the steep decline in genome copy numbers as seen in the Strong treatment.

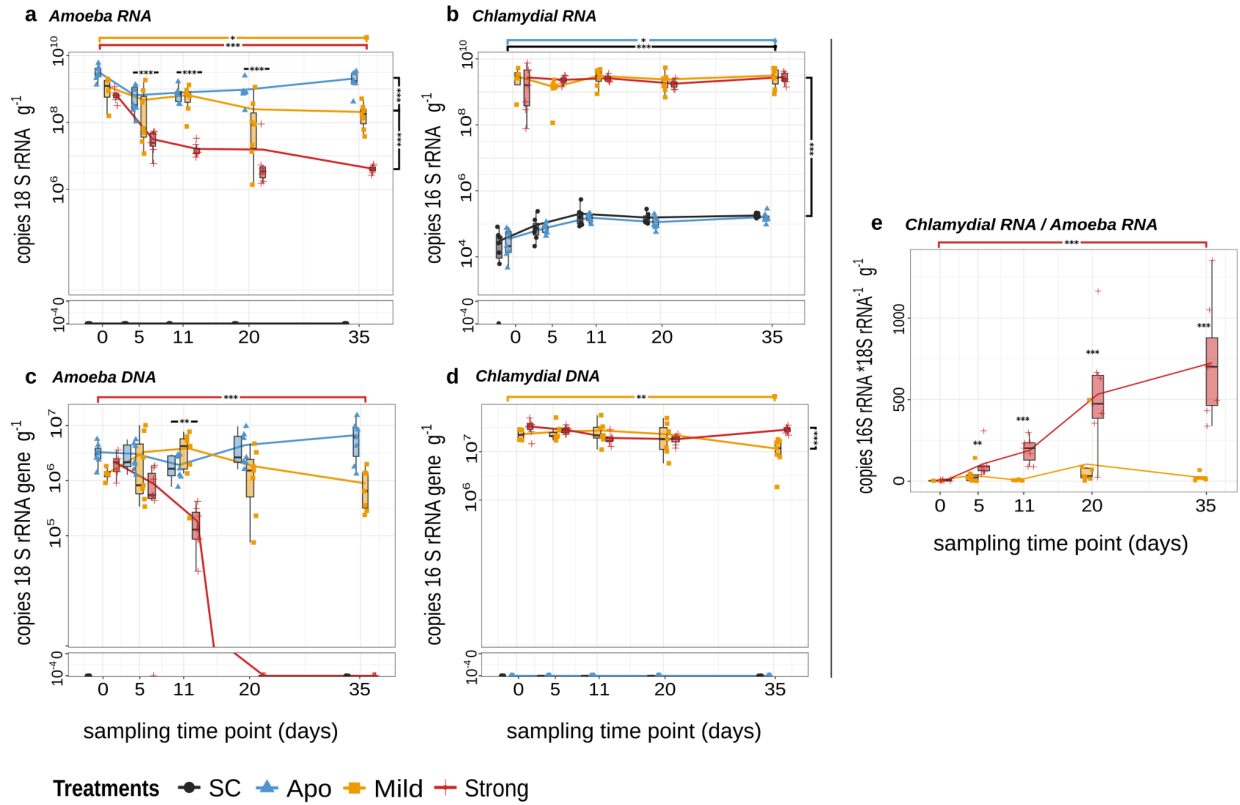


Fig. 2. Symbiont survival and impact on host survival. **a)** Absolute copy number of host 18S ribosomal RNA transcript (RNA) and **c)** 18S rRNA gene (DNA) as well as **b)** chlamydial 16S rRNA and **d)** 16S rRNA gene, quantified via droplet digital PCR (ddPCR). **e)** Ratio of chlamydial 16S rRNA to host 18S rRNA transcript copy numbers. Boxes indicate the interquartile range, the line indicates the median, whiskers indicate minimum and maximum values, respectively. At each sampling time point seven independent replicate microcosms were measured per treatment. Statistical analyses are based on GLM and post-hoc EMMs comparing treatment specific temporal changes and between treatment differences. Stars indicate statistical significance (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Amoeba-symbiont interactions shape the active bacterial soil community

Protists have well established abilities in shaping soil and rhizosphere microbiomes, typically leading to a decrease in total bacterial biomass, while stimulating microbial activity at the same time (Adl and Gupta 2006; Asiloglu et al. 2021; Gao 2020; Gao et al. 2019; Geisen et al. 2021). To assess how the symbionts influence the host impact on soil bacterial community composition and activity, we monitored both bacterial 16S rRNA gene and transcript levels across the four treatments (SC, Apo, Mild and Strong) using ddPCR with a universal bacterial/archaeal primer pair (Apprill et al. 2015; Parada et al. 2016) (Figure 3ab). As we expected most of the predation to happen on the more active, fast-growing bacterial community (typically comprised of the more palatable, Gram-negative, r-strategists (Thompson et al. 2021)), we focussed the analysis of bacterial community composition on the RNA based fraction using amplicon sequencing of 16S rRNA transcripts (J. Li et al. 2021; Che et al. 2015; Hausmann et al. 2016). Normalization with total copy numbers from the ddPCR assays allowed us to infer absolute bacterial transcript numbers rather than relative figures (Figure 3c).

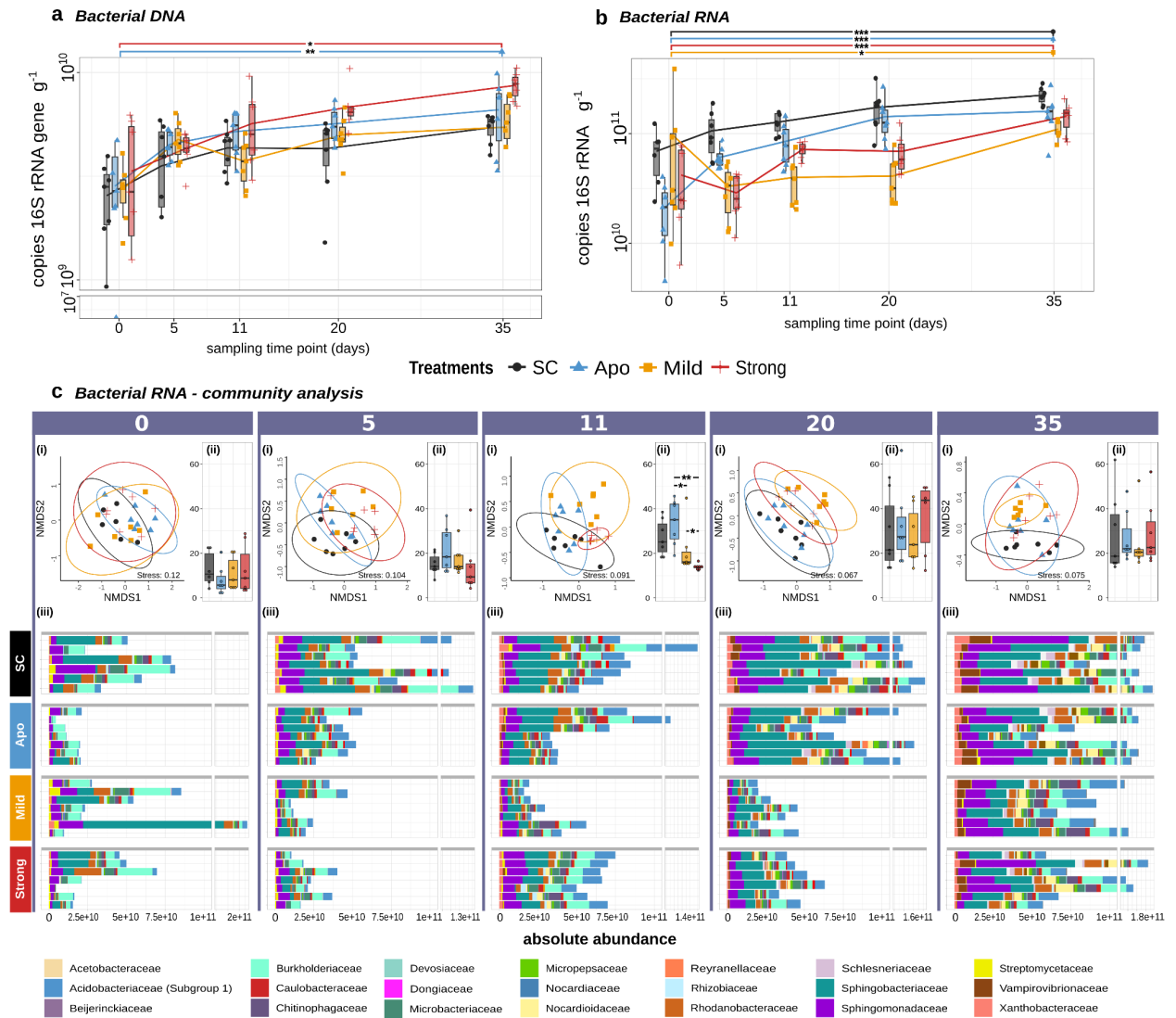


Fig. 3. The effect of bacterial symbionts on amoeba predation and bacterial community composition. Absolute quantification of **a**) bacterial 16S rRNA transcript (rRNA) and **b**) 16S rRNA gene copy (rDNA) numbers via droplet digital PCR (ddPCR), at each sampling time point seven independent replicate microcosms were analyzed per treatment. **c**) Microbiome diversity and composition of active bacteria. Each panel shows data based on sequencing of total 16S rRNA of all sampling timepoints (0-35) and treatments (n=7 per timepoint): **(i)** Beta diversity of soil bacterial communities in the four different treatments based on Bray-Curtis dissimilarity matrices, visualized with non-metric multidimensional scaling (NMDS) ordination. **(ii)** Box plots illustrating alpha diversity within treatments using inverse Simpson index based on bacterial richness and evenness. **(iii)** Absolute abundances of the 21 most abundant bacterial families across all time points. Boxes indicate the interquartile range, the line indicates the median, whiskers indicate minimum and maximum values, respectively. All ddPCR statistical analyses are based on GLM and post-hoc EMMs comparing treatment specific temporal changes (a, b), Wilcoxon rank sum test was used for alpha diversity statistical analyses (c (ii)). Stars indicate statistical significance (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Symbionts enhance grazing activity of amoeba. Monitoring bacterial 16S rRNA gene copy number - as a proxy for bacterial/archaeal cell counts and growth potential - revealed a steady, but shallow increase of bacterial abundance across all treatments over time (Figure 3a). Significant increases are only observed in the Apo and Strong treatments (GLM, Apo: $p=0.001$; Strong: $p=0.017$). More variation could be observed in the RNA based bacterial community. By monitoring 16S rRNA transcript levels (Figure 3b) we found bacterial abundance in all treatments increases significantly over time (GLM, SC: $p=0.0004$; Apo: $p<0.0001$; Strong: $p<0.0001$; Mild: $p=0.046$). However, bacterial abundance in the Mild treatment does not increase until day 20 compared to the upwards trend seen in both the SC and Apo treatments and from day 11 also in the Strong treatment. In both symbiont treatments bacterial abundance remains below that of the other two treatments throughout the incubation period. Overall effects of the treatments were mainly confined to the RNA based bacterial community, while community genome copy abundance may be more reflective of the bacterial biomass accumulation.

Symbionts affect microbial community composition. Next we characterized how predation affected changes in the diversity and composition of the RNA based bacterial community (Figure 3c). Across all treatments, the RNA based bacterial communities were dominated by four bacterial families (*Sphingobacteriaceae*, *Sphingomonadaceae*, *Burkholderiaceae* and *Acidobacteriaceae*) which were consistently above 8% relative abundance and typically ranged around 15% across all treatments and time points (table S1; Figure 3c(iii)). We then used non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarity to visualize differences across treatments (beta diversity) (Figure 3c(i)). This showed that bacterial communities had already started to diverge by day 5 into two major clusters composed of either the SC and Apo treatments or the Mild and Strong symbiont treatments (PermANOVA; $p=0.001$). These clusters remained stable until days 11 (PermANOVA; $p=0.001$) and 20 (PermANOVA; $p=0.001$). We also compared within-treatment (alpha) diversity using primarily the Inverse Simpson Index based on species richness and evenness (Figure 3c(ii)), but also Shannon and Simpsons evenness as well as richness and dominance based indices including Chao1 (Fig. S2). Both symbiont treatments showed a significantly lower alpha diversity only on day 11 (Inverse-Simpson) - most prominently in the Strong treatment compared to the non-symbiont treatments (Wilcoxon test; SC ~ Strong $p=0.002$; Apo ~ Strong $p=0.002$; Apo ~ Mild $p=0.04$; Mild ~ Strong $p=0.01$). Notably, the total abundance of the RNA based bacterial communities was also significantly lower in the symbiont treatments on days 5, 11 and 20 (Figure 3bc(iii)).

We next categorized the 16 most abundant bacterial families across time points into existing “r” or “K” bacterial life-history traits based on our review of scientific literature (table S2). These categorizations follow the assumption that r-strategist bacteria thrive in a copiotrophic environment, have short life-cycles, large nutrient requirements and are typically Gram-negative, while K-strategist

bacteria thrive under oligotrophic conditions, show slower growth rates, but have high substrate affinities. They tend to persist in environments through adaptations like spore formation and secondary metabolite production and are typically Gram-positive (Peng et al. 2022; Fierer et al. 2007). R-strategist taxa in our microcosms decrease in relative abundance in all treatments throughout the incubation period, while K-strategist taxa increase in all treatments only from day 0 to 11, while (Fig. S3). K strategists show a significant spike in abundance on day in the Strong treatment (Wilcoxon test $p=0.023$), but return to previous values already by day 20 (Fig. S3).

Early changes affect trajectories of bacterial community shifts. To gain more understanding of how genus-level taxa and amplicon sequence variants (ASVs) are affected by the symbiont treatments, we performed differential abundance analysis, comparing against the symbiont-free Apo (Figure 4a) and host-free SC (Fig. S4a) treatments.

We found the majority of differentially abundant genera to have overall reduced abundance in the Mild and Strong treatments compared to the SC and Apo treatments, between days 5 and 20 and most pronounced on day 11 (Figure 4a, Fig. S4a-c, table S3). The genera most negatively affected in differential abundance in the Mild and Strong treatments include the three highly abundant genera *Burkholderia-Caballeronia-Paraburkholderia*, *Mucilaginibacter* and *Granulicella* and the four lesser abundant genera *Chthoniobacter*, *Edaphobaculum*, *Chitinophaga*, *Pseudomonas*, and *Reyranella* (all highlighted in bold in Figure 4a). The highly abundant genus *Sphingomonas* had one of the highest numbers of differentially abundant ASVs, however entirely mixed between positively and negatively affected ones in the Mild and Strong treatments compared to Apo (highlighted in bold in Figure 4a). When comparing the Apo to the SC treatment, a similar, but less pronounced reduction in abundance could be found for the taxa *Chthoniobacter* and *Burkholderia-Caballeronia-Paraburkholderia* and abundance of *Sphingomonas* is much more negatively affected (Fig. S4b).

We then further investigated the effect of symbiont treatments compared to Apo by looking at discrepancies between the number of affected ASVs and abundance within a genus. We noticed, the genera *Chthoniobacter* and *Mucilaginibacter* both showed the highest amounts of negatively affected ASVs, most pronounced in the Strong treatment and on day 11 (Figure 4b) (Strong: 15 and 36 respectively; Mild: 2 and 7 respectively (Figure 4c)). At the same time the genera *Pseudomonas* and *Reyranella* had few negatively affected ASVs, which however made up the bulk of the genus' abundance, thereby causing a strong decline in overall abundance of the genus, again most pronounced on day 11, this time in both the Mild and Strong treatments (Figure 4b). Both effects are visualized as each genus' abundance and log2 fold change compared to Apo (Figure 4b).

Overall most shifts in abundance of the RNA based bacterial community occur on day 11, when both symbiont treatments show a spike in differentially abundant taxa (Figure 4c).

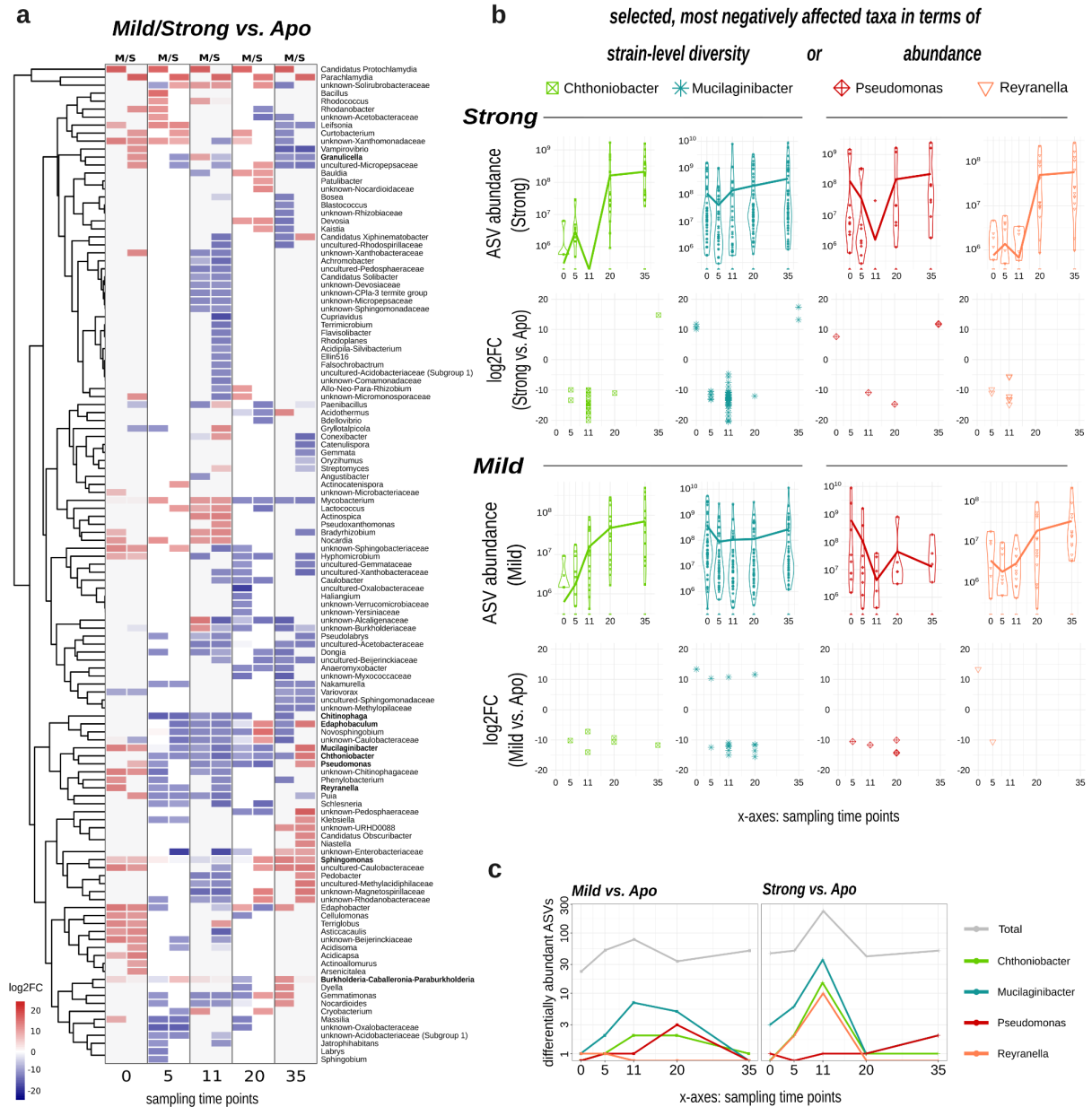


Fig. 4. Response of bacterial taxa in the presence of hosts carrying bacterial symbionts. **a)** Heatmap (using hierarchical clustering based on euclidean distances) depicting differential abundance of the active bacterial community of either *Parachlamydia acanthamoeba* UV7 (Strong, S) or *Protochlamydia amoebophila* UWE25 (Mild, M) parasite treatments compared to the aposymbiotic host (Apo) treatment, at each sampling time point (0-35) as based on sequencing of the 16S rRNA transcripts (n=7 per timepoint). Red: increased abundance, blue: decreased abundance. **b)** The four selected genera were found to be most affected in the symbiont treatments either in the amount of negatively affected ASVs (Chthoniobacter and Mucilaginibacter), or in terms of an abundance drop stemming from few ASVs making up the majority of the genus' abundance (Pseudomonas and Reyranella). Violin plots show absolute abundance of ASVs in each genus under either the Mild or Strong treatment, violin width represents the frequency of data points. log2-fold changes (log2FC) represent ASV abundance in each genus compared to the Apo treatments. **c)** Number of differentially abundant ASVs for the treatments Mild and Strong compared to the Apo treatment. Total amount in grey and for each of the four genera shown in 4b.

In summary, amoebae carrying a symbiont had stronger effects on bacterial community abundance and composition than aposymbiotic amoebae. The observed effects were concentrated at day 11 and lead to lasting changes in the RNA based bacterial community. They were also shaped by symbiont identity, with more reduction of total bacterial abundance over time in the Mild treatment, and more specific effects on community composition and differential abundance of certain taxa by the Strong treatment.

Symbiont-host interactions affect soil ecosystem processes

Stimulation and increased turnover of bacterial biomass by predation from protists has been shown to lead to an increase in microbial respiration, nutrient turnover and mineralization of nitrogen and carbon (Adl and Gupta 2006; Asiloglu et al. 2021; Gao 2020; Gao et al. 2019; Geisen et al. 2021). We therefore set out to capture the effects of altered predation in our symbiont treatments on these processes. Microbial respiration and growth as a measure for microbial activity and gross nitrogen mineralization to estimate nutrient release from predation were measured on the incubation days 5 and 20 (table S4). Analysis of C-based measurements was not possible for day 5 (see Methods), only nitrogen mineralization data was included in the analysis for that day.

Pronounced changes in microbial biomass, growth and respiration. Amoeba predation in the Strong treatment appears alleviated by day 20 through the vastly reduced host population (Figure 2 ac), thus enabling microbial biomass to recover. This is supported by measurements of microbial biomass carbon (C_{mic}) on that day, which was highest in the Strong and SC treatments and lowest in the Mild treatment (Tukey's HSD test $p=0.019$ and $p=0.044$ respectively) (Figure 5a). This is consistent with the low bacterial abundance (Figure 3c(iii)) and the retained activity of the amoeba host population in the Mild treatment on that day (Figure 2 ac). At the same time the Apo treatment shows high variability in microbial biomass, with some replicates reaching values as low as the Mild treatment. A contrasting pattern can be seen both in microbial growth and respiration rates, where Strong and SC treatments have similar values while the Mild treatment has significantly higher growth (Tukey's HSD test Strong: $p=0.0001$; SC: $p=0.0001$) and respiration rates compared to Strong and SC (Tukey's HSD test Strong: $p=0.004$; SC: $p=0.009$) (Figure 5bc). Again, the Apo treatment shows growth and respiration rates in between Strong/SC and Mild, with a significantly lower growth rate than the Mild treatment (growth: Tukey's HSD test $p=0.011$). Together, the impact seen by the Mild treatment had overall the largest decreasing effect on microbial biomass, thus promoting activity, leading to increased microbial growth and respiration rates.

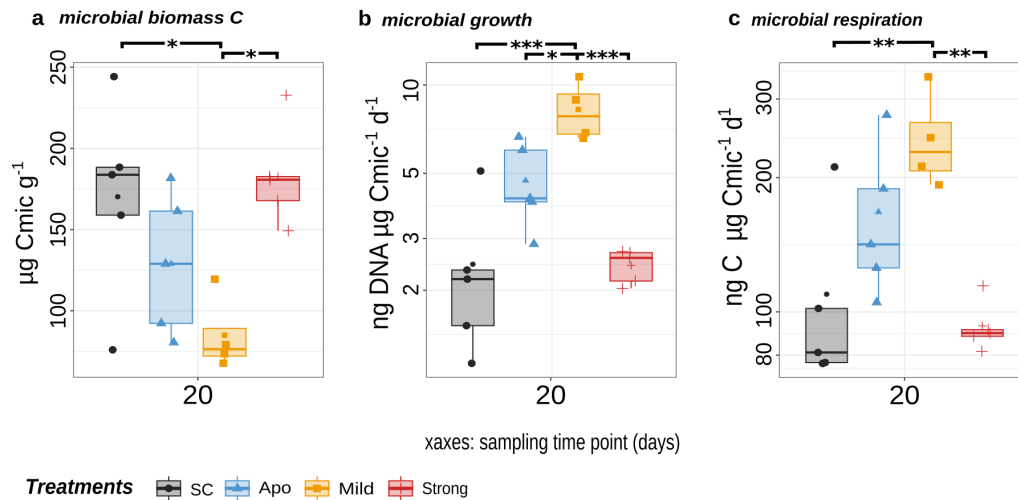


Fig. 5. Impact of amoeba symbionts on ecosystem processes. **a** Microbial biomass carbon (Cmic) per g soil. **b** Estimation of microbial growth measured as ¹⁸O enrichment of DNA produced per Cmic per day. **c** Microbial respiration shown as ng mineralized C per Cmic per day. At each sampling timepoint at least four independent replicate microcosms were measured per treatment. Boxes indicate the interquartile range, the line indicates the median, whiskers indicate minimum and maximum values, respectively. Stars indicate statistical significance (* p<0.05; ** p<0.01; *** p<0.001), ANOVA and post-hoc Tukey's HSD were used for all statistical analyses.

Enhanced nitrogen mineralization. To determine if microbial community shifts induced by altered amoeba grazing patterns affect nitrogen cycling in the soil microcosms, we measured gross N mineralization (Nmin), which reflects decomposition and nutrient cycling rates (Myrold and Bottomley 2008). In our microcosms, we found that on day 5 the Strong, Apo and SC treatments all have similar Nmin around 3 μg N per day that declined to around 1-2 μg N per day by day 20 (Figure 6a). In contrast, the Mild treatment displayed a significant spike in Nmin (Tukey's HSD test; ~SC: p=0.011; ~Apo: p=0.001; ~Strong: p=0.016; Figure 6a). The pulse of mineralized N released on day 5 may reflect the increased predation around this day, which can be seen as a sharp drop in bacterial abundances (average 66% drop, Tukey's HSD test p=0.24) in this treatment on day 5 (Figure 3b).

By day 20 Nmin values in the Mild treatment decline to levels similar to the other treatments. However the per biomass rate of Nmin shows a significantly higher rate still on day 20 in the Mild compared to the SC treatment (Tukey's HSD test; ~SC: p=0.013; Figure 6b).

The frequently recorded increased N mineralization from protist predation is thought to result from excess ammonium being excreted after consumption of prey organisms (Weekers et al. 1993; Woods et al. 1982; Rønn et al. 2002; Zahn et al. 2016), which tend to have a lower C/N ratio (Ekelund and Rønn 1994; Sherr et al. 1983). This in turn has been shown to stimulate nitrifying bacteria, able to oxidize the mineralized ammonium and recycle it back into microbial biomass (Verhagen and Laanbroek 1992; Alphei et al. 1996; Bonkowski 2004). However, we did not detect autotrophic nitrifying groups of the ammonia oxidizing bacteria or archaea in our amplicon data set. Alternative

heterotrophic nitrification processes are often stimulated by plant presence and reported weak in unplanted soils (Chen et al. 2025). As our microcosms were unplanted, nitrification represents a less likely growth stimulant in this community.

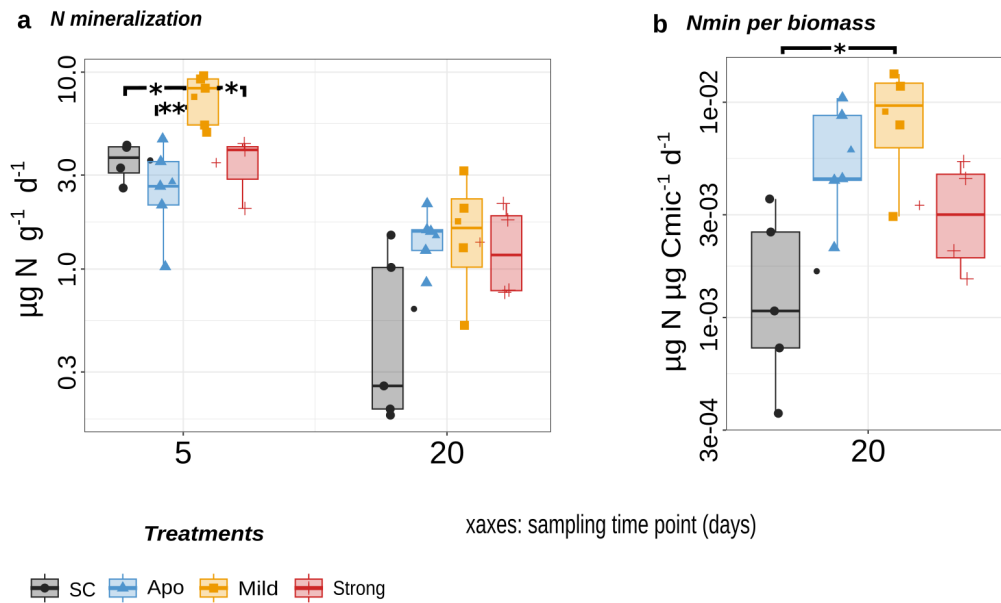


Fig. 6. Impact of amoeba symbionts on nitrogen cycling. **a** Soil gross N mineralization (Nmin) measured via ^{15}N isotope pool dilution. **b** Gross Nmin per Cmic on sampling day 20 (day 5 was not included due to carbon contamination in Cmic measurements). At each sampling timepoint at least four independent replicate microcosms were measured per treatment. Boxes indicate the interquartile range, the line indicates the median, whiskers indicate minimum and maximum values, respectively. Stars indicate statistical significance (* p < 0.05; ** p < 0.01), ANOVA and post-hoc Tukey's HSD were used for all statistical analyses.

Discussion

Chlamydial impact on the host in the context of a complex environment

Host numbers under the more complex soil microcosm conditions generally followed the pattern found under simplified axenic growth conditions (Fig. S1). Ultimately leading to a reduction, or complete demise of the host population rRNA gene copy numbers carrying either the mild or strong symbiont respectively. Curiously, in the population carrying the strong symbiont we could not observe a matching demise of rRNA transcript copy numbers, suggesting the possibility that a remaining host population persisted in a metabolically active state sufficient for rRNA detection, but below the detection limit for DNA. A potentially similar phenomenon was previously described by Leitsch et al. (Leitsch et al. 2010), who showed that a subset of *Acanthamoebae* had survived infection with the same *Parachlamydia acanthamoebae* species. The symbiont population isolated from these survivors exhibited an altered proteome that was highly reduced in the typically prominent D-subunit of NADH-ubiquinone oxidoreductase. This subunit has been linked to exploitation of host energy metabolism among human pathogens including chlamydiae (Häse et al. 2001; Liang et al. 2018), suggesting there might be a less virulent subpopulation within this species, which may enable host rescue.

On the other hand, high levels of host rRNA may also be the result of apoptosis induced by the strong symbiont. During apoptosis, DNA, proteins and mRNA are rapidly degraded (Mustafa et al. 2024), non coding RNAs such as ribosomal RNA remain stable for much longer times (Thomas et al. 2015). It is therefore likely that the host rRNA would persist for several days after apoptosis events, but whether it is stable enough to resist decomposition for 2 weeks as seen in our microcosms would likely depend on how protected this rRNA is - i.e. still bound to ribosomes inside cell membranes or some extracellular matrix.

Taken together, responses in the Strong treatment show a possibly heterogeneous symbiont population that could represent varying degrees of host parasitism. Overall the relationships between host and our chosen symbionts in the microcosms are similar to what we observe under pure culture laboratory conditions.

Symbiont-mediated shifts in grazing preferences and microbial life-history traits

Protist grazing has profound effects on soil and rhizosphere microbiomes. Typically this leads to a reduction of total bacterial biomass, but even more generalist predators like *Acanthamoeba* show selective feeding that alter bacterial community composition, often promoting abundance of predation-resistant bacteria (Adl and Gupta 2006; Asiloglu et al. 2021; Gao 2020; Gao et al. 2019;

Geisen et al. 2021). Mechanisms promoting predation resistance in bacteria can include morphological changes like filament formation, shifts in sizes, but also behavioral changes like speed of movement, biofilm formation and the synthesis of an array of secondary metabolites aimed at inhibiting, or reducing predation and sometimes even killing predatory organisms (Burian et al. 2022; Shuaibing Zhang et al. 2025; Rossine et al. 2022; Rønn et al. 2002).

Studies on wastewater systems have shown protists preferentially grazing on fast-growing, gram-negative bacteria that often fall into the life-history trait category of r-strategists. These perform well in nutrient-rich, copiotrophic environments, have high rRNA operon copy numbers and fast growth rates (Burian et al. 2022; Fierer et al. 2007). Opposite to that are K-strategists, which are characterized by high substrate affinity, slower growth, lower rRNA operon copy numbers and strong competitiveness that allows them to thrive in low-nutrient, oligotrophic environments. They are known to invest more energy into the production of secondary metabolites, often affecting growth of other microorganisms, altogether making them less attractive as food for predators (Burian et al. 2022). Additionally, Nguyen et al. (Nguyen et al. 2023) noted that increased predation pressure in soils correlates with higher abundance of antibiotic-resistance genes, an attribute often linked to K-strategists.

In our system, total bacterial abundance (16S rRNA gene copies; Figure 3a) did not significantly decline in the presence of predators. Instead, evident drops in abundance were restricted to the RNA based bacterial community (16S rRNA transcript copies; Figure 3b) and were most pronounced in the Mild and Strong treatments, indicating a feeding preference on bacteria with high ribosome content. K-strategist taxa increased in all treatments until day 11, but only showed a significant spike in the Strong treatment (Fig. S3). Thus, by potentially higher consumption of r-strategist taxa, the Strong treatment could favour the growth of K-strategists. These effects are likely reversed by day 20 when the host population is decimated by the symbiont. Specifically, both the Mild and Strong treatments lead to a shift in microbial community beta diversity and reduced richness and evenness on day 11 compared to Apo and SC (Figure 3 c; Fig. S2).

Differential abundance analysis revealed many diverse bacterial taxa were often negatively affected by the symbiont treatments and effects were concentrated between days 5 and 20. Particularly pronounced reduction of certain genera could be found on day 11 both in the case of *Chthoniobacter* and *Mucilaginibacter* which exhibited a high number of negatively affected ASVs, and in the case of *Pseudomonas* and *Reyranella* which exhibited negatively affected abundance that is made up of few affected ASVs (Figure 4b). In addition the genus *Sphingomonas* exhibited high numbers of both positively and negatively affected ASVs by the symbiont treatments.

Chthoniobacter and related species of Verrucomicrobiota are abundant, ubiquitous soil bacteria

(Rakitin et al. 2024; Ivanova and Dedysch 2023). The main recognized environmental role of Chthonibacter is in carbon cycling processes and the degradation of plant-derived biomass (Rakitin et al. 2024). While not much is known about their interaction with soil protists, their degradation of polysaccharides and extracellular polymeric substance (EPS) production might serve as an attractant to protists as it aids biofilm production, increasing predation especially by amoebae (Erktan et al. 2020; Seiler et al. 2017). Similar traits like the breakdown of complex organic materials and EPS production are also common for *Sphingomonas* (Mazoyon et al. 2023), *Mucilaginibacter* (Kumar et al. 2025, 2022) and *Pseudomonas* (Roberson and Firestone 1992). Members of the genus *Reyranella* have only recently been described in more detail. The first described member, *R. massiliensis*, was isolated in coculture with an *Acanthamoeba* species from a cooling tower (Pagnier et al. 2011). Its predation resistance and recently described quorum quenching potential at the genus level by degradation of various acyl homoserine lactones make it an interesting taxon for further characterization in foodwebs (Lee and Kim 2024). In addition, members of *Pseudomonas* have also been identified surviving in symbiosis-like states inside protist cells (Steele et al. 2023).

The negatively affected abundance of these genera in our symbiont treatments might simply be explained by a higher energy demand imposed by the chlamydiae, leading to an increased need for food consumption. Thus, many more taxa are predated on even if they typically do not represent preferred food sources. This pattern is visible in Figure 4a, which shows abundance of many bacterial taxa is negatively affected by the symbiont treatments. However, we also observed selective effects beyond just a stimulation of predation. As chlamydiae have already been shown to confer protective properties to their hosts (Arthofer et al. 2022; König et al. 2019; Ishida et al. 2014) it is conceivable that they might also enable the consumption of otherwise inedible groups of bacteria, extending the landscape of palatable prey for their hosts.

One supporting example is the genus *Pseudomonas*. Although *Pseudomonas* species in soil are often characterised as r-strategists, they exhibit a multitude of anti-predator defenses, including an array of secondary metabolites (Jousset et al. 2006), heightened motility, biosurfactant production (Amacker et al. 2020) and production of reactive oxygen species through compounds like phenazine (Price-Whelan et al. 2006). In addition, a recently identified predator sensing and toxin secretion system specifically targets and kills predatory amoebae of the species *Polysphondylium pallidum* (Shuaibing Zhang et al. 2025). Many studies have shown *Pseudomonas* abundance increasing under amoeba predation, some also showing a correlation of predator resistance and enhanced plant growth and health promoted by *Pseudomonas* species (Amacker et al. 2020, 202; Jousset et al. 2006; Weidner et al. 2017; Jousset and Bonkowski 2010). One study also showed a predator preferring a *gacS* mutant *Pseudomonas* species as a food source, which is unable to produce a number of secondary metabolites compared to the wild type (Jousset et al. 2009). In our study, we could confirm *Pseudomonas* abundance increasing under

amoeba predation in the Apo treatment (Fig. S4b). However, in stark contrast, we found strong negative effects on *Pseudomonas* abundance in both symbiont treatments (Figure 4 a,b). This negative effect lasted until day 20 in both symbiont treatments, but was most prominent and consistent over time in the Mild treatment (Figure 4 a,b; Fig. S4a). We therefore suggest that even with different parasitic impacts on their hosts' fitness, chlamydiae could potentially provide a benefit in the form of protection against anti-predator defense systems, enabling a broader consumption of prey.

These protective properties could be conferred in a direct manner, interfering with bacterial defense systems, or indirectly through induced changes in the hosts' physiology. Environmental chlamydiae encode a variety of different proteins that can either be secreted into the host cytosol via a type III secretion system, or incorporated into the host derived inclusion membrane (Inc proteins). Many of those proteins carry hydrolase, peroxidase and protease domains (Heinz et al. 2010; A. Collingro et al. 2011). If these chlamydial effectors were to degrade or neutralize small molecules (e.g. oxidizing reduced *gacS/gacA* regulated secondary metabolites) inside the host cytosol, they would effectively protect the amoeba against secreted toxins from other organisms like *Pseudomonas*. Chlamydiae have also been shown to interfere with host physiology. They are capable of interrupting amoebae encystation (Leitsch et al. 2010), can increase mitochondrial respiration (Kurihara et al. 2019) and potentially alter the host actin skeleton (Maita et al. 2018). This could indirectly serve as tolerance against systems aimed at disrupting host fecundity, speed of movement or directly lower the amount of reactive oxygen species produced via increased throughput in the respiratory chain. Host antimicrobial peptides are known to be produced in eukaryotes in response to the entry and establishment of endosymbionts (Mergaert et al. 2017). Chlamydiae are well equipped to handle these (Stelzner et al. 2023; A. Collingro et al. 2011), while other microbes might fall victim to the high levels of antimicrobial peptide expression from the presence of an endosymbiont.

Bacterial symbionts as hidden players in soil ecosystem processes

Bacterivorous protists play a key role as trophic links between small bacteria and larger organisms consuming protists. This holds true especially in the three-dimensional space of soil, where prey can be hidden in small aggregates or biofilms and thereby inaccessible to larger predators (Bonkowski and Clarholm 2012). The release of mineralized and organic nutrients from protist predation is thus of major importance in this environment and has been shown to have stimulating effects on both soil microbial communities as well as plant growth (Gao et al. 2019).

In our system, the mild chlamydial symbiont unfolded a clear stimulating effect on predator-induced microbial community shifts and thus nutrient cycling. This is particularly visible in some effects that are seen in the Apo treatment (i.e. lowered microbial biomass, but increased microbial growth and respiration, Figure 5) which are amplified to a significant level in the Mild treatment. A potentially enhanced predation in the Mild treatment together with nutrient release from lysis of prey organisms

could provide substrate for microbial growth, thus maintaining low microbial biomass, while overall activity is stimulated. Another impacting factor of higher microbial respiration rates is respiration by the predator itself. While this does not explain the higher rates seen in the Mild treatment it could explain why microbial respiration rates in the Strong treatment are similar to rates in the SC treatment on day 20, after most of the predator population has been removed through lysis (Figure 5c).

Gross N mineralization rates are elevated in the presence of the mild chlamydial symbiont. On day 5, significantly higher levels of Nmin rates in the Mild treatment compared to the other treatments suggest a high predatory activity, releasing large amounts of excess N. By day 20 all predator treatments (Apo, Mild and Strong) display more uniform levels of Nmin, which are slightly, but not significantly elevated compared to the predator-free SC treatment. However, on a per biomass basis, the Mild treatment still exhibits a significantly higher Nmin rate (Figure 6b) and together with low biomass and increased microbial growth and respiration rates on day 20 would point to amplified predatory activity through the symbiont.

In summary, our results suggest that predatory effects of the host are enhanced by weakly parasitic bacterial symbionts, stimulating higher overall microbial activity and nutrient cycling in the soil microbial community. Conversely, strong parasites, once the host numbers are diminished, exhibit ecosystem effects comparable to predator-free conditions, thus largely removing predator-mediated effects on ecosystem processes.

Conclusion

Within both terrestrial and marine habitats, predation of bacteria is crucial for maintaining balanced microbial communities (Gao et al. 2019). The importance of predator-prey interactions of single celled organisms has been highlighted as especially important in soil habitats due to the high density of microbial biomass and protists' disproportionate influence on nutrient cycling and ecosystem functioning (Bonkowski 2004; Geisen et al. 2018; Trap et al. 2016). Symbiotic associations within this context represent an underexplored dimension. The success of the bacterial phylum Chlamydiota in colonizing eukaryotic predators likely dates back to the chlamydial last common ancestor about 1-2 billion years ago (Dharamshi et al. 2023), and extant chlamydial symbionts can be found in association with all major eukaryotic lineages.

The functions of endosymbionts in microbial interaction networks can be difficult to discern, as isolated interactions often differ significantly from combined effects. Transmission modes have been considered a predictor of parasitism, as endosymbionts with a horizontal transmission route are thought to lead to more parasitic associations through uncoupling of host and symbiont needs, while mainly vertical transmission routes seem to favour more mutualistic associations (Drew et al. 2021). However, even reproductive parasites like *Wolbachia*, have been shown to more easily enter and spread through a population when also conferring protective benefits against a pathogen to their host (Fenton et al. 2011). Just like mobile genetic elements and viruses, microbial endosymbionts can parasitically spread through populations and confer facultative benefits for the host depending on environmental stressors or needs (Drew et al. 2021). Chlamydiae have often been considered professional parasites, sharing a core genome that requires energy, amino acid, and nucleotide parasitism from the host and commonly horizontal transmission routes (Elwell et al. 2016). However, the discovery of environmental chlamydiae and their wide distribution among hosts together with a more complex metabolism and higher frequency of vertical transmission, suggested that chlamydiae could play many more roles than previously assumed (Köstlbacher et al. 2021).

This study shows how two chlamydial symbionts of the same host, with different levels of host parasitism can cause a multitude of effects on their immediate symbiont-host and broader host-environment systems. Both the predator and the bacterial community it predated on were heavily influenced by symbiont presence. This resulted in shifts in the abundance of the RNA based bacterial community composition, but also on microbial growth rates, nitrogen cycling and respiration rates. However, the presence of symbionts not only increased predation, but also potentially broadened the range of prey that was consumed, pointing to a symbiont-mediated extension of host trophic capacity.

While we offer a glimpse into the environmental impact of protist endosymbionts and suggest their presence should be considered in future studies, many questions still remain. They include: What is the contribution of nutrient cycling coming directly from the protist hosts, and

how is that changed by presence of different symbionts? How does the interactome of a protist change upon infection with an endosymbiont - i.e. under simplified controlled settings is there increased consumption and/or a widened food palette? What are the effects of a system with a multitude of host-symbiont combinations? How are soil aggregate formation, stability and breakdown influenced by a possible differential impact on r and K strategist communities?

Materials and Methods

Amoeba cultures and infection with symbionts

Acanthamoeba terricola Neff (ATCC 50373) was maintained at 20°C in trypticase soy yeast extract (TSY) medium (30 g/l trypticase soy broth, Oxoid, 10 g/l yeast extract; pH 7.3). To generate isogenic amoeba populations infected with chlamydial symbionts, elementary bodies (EBs) frozen at -80°C of the strains *Protochlamydia amoebophila* UWE25 (ATCC PRA-7) and *Parachlamydia acanthamoebae* UV7 (DSM 27688), respectively, were thawed in a water bath at 37°C for approximately 2 minutes. Symbiont-free amoebae were harvested shortly beforehand and transferred into 25 cm² cell culture flasks (Nunclon delta-surface, Thermoscientific, St Leon Rot, Germany) filled with 10 ml TSY medium. Aliquots of the chlamydial EB suspensions were added and the flasks were centrifuged at 150 x g for 15 minutes at room temperature. Cultures were screened for successful infection and to exclude contamination after two weeks by fluorescence *in situ* hybridization and 4',6'-diamidino-2-phenylindole (DAPI) staining (0.1 µg/ml) and from there on regularly until the start of the microcosm experiment.

Before addition to the soil microcosms, cultures of *Acanthamoeba terricola* NEFF with or without either chlamydial symbiont were harvested by centrifugation at 2000 x g for 10 minutes at room temperature. They were resuspended in Page's amoeba saline (PAS, 120 mg/l NaCl, 4 mg/l MgSO₄*7 H₂O, 4 mg/l CaCl₂*2 H₂O, 142 mg/l Na₂HPO₄, 136 mg/l KH₂PO₄), centrifuged again and resuspended again in PAS to remove nutrients from the cellular fraction. Cell concentration was determined using the Cellometer K2 fluorescent cell counter (Nexcelcom Biosciences). Cell suspensions were then diluted to reach a cell density of 1.5*10⁶ cells per 500 µl (which was the volume added to microcosms).

Amoeba growth curve experiment

To test the effect of the two chlamydial strains used in this study on an isogenic strain of the *Acanthamoeba* strain used in this study, a growth experiment under axenic conditions was conducted. For this EBs of two continuous cultures of the *Acanthamoeba terricola* NEFF strain harbouring either the of the two symbiont strains *P. amoebophila* UWE25, or *P. acanthamoebae* UV7 were used to infect isogenic cultures of the *A. terricola* NEFF strain. Briefly naive amoeba cells were transferred into 12-well plates, supplemented with 2 ml of TSY medium, at a density of 10⁵ cells per well and were infected with 10⁶ chlamydial cells per well, resulting in a multiplicity of infection (MOI) of 10. Plates were centrifuged at 150 x g for 15 minutes at room temperature. As a control naive amoebae cells were co-incubated in the same manner as the infections. Cells were quantified on days 2, 4, 6, 8, 10, 15, 20 and 30, by destructively sampling 3 wells per treatment and quantifying amoeba cells in a

Luna FX7 automated cell counter.

Fluorescence in situ hybridization

Fluorescence *in situ* hybridization (FISH) using different combinations of three labeled probes targeting *Pr. amoebophila* or *P. acanthamoebae*, all bacteria and eukaryotes, respectively (E25-454 (Cy3) (Sixt et al. 2013); UV7-763 (Cy3) (Astrid Collingro et al. 2005); EUBI+II+III (Holger Daims et al. 2005; FLUOS-Double Labelled Oligonucleotide Probe); EUK516 (Amann et al. 1990) Biomers, Ulm, Germany) was performed as described in (Holger Daims et al. 2005). In brief FISH was performed by collecting amoebae trophozoites and fixing them on teflon-coated microscopy slides, using an on-slide 4% formaldehyde incubation for 10 minutes. After rinsing samples in milliQ and dehydration in 50%, 75% and 100% ethanol for each 3 minutes, slides were air-dried and directly used for hybridization with the probes. Hybridizations and subsequent washing were performed using hybridization (90 mM NaCl, 20 mM Tris-HCl, 0.01% SDS, 25% formamide) and washing (1 mM Tris-HCl, 0.25 mM EDTA and 0.149 M NaCl) buffers. Slides were hybridized for 2 hours at 46°C and washed for 13 minutes at 48°C. Slides were ultimately washed for 10 seconds in cold deionized water (~4°C), air-dried and then stained using DAPI (0.1 µg/ml) for 5 minutes. Images were recorded using a confocal laser-scanning microscope (Leica SP8), together with the software Leica LasX.

Soil sample collection

Beech forest soil was collected at one location in Neuwaldegg, Vienna, Austria (48.24782°N, 16.26496°E), on the 11. October 2021. In total, 5 kg of forest soil were collected by first removing plants, litter and organic layers on the surface by hand and only collecting soil from the upper 10 cm, which was directly sieved through a 2 mm mesh. Collected soil was brought back to the laboratory in Vienna and 1.7 kg were stored at 4°C in closed plastic bags for 11 days until bacterial community isolation at the start of the microcosm experiment on day -14 (Figure 1b).

Isolation of bacterial soil community

For isolation of the protist-free bacterial soil community we distributed 200 g of the native soil stored at 4°C on the day of collection into twenty 50 ml tubes (Greiner bio-one) each containing 10 g of soil. Each of these tubes were then filled up to 50 ml with PAS buffer. Tubes were vortexed at full speed for 2 minutes, then left to settle for 5 minutes. To separate bacterial cells, slurries were then filtered through a 40 µm cell strainer (Sigma-Aldrich). The resulting filtrate was centrifuged to remove larger particles at 5000 x g for 1 minute at room temperature. The supernatant was filtered through 5 µm and then 1.2 µm syringe filters to remove protists (Sartorius Minisart). Finally cells were concentrated by centrifugation at 5000 x g for 20 minutes at 4°C. The pellets were resuspended in PAS, united in one

tube and the cell concentration was determined using optical density (OD), by measuring OD₆₀₀ of the suspension in a spectrophotometer (Biorad SmartSpec 3000).

Soil sterilization

The collected forest soil was sterilized in three stages. First, after collection three bags filled with 1.7, 1.5 and 1.1 kg of soil were sterilized by autoclaving at 121°C for 20 minutes. Autoclaved soils were then combined in one mesh bag (poresize around 50 µm) to which 4 litres of similarly sterilized tap water were added to elute released nutrients from the soil. This slurry inside the mesh was left on a grid to rinse extant water overnight. Rinsed soil was then transferred into small plastic bags (30 x 45 cm) containing multiple holes to allow for water to evaporate while placed at 65°C overnight. Soil was then distributed in fresh bags that were pressed flat to reach a height of not more than 3 cm. Bags were submitted like this for the second sterilization, using gamma-ray irradiation at the Mediscan GmbH & Co KG facility in Seibersdorf, Austria. Soil was irradiated with 25 kGy using a Cobalt 60 Gammatron 1500 device. After irradiation soil was further dried under semi-sterile conditions in a fume hood by spreading the soil over a wide sterile surface and manually breaking up larger clumps. Soil was left like this for three days, until it had almost entirely dried out. The soil was then transferred again into plastic bags, all clumps were broken up until a homogeneous, almost powder-like texture was reached. To this soil microcrystalline cellulose (Alfa Aesar, 1 mg/g dry soil) was added and evenly distributed to provide a stable Carbon (C) source in addition to the labile C mineralized by the sterilization procedures (Berns and Philipp 2008). To increase the moisture content for the third round of sterilization, sterilized tap water was added to reach an approximate water content of 19%. The soil and cellulose mixture was again submitted for sterilisation using the Cobalt 60 Gammatron 1500 device at Mediscan in Seibersdorf. After collection the soil was immediately used to start the microcosm setup (see below). Three aliquots of 0.5 g of the sterilized soil were immediately transferred into lysing matrix E (2 ml) tubes (MP biomedical, Eschwege, Germany) and frozen at -80°C for DNA and RNA extraction to serve as sterile soil controls before the start of the microcosm experiment.

Preparation of soil microcosms

To set up the soil microcosms, under sterile conditions, we placed 15 g of sterilized wet soil (see sterilization procedure above) with a water content of approximately 22% into 100 ml Schott bottles that had been washed with 10% HCl and sterilized by autoclaving at 121°C for 20 minutes. A total of 140 bottles were filled with sterile soil in this manner. To each of these microcosms we then added a bacterial soil community that was derived as described above from the same unsterilized soil. 2 ml of the isolated bacterial cell suspension were added to each of the soil microcosms by pipetting the suspension evenly over the soil without mixing to avoid formation of clumps. Total estimated cell

density per microcosm was 2.4×10^8 bacteria, amounting to 1.6×10^7 cells per gram wet soil. Before the addition of amoebae, microcosms were stored with slightly unscrewed lids to allow gas exchange for 13 days in the dark at room temperature.

The different treatments considered in our microcosm experiments were (i) no addition (500 μ l PAS buffer addition, only protist-free bacterial community), (ii) aposymbiotic amoeba (*Acanthamoeba terricola* NEFF) addition, (iii) amoeba infected with *Protochlamydia amoebophila* UWE25 addition, (iv) amoeba infected with *Parachlamydia acanthamoebae* UV7 addition (Figure 1b). Microcosms were set-up in seven replicates for each treatment and timepoint. Amoeba cell suspensions were added to pre-incubated soil microcosms by pipetting 500 μ l evenly across the soil in each microcosm without mixing of the soil to reach a final concentration of 10^5 amoeba cells per g soil. Microcosms were then covered with a Micropore™ tape to allow gas exchange and placed in the dark at room temperature. Sampling took place destructively at each time point (0 days, 5 days, 11 days, 20 days and 35 days) by first mixing the soil in each vessel to homogenize and then immediately freezing 500 mg per microcosm in lysing matrix E (2 ml) tubes (MP biomedical, Eschwege, Germany) at -80°C for later nucleic acid extraction. Additionally on days 5 and 20, soil was also used further to measure ecosystem processes including determination of soil biomass, respiration, nitrogen mineralization and DNA turnover.

Characterization of the microbial soil community

DNA/RNA extraction

DNA and RNA were extracted simultaneously from samples frozen at -80°C in 500 mg aliquots in lysing matrix E (2 ml) tubes (MP biomedical, Eschwege, Germany), using the ZymoBIOMICS DNA/RNA Miniprep Kit (Zymo Research) with adaptations to the DNA/RNA parallel purification protocol. In brief, samples in lysing matrix E tubes were filled with 750 μ l of DNA/RNA Shield™ and processed in a bead beater (MP Bio Fastprep-24) at 6.5 m/s for 30 seconds. Further steps were done according to the manufacturer's instructions, DNase I treatment was performed at 37°C for 15 minutes, DNA was eluted in 100 μ l and RNA in 50 μ l of the elution buffer. Nucleic acid concentrations were quantified on a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) and Qubit™ 4 Fluorometer (Invitrogen) using dsDNA BR and RNA HS Assay Kits (all Thermo Fisher Scientific). Purity of RNA was assessed by PCR of extracts using general bacterial primers 515F and 806R primers modified by (Apprill et al. 2015; Parada et al. 2016).

cDNA was generated from RNA using the SuperScript IV reverse transcriptase with random hexamer primers (both Thermo Fisher Scientific). DNA extracts and cDNA served as templates for amplification of 18S and 16S rRNA genes with droplet digital PCR (ddPCR) as well as for 16S rRNA gene amplicon sequencing.

Quantification of amoebae, chlamydiae and total bacteria

At each sampling time point of the microcosm experiment, we measured 16S rRNA and rRNA gene copy numbers using ddPCR. For this three different primer pairs were used, targeting either specifically acanthamoebae (primers AcantF900 and Acant1100 (Qvarnstrom et al. 2006)), chlamydiae (primers Chl40F (Haider et al. 2008) and Chl523R), or broadly all bacteria (primers 515F (Parada et al. 2016) and 806R (Apprill et al. 2015)). ddPCR reactions were generated and quantified using the QX200 droplet generator and QX200 droplet reader (Bio-Rad). Samples with less than 10.000 droplets were repeated. Samples that generated below 5 droplets were regarded as below the limit of quantification (as described in the ddPCR Applications Guide, Biorad) and therefore regarded as zero. The limit of 5 was determined by the maximum amount of droplets that were found at high amplitude in negative control samples, that underwent the cDNA synthesis procedure and thus more often generated positive droplets. Analysis and calculation of gene copy numbers was performed with the software Quantasoft (v.1.7.4.0917, Bio-Rad). The resulting gene copy numbers for amoebae and bacteria served as a proxy for actual cell numbers, because the exact copies of 18S and 16S rRNA genes for the organisms are unknown.

Amplicon sequencing and analyses

Amplification of the reverse transcribed 16S rRNA transcript and amplicon sequencing were performed at the Joint Microbiome Facility of the Medical University of Vienna and the University of Vienna. The V4 region of the bacterial 16S rRNA transcript was amplified (30 cycles) from the cDNA using the modified primer pair 515F/806R (Apprill et al. 2015; Parada et al. 2016), and barcoded (8 cycles) in a unique dual barcoding setup (UDB-H12). Barcoded samples were purified and normalized over a SequelPrep™ Normalization Plate Kit, and further pooled and concentrated on columns (Analytik Jena). Indexed sequencing libraries were prepared with the Illumina TruSeq Nano Kit as described previously (Pjevac et al. 2021), and sequenced in paired-end mode (600 cycles; V3 chemistry) on an Illumina MiSeq following the manufacturer's instructions. The workflow systematically included four negative controls (PCR blanks, i.e., PCR-grade water as template) for each 90 samples sequenced. Also, a ZYMObiomics mock community was sequenced and analyzed at the sequencing facility as part of their established quality control routine (Pjevac et al. 2021).

Amplicon pools were extracted from the raw sequencing data using the FASTQ workflow in BaseSpace (Illumina) with default parameters. Demultiplexing was performed with the python package demultiplex (Laros JFJ, github.com/jfjlaros/demultiplex) allowing one mismatch for barcodes and two mismatches for linkers and primers. Amplicon sequence variants (ASVs) were inferred using the DADA2 R package version 1.14.1, applying the recommended workflow (Callahan et al. 2016). FASTQ reads 1 and 2 were trimmed at 220 nt and 150 nt with allowed expected errors of 2. SSU rRNA gene sequences were classified using SINA version 1.7.2 (Pruesse et al. 2012) and the

SILVA database SSU Ref NR 99 release 138.1 (Quast et al. 2012). 16S rRNA transcript counts were transformed via centered log ratio (clr) transformation using the R package mia (Tuomas Borman et al. 2025), and samples below a read count of 5000 were removed. Transformed and filtered counts were multiplied by the ddPCR transcript counts to generate total 16S rRNA transcript abundance counts. Count based differential abundance analysis of ASVs between treatments was performed using DESeq2 (Love et al. 2014). For data visualization of the effect of treatments on microbial beta diversity we ran non-metric multidimensional scaling (NMDS) analysis using the function metaMDS of the R package vegan (Jari Oksanen et al. 2025, 22025) calculated based on Bray-Curtis dissimilarity. The Simpson index, Inverse-Simpson index, Shannon-diversity index, Chao richness index, observed richness and relative dominance were chosen for estimating microbial alpha diversity, capturing both species richness and evenness and visualised using the bioconductor package mia (Tuomas Borman et al. 2025).

Determination of soil properties and ecosystem processes

Soil physical and chemical properties

Soil water content was determined gravimetrically, by drying fresh soils in aluminium dishes for 24 hours at 70°C in a drying oven (UF260 plus, Memmert). Water holding capacity was measured by placing fresh soils (2 g) into a funnel lined with a filter paper (Whatman, 110 mm, Grade 40) that had been saturated with double deionized water (milliQ). To this soil milliQ was added in 100 µl increments until a drop of water could be seen dropping from the filter. The filters were then covered with parafilm and left on room temperature overnight to allow water exceeding the water holding capacity of the soil to drain off the soil. After 24 hours maximum soil water holding capacity was measured by taking 1 g of the saturated soil and measuring soil water content gravimetrically by drying it in aluminium dishes for 24 hours at 70 °C.

Dissolved (extractable) C and N contents were measured by extraction of fresh soil with a 1 M KCl solution (1:7.5 (w:v)). Soil extracts were shaken for 30 min on an orbital shaker (KS 501 digital, IKA) and subsequently centrifuged at 11.000 x g for 5 minutes. Total dissolved N (TDN) and dissolved organic C (DOC) (Fig. S5d,e) contents were measured with a TOC/TN analyzer (TOC-VCPH/CPN/TNM-1, Shimadzu, Austria). Microbial biomass C and N (C_{mic} and N_{mic}, Fig. S5b) were both determined by chloroform fumigation of soil described by (Vance et al. 1987). Briefly, an aliquot of soil was fumigated with chloroform for 48 hours and subsequently extracted with 1 M KCl as described above. C_{mic} and N_{mic} were calculated by subtracting the C and N values from unfumigated samples from the corresponding fumigated samples and correcting the resulting values for a 45% efficiency of the fumigation. NH₄⁺ concentrations were quantified from the same KCl extracts using a colorimetric method described by (Hood-Nowotny et al. 2010).

Mass-specific respiration and growth rates

Measurements for microbial respiration and growth were conducted in five replicates per treatment using slight modifications of the methods described in (Spohn et al. 2016; Zheng et al. 2019). In brief, duplicates of 400 mg of soil were weighed into 1.2 ml cryovials and placed separately and without lid into 27 ml glass headspace vials. Soil water of the sample was then enriched to 20atom% ^{18}O by adding ^{18}O enriched water (Campro 97at%, Campro Scientific, Netherlands) directly onto the soils using a gas tight syringe while the second duplicate received an equal amount of ultrapure water (milliQ). Headspace vials were then sealed with airtight caps with a butyl rubber septum. Directly after sealing the vial a gas sample (5 ml) was taken and the CO_2 concentration was measured on an infrared gas analyser (EGM4, PP systems). Extracted air was replaced by the same amount of synthetic air with a known CO_2 concentration. Another gas sample was taken and measured after 24 hours of incubation. The microbial respiration rate (CO_2Flux , Fig. S5f) was then calculated as the difference in CO_2 concentration measured between those two time points, additionally accounting for the replaced air, divided by the incubation period. Microbial specific respiration per biomass was calculated by dividing CO_2Flux with c_{Mic} (calculation described above).

Based on the incorporation of ^{18}O from the soil water into genomic DNA, we could estimate DNA production and microbial growth rate. For this, DNA was extracted using the FastDNA™ SPIN Kit for Soil (MP Biomedicals) following the manufacturer's instructions. DNA concentration of each extraction was measured using the Qubit™ dsDNA BR Assay Kits (Thermo Fisher Scientific). Total O content and ^{18}O enrichment of the extracted DNA were further measured using a high temperature conversion elemental analyser (TC/EA, Thermo Fisher) coupled via a Conflo III open split system to an isotope ratio mass spectrometer (Delta V Advantage, Thermo Fisher). DNA production rate ($\text{DNA}_{\text{production}}$ ng DNA $\text{g}^{-1} \text{h}^{-1}$) was calculated as described by (Schnecker et al. 2023). Microbial growth was then calculated as follows:

$$\text{microbial growth} = \frac{\text{DNA}_{\text{production}} * 24}{c_{\text{mic}}}$$

Gross N mineralization

Gross N mineralization was estimated using a N isotope pool dilution technique described by (Shasha Zhang et al. 2019; Mooshammer et al. 2014). This method is conducted by enriching the NH_4^+ pool of a soil sample with a ^{15}N tracer, which is diluted by unlabelled NH_4^+ produced by mineralization of organic N (N_{min}). N_{min} is then calculated based on the differences in isotopic composition and pool size of NH_4^+ between two time points. NH_4^+ additions were limited to below 20% of the initial pool size and enrichments to below 10 at%. Pool sizes were quantified from KCl extracts using a colorimetric

method described by (Hood-Nowotny et al. 2010).

N mineralization was determined following the protocol described in (Shasha Zhang et al. 2019) with slight modifications. In brief 2 g of soil on sampling day 5 or 20 were weighed into 50 ml polypropylene vials in duplicate. The tracer ($^{15}\text{NH}_4$) $_2\text{SO}_4$ (98 atom% ^{15}N , Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was added at 10 atom% enrichment. After addition soils were incubated for either 4 hours (t1) or 24 hours (t2), followed by extraction with 15 ml KCl solution (60 minutes of shaking at room temperature) and finally filtration through an ashless cellulose filter paper (110mm, Grade 40, Whatman) (for samples on day 5) or centrifugation at 11000 x g for 5 minutes and harvesting of soil particle free supernatant (for samples on day 20).

In order to determine the enrichment of the ^{15}N label in the NH_4^+ pool, we employed a micro-diffusion method, which captures NH_4^+ on an acid trap, as described previously (Mooshammer et al. 2014; Shasha Zhang et al. 2019). Briefly, 5 ml of 2M KCl extract were transferred into polypropylene scintillation vials. Acid traps consisting of a 4 mm cellulose disk soaked with 2.5 M KHSO_4 encased in thin teflon sheets were added to the vials together with 50 mg of MgO powder (raising the pH over 9.5). Vials were immediately closed and shaken at 100 rpm at room temperature for 2 days. The acid-traps, were then opened and the filter discs containing the trapped NH_4^+ were dried at room temperature over concentrated sulfuric acid. Samples were measured on a continuous-flow IRMS consisting of an elemental analyzer (EA 1110, CE Instruments) coupled via a ConFlo III interface (Finnigan MAT, Thermo Fisher) to an IRMS (DeltaPLUS, Finnigan MAT, Thermo Fisher).

Statistical analyses

Data point distributions were first investigated in density plots and subsequently normality of the data was tested using the Shapiro-Wilk test. One-way Anova and post hoc Tukey HSD test were used to compare snapshot differences at single timepoints between individual treatments. In the case of non-normal distribution samples were tested with the Kruskal-Wallis test followed by Wilcoxon rank sum tests. Temporal trends like such as differences in abundance of amoeba, symbionts and microbial community over time within each treatment were compared by log-transforming abundances ($\log[\text{abundance} + 1]$) and modeled using a generalized linear model (GLM) with the following design:

$$\log(\text{abundance}+1) \sim \text{treatment} * \text{day}$$

Treatment specific effects using pairwise comparisons were estimated post-hoc with estimated marginal means (EMMs) using the emmeans package (v.1.11.1) in R (Lenth 2025) with multiple comparisons adjusted using Tukey's method. A treatment was considered statistically significant if it had $P < 0.05$.

Differences in beta-diversity patterns between treatments were estimated using PERMANOVA with

the `adonis2` function from the `vegan` R package (v.2.7-1). Data were transformed if necessary using centered log ratio (clr) transformation. All statistical analyses were performed using R version 4.4.3 (R Core Team 2024) with packages `rstatix` (Alboukadel Kassambara 2023) and `vegan` (Jari Oksanen et al. 2025).

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Supplementary Information

Supplementary Figures

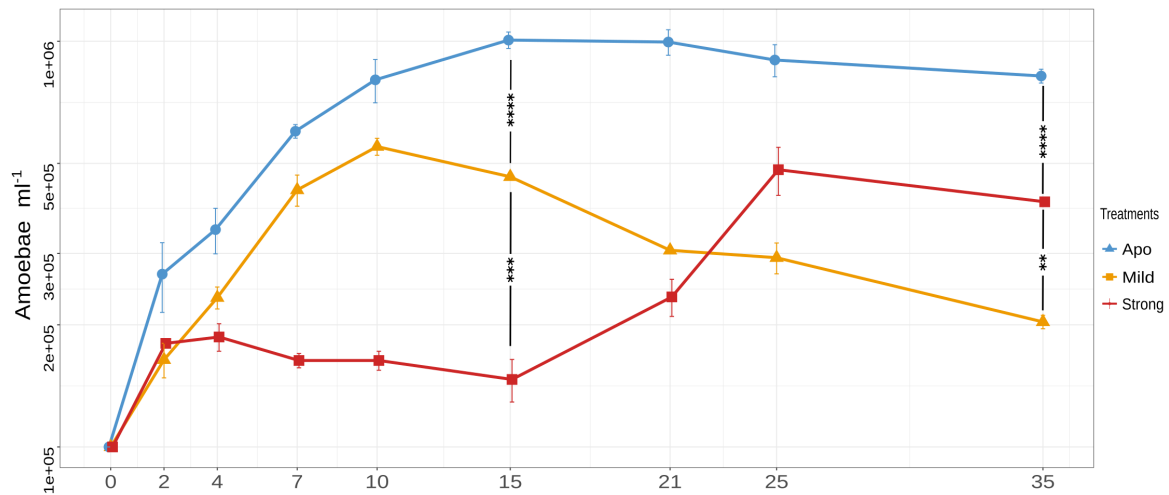


Fig. S1. Chlamydial impact on host survival in culture. Triplicate cultures of *Acanthamoeba terricola* NEFF either uninfected (Apo) or infected with chlamydiae of the species *Protochlamydia amoebophila* UWE25 (Mild), or *Parachlamydia acanthamoebae* UV7 (Strong) were grown in axenic TSY medium at a starting density of 10^5 cells/ml. Growth of host cells was followed by determining cell numbers on eight timepoints spanning from two to 35 days, using the Luna-FX7tm cell counter. Error bars indicate the standard deviation. At each sampling time point three independent replicate cell cultures were measured per treatment. Stars indicate statistical significance (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$), ANOVA and post-hoc tuckey's HSD were used for all statistical analyses.

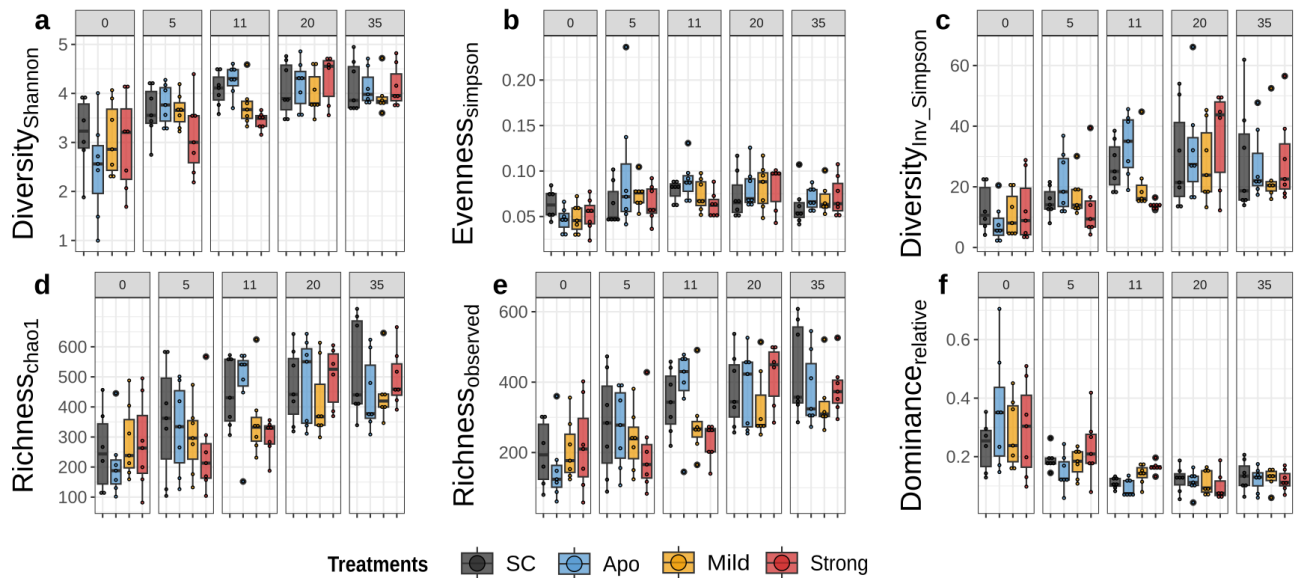


Fig. S2. Different alpha diversity indices for the soil microcosm treatments. Active bacterial community was quantified by sequencing of 16S rRNA amplicons of seven replicate microcosms per treatment and time point. Alpha diversity indices capturing a combination of richness and evenness include **a** Shannon diversity, **b** Simpson's evenness and **c** Inverse Simpson; only richness **d** Chao1 and **e** species Observed; **f** relative species dominance. Alpha diversity calculations were performed using the R package mia.

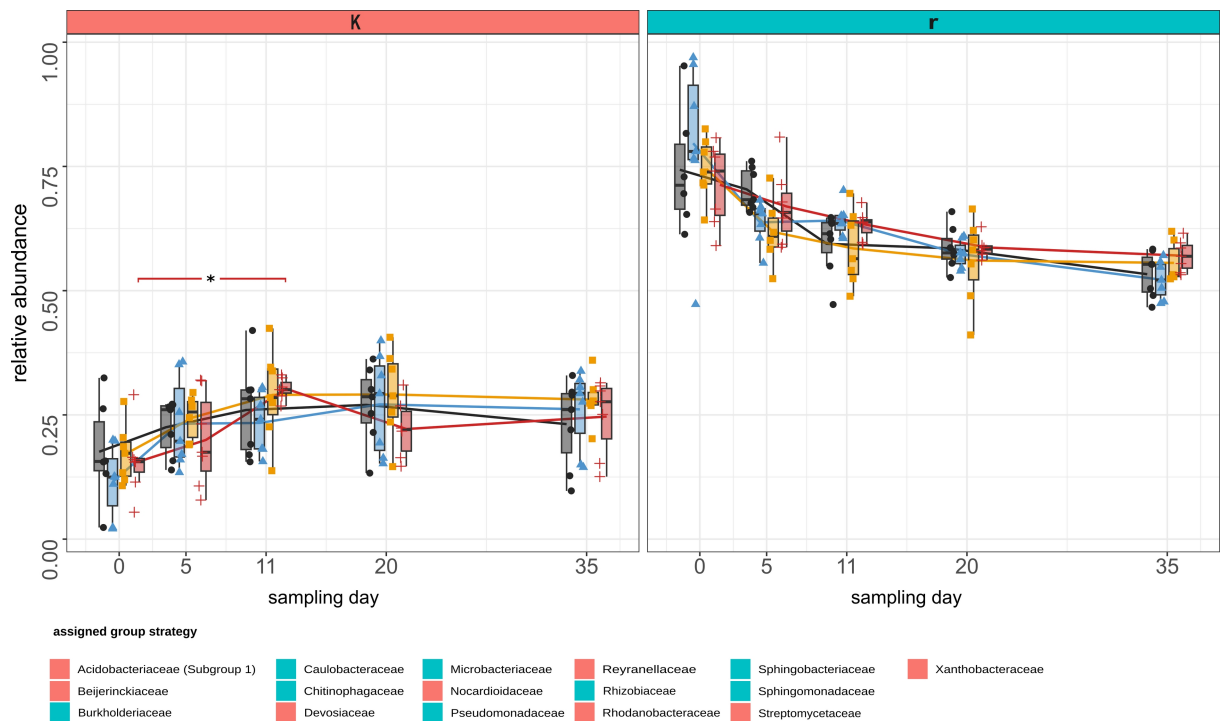


Fig. S3. Relative abundances of K and r strategist taxa. 16 Bacterial families were categorized into “r” (cyan) or “K” (coral) life-history traits (strategy) based on scientific literature (table S2). Plots are based on seven independent replicate microcosms per treatment and time point. Statistical analyses are based on GLM and post-hoc EMMs comparing treatment specific differences. Kruskal-Wallis and post-hoc Wilcoxon rank-sum test were used to compare between individual timepoints. Stars indicate statistical significance (* $p < 0.05$),

Bacterial RNA - differential abundance analysis

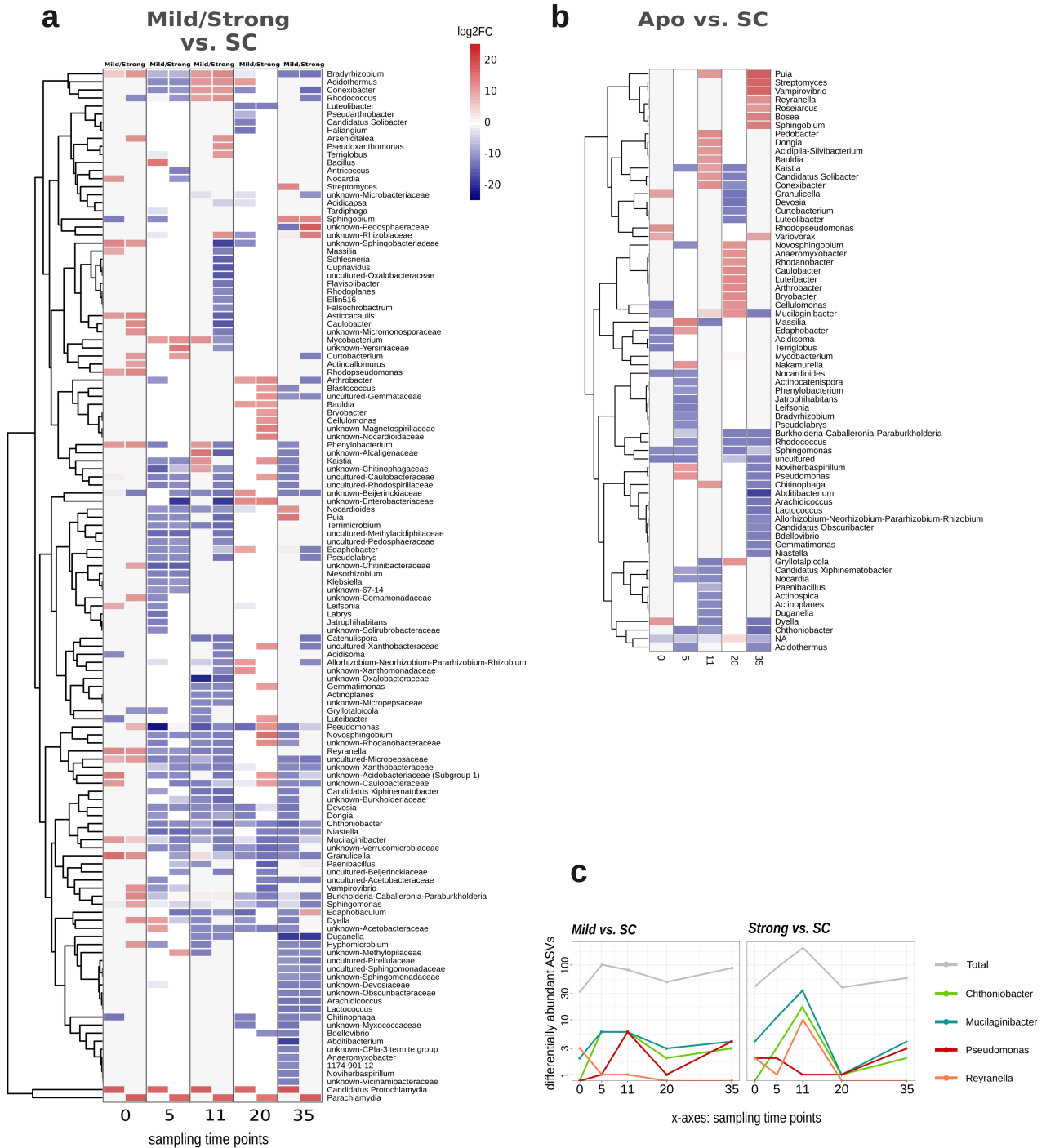


Fig. S4. Differential abundance of bacterial taxa in the presence of host carrying chlamydial symbionts and aposymbiotic host, compared to a control bacterial community. Heatmaps depicting differential abundance of the active bacterial community of either **a** symbiont treatment - *Parachlamydia acanthamoeba* UV7 (Strong) or *Protochlamydia amobophila* UWE25 (Mild) - or **b** aposymbiotic host – *Acanthamoeba terricola* NEFF (Apo) – treatment, compared to the control host-free bacterial soil community (SC) at each sampling time point (0-35) as based on sequencing of the 16S rRNA transcripts (n=7 per timepoint).

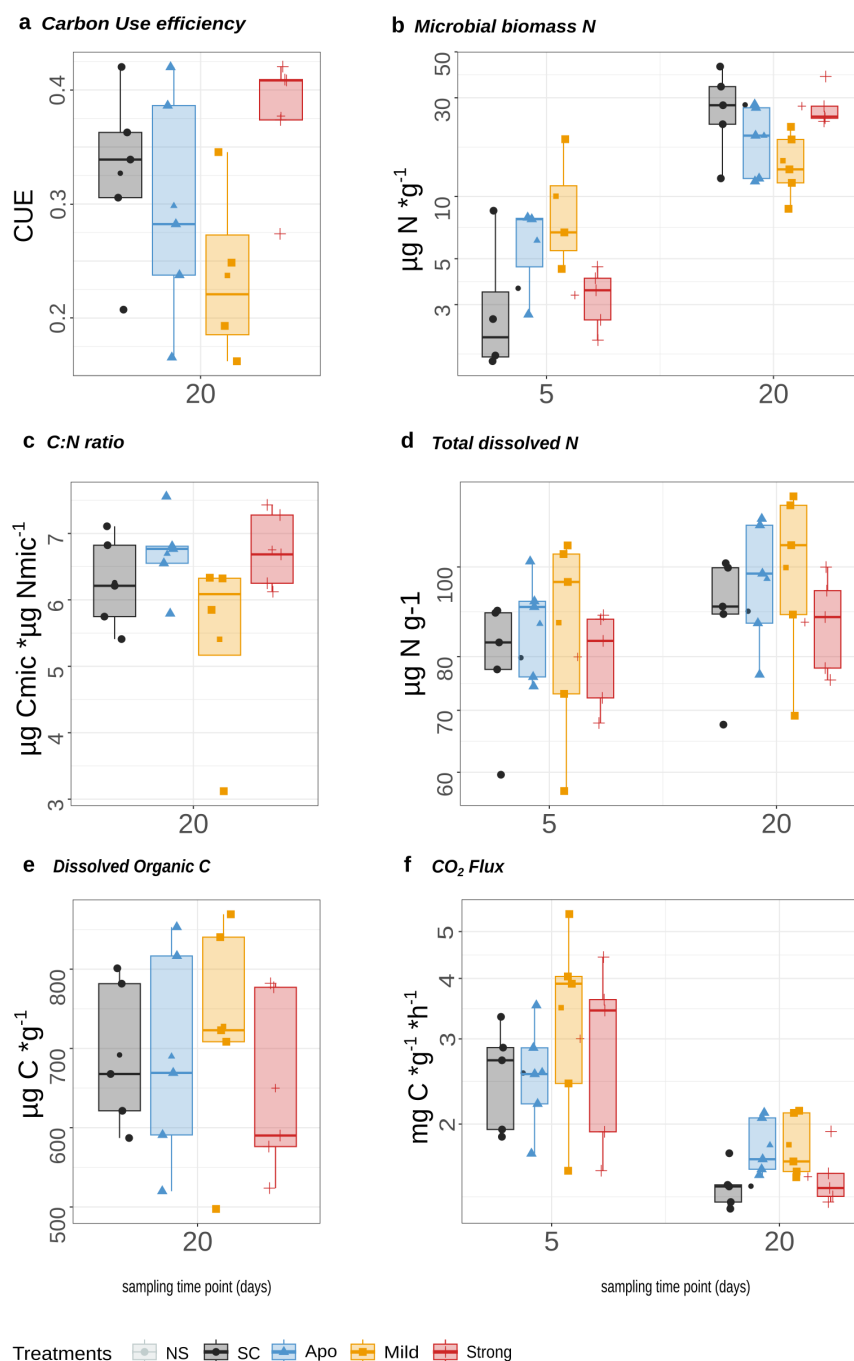


Fig. S5. Extended environmental data. **a** Carbon use efficiency, calculated according to Manzoni et al. 2012. **b** Microbial biomass N (Nmic) per g dry soil. **c** C to N ratio calculated by dividing Cmic by Nmic. **d,e** Total dissolved N and dissolved organic C, derived directly from KCl extracts from soil. **f** CO_2 flux, shows values of CO_2 as measured directly from headspace vials. At each sampling time point five independent replicate microcosms were measured per treatment (data see table S5). ANOVA and post-hoc tuckey's HSD were used for all statistical analyses on each day.

Reference

Manzoni, Stefano, Philip Taylor, Andreas Richter, Amilcare Porporato, and Göran I. Ågren. 2012. "Environmental and Stoichiometric Controls on Microbial Carbon-use Efficiency in Soils." *New Phytologist* 196 (1): 79–91. <https://doi.org/10.1111/j.1469-8137.2012.04225.x>.

Supplementary Tables

Table S1. Most abundant (>5%) taxa per sampling day

Day 0				
Species	Domain	Phylum	Class	Relative_Count (percent)
Apo	Bacteria	Proteobacteria	Burkholderiaceae	39.22
Mild	Bacteria	Proteobacteria	Burkholderiaceae	28.49
Strong	Bacteria	Proteobacteria	Burkholderiaceae	22.31
SC	Bacteria	Proteobacteria	Burkholderiaceae	22.06
Apo	Bacteria	Proteobacteria	Sphingomonadaceae	20.45
Strong	Bacteria	Proteobacteria	Sphingomonadaceae	16.45
Mild	Bacteria	Proteobacteria	Sphingomonadaceae	16.43
SC	Bacteria	Bacteroidota	Sphingobacteriaceae	14.90
Strong	Bacteria	Bacteroidota	Sphingobacteriaceae	14.55
SC	Bacteria	Proteobacteria	Sphingomonadaceae	13.89
Mild	Bacteria	Bacteroidota	Sphingobacteriaceae	12.58
Strong	Bacteria	Verrucomicrobiota	Parachlamydiaceae	7.95
SC	Bacteria	Proteobacteria	Rhodanobacteraceae	7.62
Apo	Bacteria	Bacteroidota	Sphingobacteriaceae	7.45
Mild	Bacteria	Actinobacteriota	Microbacteriaceae	6.86
Strong	Bacteria	Proteobacteria	Rhodanobacteraceae	6.74
Strong	Bacteria	Actinobacteriota	Microbacteriaceae	6.68
Strong	Bacteria	Proteobacteria	Pseudomonadaceae	6.27
Mild	Bacteria	Proteobacteria	Rhodanobacteraceae	6.19

Day 5				
Species	Domain	Phylum	Class	Relative_Count (percent)
Strong	Bacteria	Proteobacteria	Burkholderiaceae	23.81
Apo	Bacteria	Bacteroidota	Sphingobacteriaceae	23.34
SC	Bacteria	Bacteroidota	Sphingobacteriaceae	20.31
Strong	Bacteria	Proteobacteria	Sphingomonadaceae	18.65
SC	Bacteria	Proteobacteria	Burkholderiaceae	17.24
Mild	Bacteria	Proteobacteria	Burkholderiaceae	16.35
Mild	Bacteria	Bacteroidota	Sphingobacteriaceae	15.15
SC	Bacteria	Proteobacteria	Sphingomonadaceae	14.80
Apo	Bacteria	Acidobacteriota	Acidobacteriaceae (Subgroup 1)	12.29
Apo	Bacteria	Proteobacteria	Burkholderiaceae	11.71
SC	Bacteria	Acidobacteriota	Acidobacteriaceae (Subgroup 1)	10.33
Mild	Bacteria	Acidobacteriota	Acidobacteriaceae (Subgroup 1)	9.96
Apo	Bacteria	Proteobacteria	Sphingomonadaceae	9.79
Strong	Bacteria	Bacteroidota	Sphingobacteriaceae	9.37
Strong	Bacteria	Actinobacteriota	Microbacteriaceae	8.97
Strong	Bacteria	Acidobacteriota	Acidobacteriaceae (Subgroup 1)	8.26
Mild	Bacteria	Proteobacteria	Sphingomonadaceae	8.18

Day 11				
Species	Domain	Phylum	Class	Relative_Count (percent)
Apo	Bacteria	Bacteroidota	Sphingobacteriaceae	29.02
SC	Bacteria	Bacteroidota	Sphingobacteriaceae	26.95
Mild	Bacteria	Bacteroidota	Sphingobacteriaceae	17.81

Strong	Bacteria	Acidobacteriota	Acidobacteriaceae (Subgroup 1)	16.66
Strong	Bacteria	Proteobacteria	Sphingomonadaceae	16.63
Mild	Bacteria	Acidobacteriota	Acidobacteriaceae (Subgroup 1)	14.99
SC	Bacteria	Acidobacteriota	Acidobacteriaceae (Subgroup 1)	14.93
Strong	Bacteria	Bacteroidota	Sphingobacteriaceae	14.92
Strong	Bacteria	Proteobacteria	Burkholderiaceae	14.79
Mild	Bacteria	Proteobacteria	Sphingomonadaceae	13.73
Apo	Bacteria	Acidobacteriota	Acidobacteriaceae (Subgroup 1)	11.55
SC	Bacteria	Proteobacteria	Burkholderiaceae	10.39
Mild	Bacteria	Proteobacteria	Burkholderiaceae	10.37
Apo	Bacteria	Proteobacteria	Burkholderiaceae	9.31
Apo	Bacteria	Proteobacteria	Sphingomonadaceae	8.64
SC	Bacteria	Proteobacteria	Sphingomonadaceae	7.71

Day 20				
Species	Domain	Phylum	Class	Relative_Count (percent)
Apo	Bacteria	Bacteroidota	Sphingobacteriaceae	24.64
SC	Bacteria	Bacteroidota	Sphingobacteriaceae	21.44
Strong	Bacteria	Bacteroidota	Sphingobacteriaceae	19.22
Mild	Bacteria	Bacteroidota	Sphingobacteriaceae	18.28
Mild	Bacteria	Acidobacteriota	Acidobacteriaceae (Subgroup 1)	18.18
SC	Bacteria	Acidobacteriota	Acidobacteriaceae (Subgroup 1)	15.12
Apo	Bacteria	Acidobacteriota	Acidobacteriaceae (Subgroup 1)	14.33
SC	Bacteria	Proteobacteria	Sphingomonadaceae	12.60
Mild	Bacteria	Proteobacteria	Sphingomonadaceae	11.40
Strong	Bacteria	Acidobacteriota	Acidobacteriaceae (Subgroup 1)	11.07
Mild	Bacteria	Proteobacteria	Burkholderiaceae	10.61
SC	Bacteria	Proteobacteria	Burkholderiaceae	10.61
Strong	Bacteria	Proteobacteria	Sphingomonadaceae	10.42
Apo	Bacteria	Proteobacteria	Burkholderiaceae	9.85
Apo	Bacteria	Proteobacteria	Sphingomonadaceae	9.02
Strong	Bacteria	Proteobacteria	Burkholderiaceae	8.30

Day 35				
Species	Domain	Phylum	Class	Relative_Count (percent)
Strong	Bacteria	Bacteroidota	Sphingobacteriaceae	22.40
SC	Bacteria	Bacteroidota	Sphingobacteriaceae	19.10
Apo	Bacteria	Bacteroidota	Sphingobacteriaceae	16.25
Mild	Bacteria	Bacteroidota	Sphingobacteriaceae	14.99
Apo	Bacteria	Proteobacteria	Sphingomonadaceae	13.99
Mild	Bacteria	Acidobacteriota	Acidobacteriaceae (Subgroup 1)	13.62
Apo	Bacteria	Acidobacteriota	Acidobacteriaceae (Subgroup 1)	12.98
SC	Bacteria	Proteobacteria	Sphingomonadaceae	12.91
Strong	Bacteria	Acidobacteriota	Acidobacteriaceae (Subgroup 1)	12.50
Mild	Bacteria	Proteobacteria	Sphingomonadaceae	12.21
SC	Bacteria	Acidobacteriota	Acidobacteriaceae (Subgroup 1)	11.19
Strong	Bacteria	Proteobacteria	Sphingomonadaceae	10.98
Strong	Bacteria	Proteobacteria	Burkholderiaceae	10.41
Apo	Bacteria	Proteobacteria	Burkholderiaceae	9.42
SC	Bacteria	Proteobacteria	Burkholderiaceae	8.32
Mild	Bacteria	Proteobacteria	Burkholderiaceae	8.02

Table S2. Categorization of abundant soil taxa into r or K strategists

Phylum/subphylum	Family	r/K Strategy	Role in Soil & Reference(s)
Acidobacteriota (Subdivision 1)	Acidobacteriaceae (sub 1)	K-strategist (oligotroph)	(Kielak et al., 2016)
Proteobacteria – Alphaproteobacteria	Beijerinckiaceae	K-strategist (oligotroph)	(Cyle et al., 2020)
	Caulobacteraceae	r-strategist (copiotroph)	(Cyle et al., 2020)
	Devosiaceae	K-strategist (oligotroph)	(Talwar et al., 2020)
	Reyraneliaceae	K-strategist (oligotroph)	(Bartelme et al., 2020)
	Rhizobiaceae	r-strategist (copiotroph)	(Cho et al., 2024)
	Sphingomonadaceae	r-strategist (copiotroph)	(Sorouri et al., 2023; Zhan et al., 2021)
	Xanthobacteraceae	K-strategist (oligotroph)	(Bárta et al., 2017)
Proteobacteria – Betaproteobacteria	Burkholderiaceae	r-strategist (copiotroph)	(Chiba et al., 2021)
Proteobacteria – Gammaproteobacteria	Pseudomonadaceae	r-strategist (copiotroph)	(Martinez Alvarez et al., 2022)
	Rhodanobacteraceae	K-strategist (oligotroph)	(Martinez Alvarez et al., 2022)
Bacteroidota	Chitinophagaceae	r-strategist (copiotroph)	(Wieczorek et al., 2019; Zhan et al., 2021)
	Sphingobacteriaceae	r-strategist (copiotroph)	(Cho et al., 2024; Mastný et al., 2021)
Actinobacteriota	Microbacteriaceae	r-strategist (copiotroph)	(Barnett et al., 2023)
	Nocardiodaceae	K-strategist (oligotroph)	(Bartelme et al., 2020; Ma et al., 2023; Zhan et al., 2021)
	Streptomycetaceae	K-strategist (oligotroph)	(Fierer et al., 2007)

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Table S3. Number of differentially abundant ASVs per genus (with minimum of 3)

Highlighted in bold are genera with highest numbers of affected ASVs (same as Figure 4): Burkholderia-Caballeronia-Paraburkholderia, Mucilaginibacter, Chthoniobacter, Edaphobaculum, Chitinophaga, Pseudomonas, and Reyranella

compared to Apo	T0	T0	T5	T5	T11	T11
Group	Mild~ Apo	Strong~ Apo	Mild~ Apo	Strong~ Apo	Mild~ Apo	Strong~ Apo
Burkholderia-Caballeronia-Paraburkholderia		3	3	5	4	9
Chitinophaga						4
Chthoniobacter						15
Conexibacter					4	
Dongia						4
Edaphobacter						3
Edaphobaculum						9
Granulicella		3				5
Mucilaginibacter		3		6	7	36
Mycobacterium					3	
Reyranella						10
Phenylobacterium						8
Pseudomonas						
Puia						3
Ca. Solibacter						3
Sphingomonas	3	4	7	5	7	10

compared to Apo	T20	T20	T35	T35
Group	Mild~ Apo	Strong~ Apo	Mild~ Apo	Strong~ Apo
Acidothermus	3			
Burkholderia- Caballeronia- Paraburkholderia				
Chthoniobacter				
Edaphobaculum				
Mucilaginibacter	5			
Reyranella				
Pseudomonas	3			
Sphingomonas	4			

compared to SC	T0	T0	T0	T5	T5	T5
Group	Mild~ SC	Strong~ SC	Apo~ SC	Mild~ SC	Strong~ SC	Apo~ SC
Bradyrhizobium	3					
Burkholderia- Caballeronia- Paraburkholderia				6	8	5
Chitinophaga						
Chthoniobacter				6	3	
Curtobacterium				3		
Edaphobaculum						
Granulicella					3	
Mucilaginibacter		4		6	11	
Nocardioides				5		
Reyranella	3					
Rhodococcus				3		
Pseudomonas						4
Sphingomonas		4		10	7	4

compared to SC	T11	T11	T11	T20	T20	T20
Group	Mild~ SC	Strong~ SC	Apo~ SC	Mild~ SC	Strong~ SC	Apo~ SC
Burkholderia- Caballeronia- Paraburkholderia	5	6		4		
Chthoniobacter	6	17				
Edaphobaculum		9				
Granulicella	4	4				
Hyphomicrobium						
Mucilaginibacter	6	34		3		
Reyranella		10				
Phenyllobacterium		7				
Pseudomonas						
Sphingomonas	6	8	4			
Terrimicrobium		3				
Ca. Xiphinematobacter		4				

compared to SC	T35	T35	T35
Group	Mild~ SC	Strong~ SC	Apo~ SC
Burkholderia- Caballeronia- Paraburkholderia		3	
Chthoniobacter		3	
Edaphobaculum			
Granulicella		3	
Hyphomicrobium			3
Mucilaginibacter		4	3
Reyranella			
Pseudomonas			3
Sphingomonas		3	4

Table S4. Ecosystem process data

DAY 20	Microbial respiration	Microbial growth	Microbial biomass (Cmic)	Gross N mineralization
	[µg / mg Cmic / d]	[µg / mg Cmic / d]	[µg Cmic / g dm]	[µg N / g dm / day]
SC-1	3.208	1.515	244.16	0.1947
SC-2	8.803	5.093	75.97	0.1811
SC-3	3.379	2.183	183.74	1.0189
SC-4	4.241	2.344	158.87	
SC-5	3.193	1.128	188.38	1.4872
Apo-1	5.228	3.999	181.67	0.8515
Apo-2	5.903	4.110	128.96	1.2429
Apo-3	4.376	2.873	161.39	1.5829
Apo-4	7.867	6.001	92.27	2.1452
Apo-5	11.512	6.655	80.51	1.5536
Mild-1	8.027	8.904	119.47	3.1505
Mild-2				
Mild-3	14.012	10.666	67.74	2.0364
Mild-4	10.239	6.878	73.58	1.2825
Mild-5	8.835	6.584	79.09	0.5177
Strong-1	3.674	2.577	180.68	1.7767
Strong-2	3.400	2.149	182.63	2.1525
Strong-3	4.768	2.029	149.29	
Strong-4	3.734	2.711	232.76	0.7819
Strong-5	3.802	2.686	167.80	0.7612
DAY 5	Gross N mineralization			
	[µg N / g dm / day]			
SC-1	3.26			
SC-2	4.26			
SC-3	4.15			
SC-4				

SC-5	2.58
Apo-1	2.12
Apo-2	3.51
Apo-3	2.63
Apo-4	1.03
Apo-5	4.57
Mild-1	5.39
Mild-2	9.23
Mild-3	9.58
Mild-4	4.95
Mild-5	8.29
Strong-1	2.03
Strong-2	4.34
Strong-3	4.03
Strong-4	
Strong-5	

Table S5. Extended ecosystem process data

DAY 20	Carbon Use Efficiency	Microbial biomass N (Nmic)	C:N ratio	Total dissolved nitrogen	Dissolved organic carbon	CO2 flux
		[µg N / g dm]	[Cmic / Nmic]	[µg N / g dm]	[µg C / g dm]	[ng C / g dm/ h]
SC-1	0.339	42.48	5.75	88.94	670.80	1740.57
SC-2	0.207	12.23	6.21	100.94	755.36	1486.24
SC-3	0.420	33.96	5.41	99.81	739.30	1379.83
SC-4	0.306	22.35	7.11	67.55	493.18	1497.44
SC-5	0.363	27.61	6.82	90.62	700.49	1336.66
Apo-1	0.420	26.85	6.77	110.94	816.66	2110.45
Apo-2	0.282	19.68	6.55	98.37	669.18	1691.73
Apo-3	0.386	27.85	5.79	112.74	853.04	1569.39
Apo-4	0.238	12.21	7.56	76.50	519.92	1613.09
Apo-5	0.165	11.83	6.81	86.95	590.89	2059.59
Mild-1	0.346	18.86	6.33	119.20	869.23	2131.12
Mild-2		8.72		116.58	840.38	1595.59
Mild-3	0.162	21.71	3.12	88.83	708.45	2109.36
Mild-4	0.193	11.64	6.32	69.07	497.54	1674.16
Mild-5	0.249	13.52	5.85	105.57	722.94	1552.89
Strong-1	0.408	24.32	7.43	99.98	777.13	1475.15
Strong-2	0.409	27.32	6.68	94.28	782.16	1379.98
Strong-3	0.274	23.89	6.25	75.49	523.82	1581.70
Strong-4	0.420	38.02	6.12	77.76	590.15	1931.61
Strong-5	0.374	23.06	7.28	88.28	576.21	1417.81

DAY 5	Microbial biomass N (Nmic)	Total dissolved nitrogen	CO2 flux
	[$\mu\text{g N / g dm}$]	[$\mu\text{g N / g dm}$]	[ng C / g dm/ h]
SC-1	2.55	77.50	2709.38
SC-2		89.22	2878.09
SC-3	8.53	89.71	1949.09
SC-4	1.70	59.63	1882.57
SC-5	1.60	82.88	3332.24
Apo-1		101.40	1737.48
Apo-2		90.53	2201.93
Apo-3	7.91	91.81	2537.44
Apo-4	7.77	76.06	3519.56
Apo-5	2.69	74.30	2875.12
Mild-1	18.92	103.26	1601.44
Mild-2	6.70	105.51	3901.21
Mild-3	4.46	72.93	2426.72
Mild-4		57.26	5434.31
Mild-5		96.34	4037.94
Strong-1	2.02	88.70	4428.44
Strong-2	3.52	87.81	1927.61
Strong-3	4.02	72.19	3434.46
Strong-4	2.53	67.82	1601.74
Strong-5	4.57	83.20	3616.94

Chapter III

Environmental Chlamydiae at the Animal- Protist Interface: *A Hydra*-Associated Chlamydial Symbiont

Environmental chlamydiae at the animal-protist interface: A *Hydra*-associated chlamydial symbiont

Short title: A *Hydra*-associated chlamydial symbiont

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Abstract

Symbiotic associations between microorganisms often involve eukaryotes partnering with microbes for nutrient exchange, protection and resource acquisition. Bacterial lineages, like the Chlamydiota have evolved entirely symbiotic lifestyles, exploiting their eukaryotic hosts for energy and shelter. The study of environmental chlamydiae - outside the well-studied vertebrate host range - has revealed diverging interactions on the mutualism-parasitism spectrum. This highlights their potentially important roles in trophic networks, underscoring the relevance of obtaining isolates from diverse environments and hosts.

Here, we describe the first isolate of a chlamydial symbiont of the freshwater cnidarian *Hydra*. The symbiont can be stably maintained in insect cell lines and represents the first cultured organism of the recently described family-level lineage Chlamydiae Clade III for which we propose the name *Endochlamydiaceae*. Fluorescence and electron microscopy reveal the chlamydial morphology and endodermal location. Comparative genomics shows the isolate, named *Endochlamydia hydrae*, encodes a conserved set of genes involved in host invasion, communication and pathogenicity. Instead of displaying unique adaptations to its host, *E. hydrae* shows signs consistent with genome reorganization and streamlining, suggesting a more recent host shift. Screening for closely related 16S rRNA gene sequences in public environmental microbiomes also indicates a broader host range. Moreover, exploration of environmental *Hydra oligactis* populations revealed they might serve as host for a broader range of chlamydial species. This study highlights the evolutionary success of chlamydiae and their genomic toolkit to infect a wide range of hosts and their ecological significance by interacting with diverse organisms within trophic networks.

Introduction

Symbiotic associations shape ecosystems as well as lifestyles and evolution of organisms thriving within them. Many of these associations are ancient and provide good opportunities to study the dynamics of processes influencing them. Members of the bacterial phylum Chlamydiota that have been living an obligate endosymbiotic lifestyle for the past 1-2 billion [1, 2] are found associated with hosts from many eukaryotic clades, most notably as important human and livestock pathogens, and also have been detected in various environments [3]. They thrive as symbionts of a diverse range of protist hosts, but also as symbionts of arthropods in soil and in cnidarian hosts in marine habitats [3–7].

Chlamydiae alternate between two metabolically different stages, a replicative stage (reticulate body, RB), typically residing inside host derived vacuoles termed inclusions, and a non-replicating,

infectious stage (elementary body, EB), permitting extracellular survival and reinfection [8–10]. The chlamydial dependence on eukaryotic host organisms and the difficulty of isolating and maintaining many hosts in a laboratory, hampers the cultivation of novel chlamydial isolates [11, 12]. Thus, genomics and metagenomics are often the methods of choice when studying environmental chlamydiae. Although chlamydiae generally have small genomes of around 1 - 3 Mbp and lack many genes necessary for essential metabolic pathways, a comparatively large core gene set is conserved throughout all chlamydial clades [1]. This toolkit helps them to not only colonize and manipulate host organisms, but also to exploit them for energy, amino acids and nucleotides. Most notably genes like the highly conserved type III secretion system for host cell manipulation and nucleotide transport systems, which allow them to complement for their lack of de novo nucleotide synthesis and simultaneously facilitate energy parasitism [7, 13, 14].

The cnidarian freshwater predator *Hydra* has been well documented for its close association with other organisms. A thoroughly investigated example are *Chlorella* algae in *Hydra viridissima*, showing a close association and co-evolution between the two partners [15]. Symbiotic associations in *Hydra* have also been shown to be essential in the development and feeding behaviour of *Hydra* polyps [16, 17]. Another example involves a *Hydra oligactis* strain (SpB) for which spontaneous formation and persistence of tumors was only observed in the presence of a bacterial conflict between *Pseudomonas* and the spirochete *Turneriella* [18]. More recent findings on the microbiome of several different *Hydra* species have contributed to the understanding of innate immunity through antimicrobial peptides by controlling resident bacteria [19], which in turn provide resistance against colonization [20]. Medusozoa like *Hydra* are amongst the first animal lineages to possess Toll-like receptor-like (TLR-like) proteins, nucleotide-binding oligomerization domain-like receptors (NLR)-like proteins, and a basic NF- κ B pathway [21]. Thus, *Hydra* is used as a model organism to study the evolution of the innate immune system. *Hydra oligactis* symbiosis with bacteria of the phylum Chlamydiota has been suggested in a previous study, which investigated wild *Hydra* populations from Hungary and France [22].

In this study we present a closer description and analysis of this *Hydra*–chlamydiae symbiosis, derived from wild populations. By combining fluorescence- and transmission electron microscopy together with genome sequencing and analysis, we characterize the first isolate of a member of the recently proposed Chlamydiae clade III family [1]. Both genomic analysis and environmental distribution of this chlamydial species point to an evolutionarily recent switch to *Hydra* as a host, thus offering an exciting study model for host-symbiont evolution and the adaptation to host immune systems of two ancient organism lineages.

Results and Discussion

Stable association of a chlamydial symbiont with *Hydra oligactis*

A previous study suggested that a single chlamydial organism represents an important fraction of the microbiome of *Hydra oligactis* laboratory cultures established between 2016 and 2021 originating from environmental samples [22]. To confirm these observations and to investigate the stability of the association between *Hydra* and this chlamydial symbiont, the symbiotic *H. oligactis* strains St. Petersburg (SpB), X11/14, C2/7, Vilneuvette, and Montaud [22, 23] were monitored in culture regularly by fluorescence *in situ* hybridization (FISH) of sectioned individuals and digital PCR (dPCR) of both individuals and culture supernatant. Here, we could show the chlamydial symbionts were detected consistently within the tissue of the *Hydra* strain Montaud by FISH throughout a two-year cultivation period (Figure 1). 16S rRNA gene sequencing and dPCR using DNA isolated from individual *Hydra* polyps of all strains allowed us to verify the symbiont identity and quantify the number of chlamydiae present per animal. Of the 62 analyzed *H. oligactis* individuals 77.4% were infected with chlamydiae (Table S1). On average a single infected polyp of *H. oligactis* strain C2/7, Montaud, Spb, Vilneuvette, and X11/14 harbored 7.8×10^8 , 2.1×10^8 , 2×10^7 , 1.1×10^9 , and 6.4×10^8 chlamydiae, respectively (Figure 2A). Variance however was high between tested individuals, ranging from 766 up to 4.4×10^9 chlamydiae per polyp (Table S1, Figure 2A).

Chlamydiae reside in large inclusions within the endoderm of the *Hydra* polyp

To visualize the location of chlamydiae within *Hydra* polyps, we used FISH with chlamydiae-specific probes on thin sections of whole animals. We observed chlamydiae-containing inclusions exclusively inside epithelial cells of the *Hydra* endoderm along the entire body column of the animal, including foot and tentacles (Figure 1AB). Subsequently we confirmed the location of chlamydial cells inside the *Hydra* endoderm, appearing exclusively in densely filled inclusions with lengths of up to 25 μm , by transmission electron microscopy (TEM) (Figure 1C, Panel 1-3). We identified morphotypes characteristic of the chlamydial developmental stages: EBs were often elongated, with 300 - 520 nm in length, and located in the center of inclusions. RBs appeared round, with 480 - 860 nm in diameter, and more prevalent at the periphery of inclusions (Figure 1C, Panel 4-6). This is consistent with observations of other chlamydiae that form large inclusions like the human pathogen *Chlamydia trachomatis* [24] and the amoeba symbiont *Parachlamydia acanthamoebae* [25].

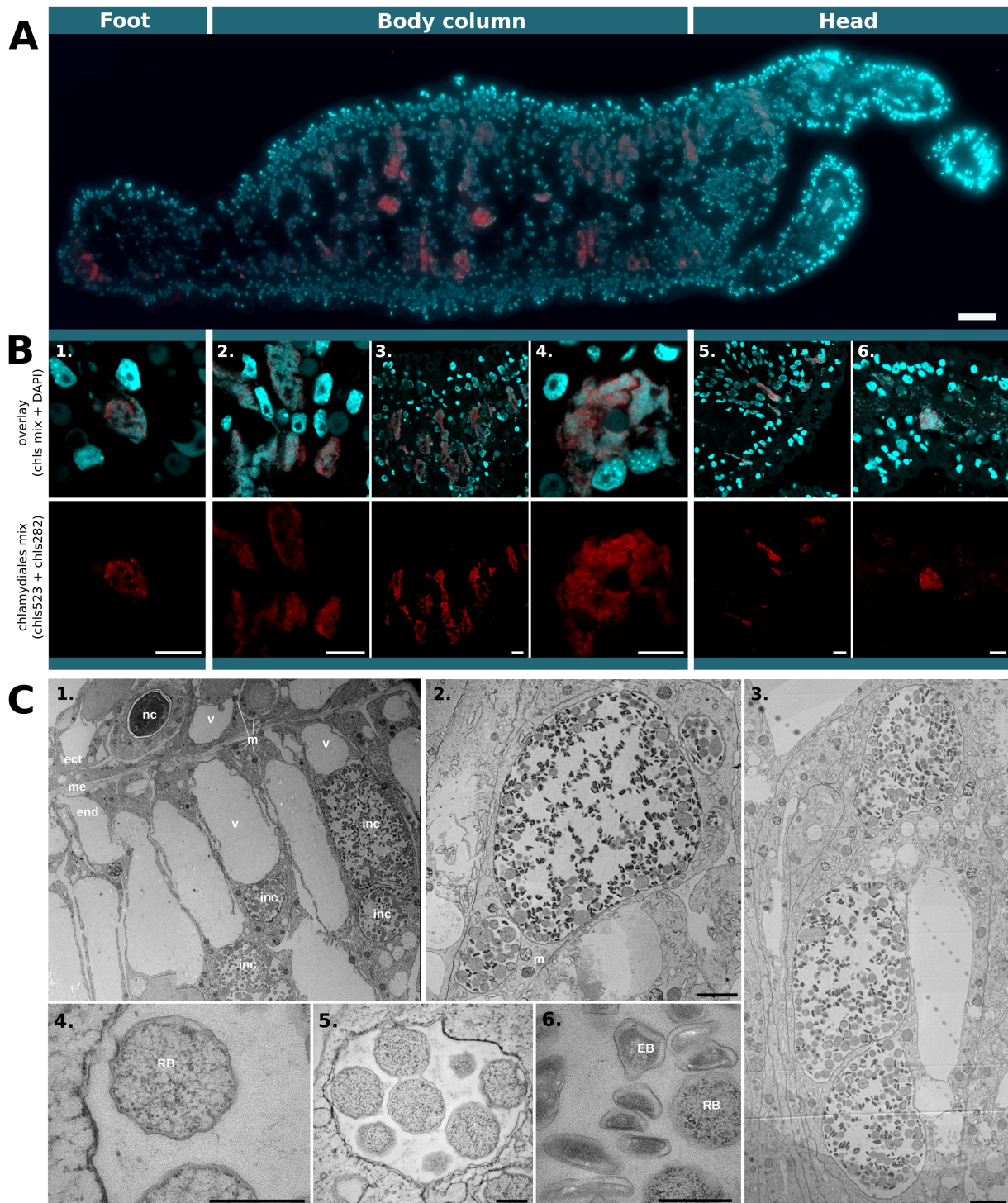


Figure 1: Chlamydiae infect the endoderm of *Hydra oligactis*. (A) Chlamydiae are located inside the endoderm along all parts of the *H. oligactis* polyp, as observed by FISH and confocal laser scanning microscopy on 4 μm sections of three different individuals of *H. oligactis* strain Montaud. (B) Zoom in on different areas (foot, body column, head + tentacles) showing chlamydiae inside the endoderm of polyps with FISH and microscopy. Red: Chlamydiae-specific oligonucleotide probes Chls523 and Chls282; cyan: DNA (DAPI staining). (C) Transmission electron micrographs showing large inclusions within the *Hydra* endodermal cells (C 1-3). Close-ups of the chlamydial reticulate bodies in small inclusions (C 4-5) and of elementary bodies in a large inclusion (C 6). Ectoderm (ect), mesoglea (me), endoderm (end), nematocyst (nc), vacuole (v), mitochondria (m), chlamydial inclusion (inc), chlamydial reticulate bodies (RB), chlamydial elementary bodies (EB). Scale bars: 50 μm (A), 10 μm (B), 2.5 μm (C 2,3), and 0.5 μm (C 4-6).

Evidence for horizontal and vertical transmission

Chlamydial EBs enter a host cell via a combination of phagocytosis and chlamydiae-induced actin remodelling at the entry site [24]. After entry and transformation to replicating RBs, they eventually transform back to EBs that are typically released via host cell lysis or inclusion extrusion [26–28], sometimes also through cell-to-cell contact [29, 30]. In this *Hydra*-chlamydiae system we could not directly observe evidence for lysis or chlamydial extrusion in *Hydra* endodermal cells in TEM. We thus tested for the presence of chlamydial DNA in the supernatant of *H. oligactis* strain Montaud cultures, using dPCR, and found on average 3.9×10^5 genome copies/ml of in the supernatant of all tested cultures (n=7), while infected polyps of the same culture had on average 7.8×10^8 copies/polyp (Figure 2, Tables S1 and S2). Although this does not directly confirm horizontal transmission between individual animals, it indicates the release of chlamydiae into the supernatant of *Hydra* cultures. As chlamydiae-free *Hydra* cultures were not available, we further studied horizontal transmission of chlamydial EBs from *H. oligactis* by establishing infection in *Spodoptera frugiperda* (Sf9) insect cells, which have been shown previously to be susceptible to chlamydial infection [4, 31]. Using chlamydiae-specific PCR and FISH, we could show that the chlamydial symbionts could stably infect Sf9 cell cultures (Figure 2).

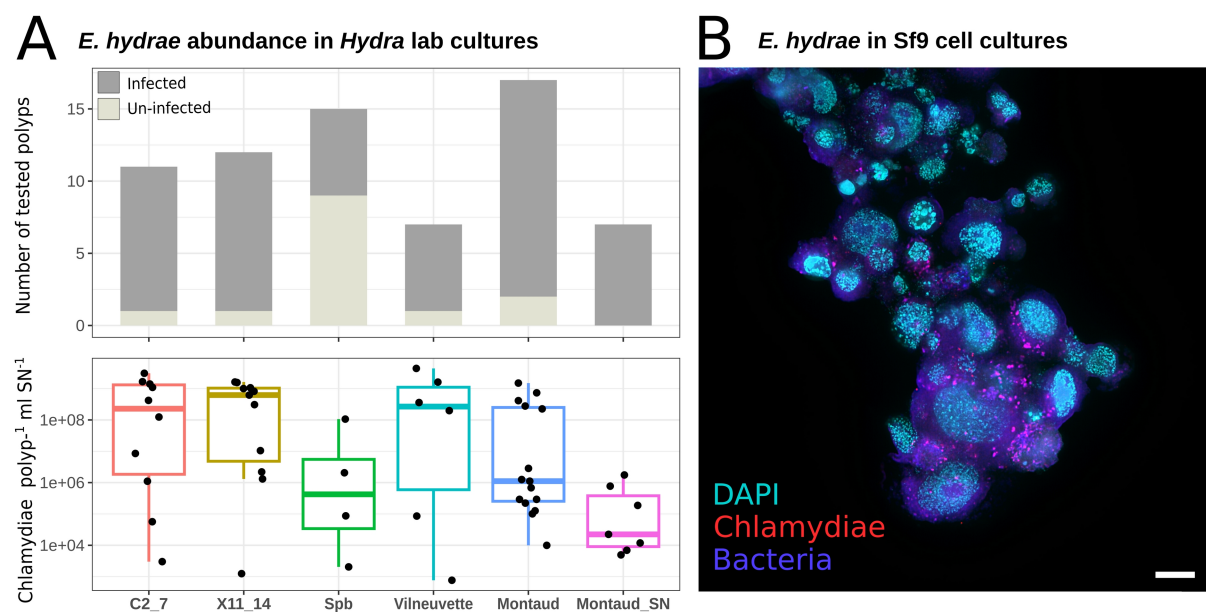


Figure 2. Abundance of chlamydiae in *Hydra* lab strains and stable reproduction in an insect cell line. (A) Number of tested positive (grey) and negative (i.e. uninfected; light-grey) individuals of each *H. oligactis* strain are shown in the bar plot. Below, total chlamydial abundance in individual polyps of the same *H. oligactis* strains or culture supernatant (SN) as measured by dPCR. Boxes indicate the interquartile range, the horizontal line indicates the median, whiskers indicate minimum and maximum values, respectively. (B) Chlamydiae are located inside Sf9 cells, observed after five months of co-cultivation with FISH and Thunder Imager. Cyan: DNA stain DAPI; Red: Chlamydiae mix (Chls523 and Chls282), Blue: Eubacteria mix (EUBI + EUBII + EUBIII). Scalebar 10 μ m.

When sufficient food is available *Hydra* mainly reproduces asexually by budding. In *H. oligactis* this mode is preferentially used over sexual reproduction, which especially in females typically results in the death of the polyp [32]. We were able to confirm vertical transmission of chlamydial symbionts to the offspring during asexual reproduction, using FISH on thin sections of *H. oligactis* Montaud polyps (Figure 3).

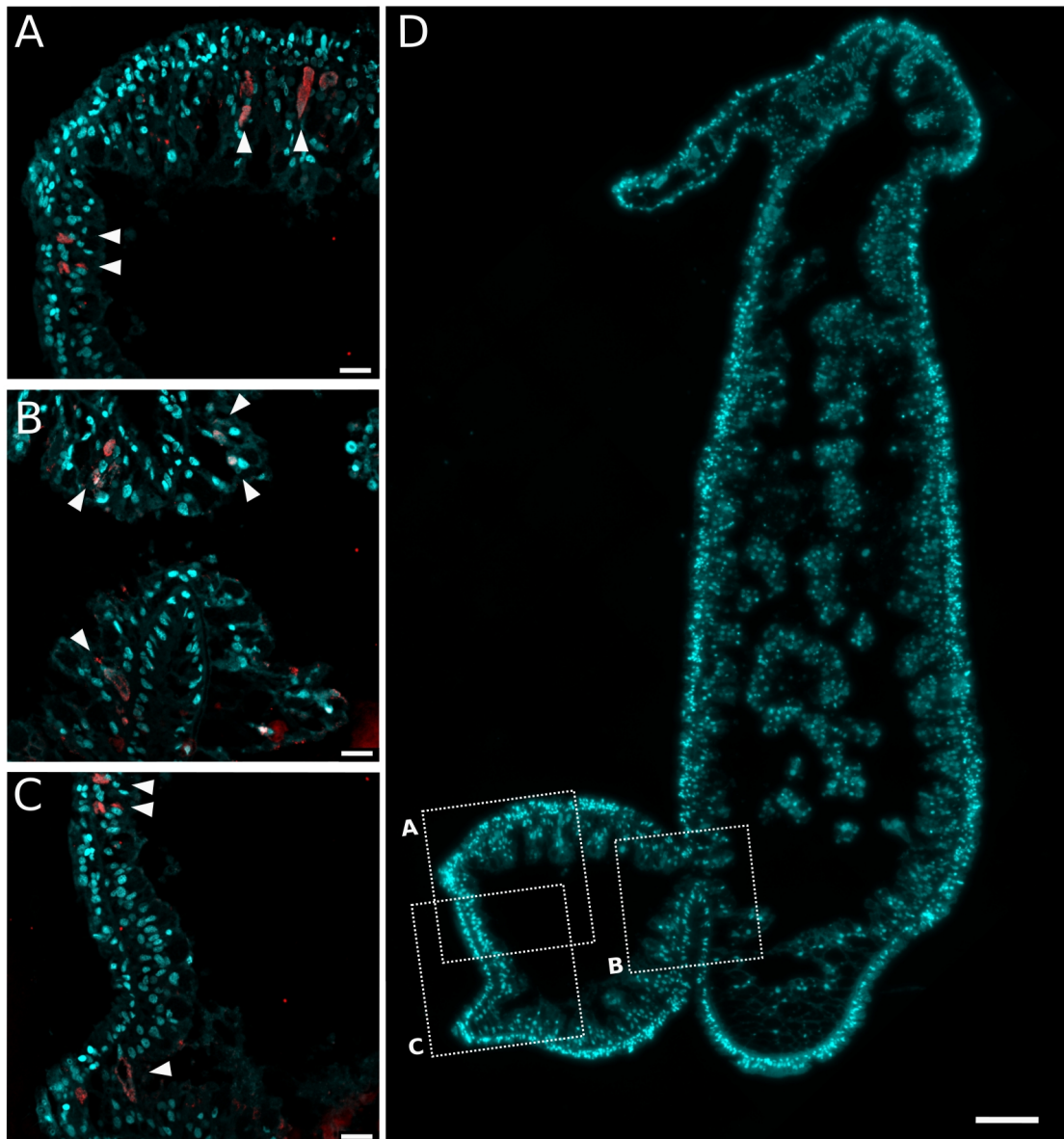


Figure 3: Symbionts are transmitted vertically through the endoderm. (A-C) Chlamydiae-containing inclusions appear throughout the endoderm of forming *Hydra oligactis* buds, as observed with FISH and confocal laser scanning microscopy. (D) Overview of the section with DAPI, as observed with FISH and Thunder Imager. Red: Mix of Chls523 and Chls282 probes; Cyan: DAPI stain. Scale bars 20 μ m (A-C) and 100 μ m (D).

Taken together, chlamydial symbionts are released into the culture supernatant from infected *H. oligactis* polyps. These extracellular chlamydiae can be propagated in insect cell cultures, suggesting horizontal transmission via the environment. In addition, chlamydial symbionts are transmitted vertically to newly forming buds during asexual reproduction of the *Hydra* polyp. While this ensures propagation and spread of the symbiont within the host population, vertical transmission generally stabilises host-symbiont associations and has been shown to create a coupling of host- and symbiont fitness, leading to decreased virulence [33].

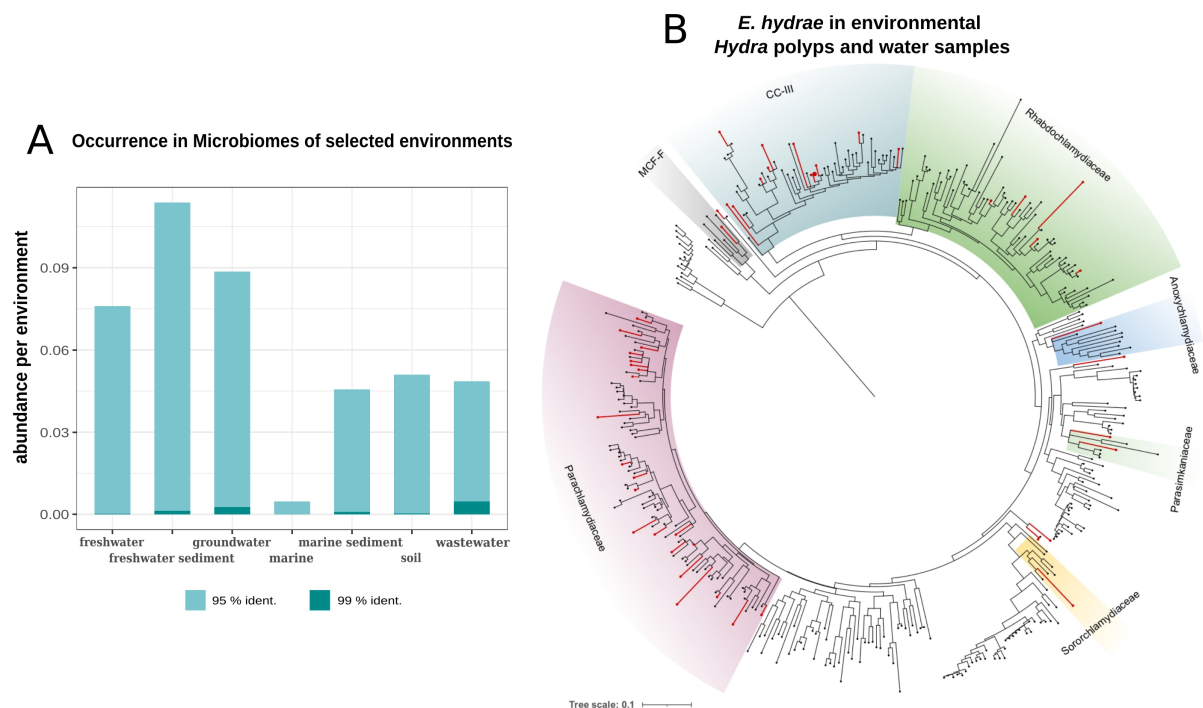


Figure 4. Diversity and distribution of *Hydra*-associated chlamydiae in the environment. (A) Occurrence of *E. hydrae* 16S rRNA gene sequence in microbiomes of selected environments at the species (99% identity) and genus (95% identity) levels. Abundance is shown as the percentage of microbiomes within the IMNGS database in which the sequence could be found at either identity level. (B) Maximum likelihood phylogeny of 16S rRNA gene (V4 region) of 51 chlamydial genus-level OTUs. Red colored branches represent taxa from which a chlamydial sequence originating from a *Hydra* polyp could be detected.

A potential environmental reservoir

To assess the environmental distribution of the *Hydra*-associated chlamydial symbiont, we searched available microbiome data sets for similar 16S rRNA gene sequences using the integrated microbial next-generation sequencing (IMNGS) database [34]. On the species level (99% sequence identity with the 16S rRNA gene of the symbiont), environments with highest relative abundances were found in wastewater, groundwater, and freshwater sediment microbiomes. On the genus level (95% sequence identity), highest relative abundances were found in freshwater sediment (0.11%), groundwater (0.09%) and freshwater (0.08%) (Figure 4A, Table S3). The detection of 16S rRNA gene sequences similar to that of the *Hydra* symbiont at the species and genus-level in *Hydra*-free environments such as groundwater and wastewater, suggests that their broad distribution might be facilitated by other hosts. Protists, common hosts of chlamydiae [3], thrive in these environments and are sometimes found colonizing the surface of *Hydra* polyps, like the ciliate *Kerona pediculus* [35].

Diverse chlamydiae associated with wild *Hydra* populations

Little is known about the association of chlamydial symbionts with wild *Hydra* populations. We thus analysed chlamydial 16S rRNA gene sequences from a microbiome dataset of wild *H. oligactis* populations and surrounding environmental water samples (Tökölyi *et al.* in revision). Chlamydial sequences identical or similar (>95% sequence identity) to the chlamydial symbiont of the lab strains were present in several wild *Hydra* polyps. Of note, chlamydial sequences detected here were not restricted to the *H. oligactis* symbiont phylotype, but were highly diverse and affiliated with several chlamydial families, including the *Parachlamydiaceae* (24 operational taxonomic units, OTUs), *Rhabdochlamydiaceae* (5 OTUs) and CC-III (12 OTUs) (Figure 4B). Most sequences, including the *H. oligactis* symbiont phylotype, were detected both in polyp and water samples (Figure S1, Table S4, Data S1).

The relative abundance of chlamydial sequences in the environmental polyps was very low (<0.0001% in most samples, with a maximum of 0.04%) (Tökölyi *et al.* (2025), in revision). This might represent an underestimate due to known biases of commonly used bacterial primers discriminating against the Chlamydiota [36, 37] and contrasts with the high (>50%) relative abundance found in *H. oligactis* Montaud lab populations [22]. The presence of chlamydial sequences in the samples from wild *Hydra* populations, however, could either represent *Hydra* symbionts or chlamydiae present in the water or in microbial eukaryotes that were not efficiently removed during sample preparation. Together, the detection of chlamydial DNA in wild *Hydra* populations identical to the symbiont of *H. oligactis* Montaud lab strains strongly suggests the presence of the symbiont in

natural environments. In addition, there is evidence for an even higher diversity of chlamydiae associated with wild *Hydra* populations and their microbiomes.

***Endochlamydia hydrae* is the first cultured representative of the *Endochlamydiaceae* (CC-III) family**

We obtained the full genome sequence of the chlamydial symbiont of *H. oligactis* through a combination of long and short read sequencing. With a size of around 2.3 Mbp and 2542 coding sequences the genome is larger than those of chlamydial vertebrate pathogens but similar in size to those of protist-associated chlamydiae. The GC content of 51.4% is at the upper limit in the phylum [1, 38] (Table S6). Based on phylogenetic analysis and average amino acid identity (AAI, Figure S2) comparison this symbiont represents a novel species within the recently described Chlamydiae Clade III (CC-III) family [1] (Figure 5). A family, so far described only based on metagenome-assembled genomes (MAGs) from environments including freshwater, groundwater and marine seawater [1]. The *Hydra* symbiont is to our knowledge the only cultured representative of this family. For this novel species we propose the name *Endochlamydia hydrae*, and consequently for the CC-III family we propose the name *Endochlamydiaceae* (for detailed descriptions see Text S1).

The genome of *E. hydrae* contains most of the well conserved hallmark genes typically found in the Chlamydiota (Table S5) [1]. It encodes genes and complete pathways for energy parasitism (nucleotide scavenging genes *tlcA-C*) [13, 39, 40], virulence genes like the type III secretion system, many adhesin proteins (n=13) and several serine-threonine kinases (*pknD*, *pknI*, *prkC*, *RIPK4*) (Table S5) [38].

It has full pathways for menaquinone synthesis and shikimate, enabling the synthesis of aromatic amino acids. Genes for the production of secondary metabolites are usually organized in biosynthetic gene clusters (BGCs) [41] and have been found to be widespread across the Chlamydiota [42]. In *E. hydrae* five such BGCs were predicted using antiSMASH [43]. They include a cluster for NRPS-like (Nonribosomal Peptide Synthetase) [44], a cluster for the production of proteusins [45], type III polyketide synthases (T3PKS) [46], and two terpene precursor clusters (Table S7), which all potentially offer defense against other microbes infecting *Hydra* by the production of anti-microbial substances.

E. hydrae is capable of acetogenesis by the oxidative carboxylation of pyruvate to acetate and carbon dioxide, using the pyruvate:ubiquinone oxidoreductase. In addition, we found a complete de-novo pyrimidine synthesis pathway, a feature present only in two other environmental chlamydiae [47, 48] that would enable greater independence from any host [13].

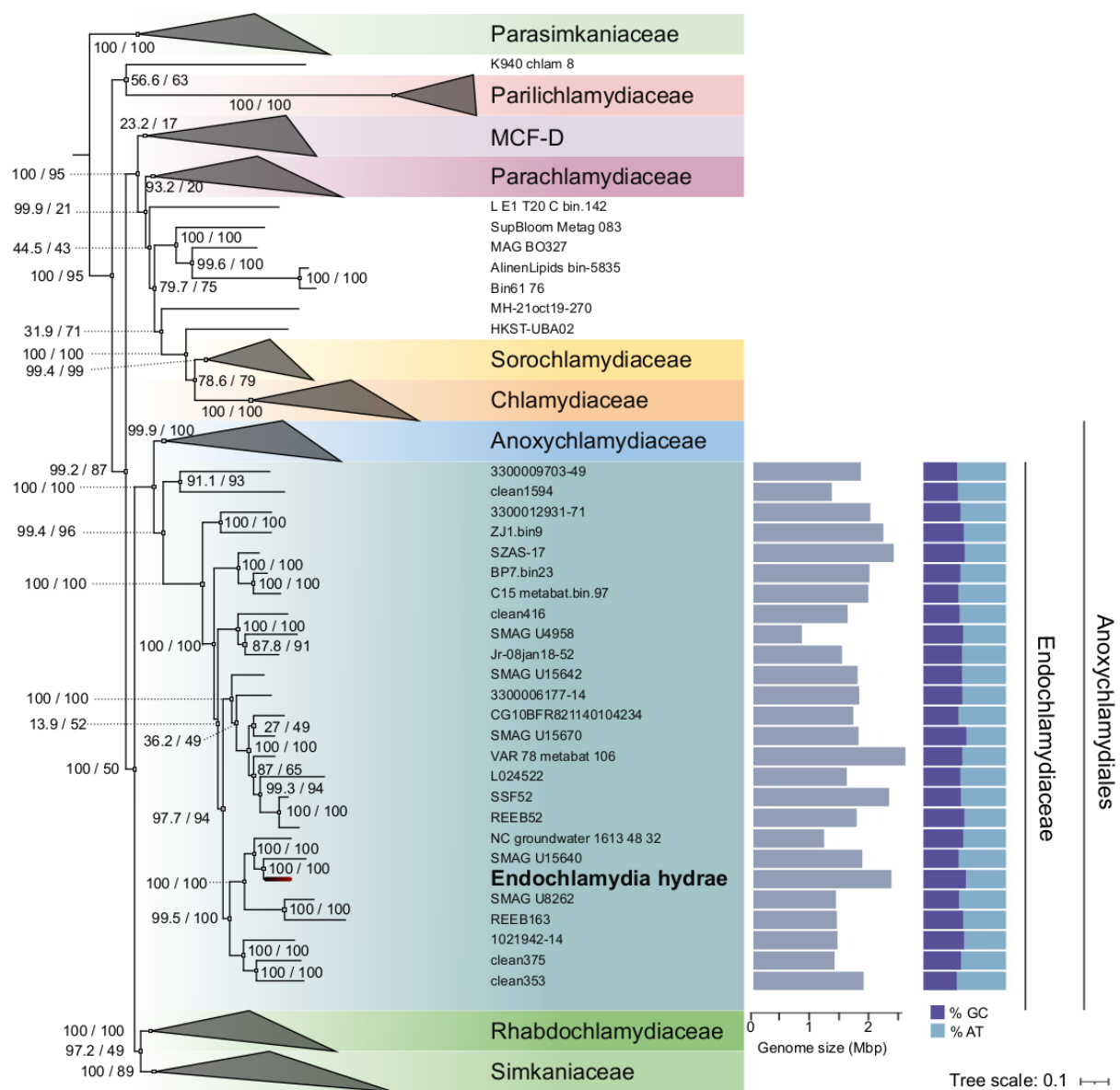


Figure 5. Phylogenetic tree showing the relationship of the *H. oligactis* symbiont *Endochlamydia hydrae* in the phylum Chlamydiota. The symbiont represents the first isolate of the family *Endochlamydiaceae* (formerly Chlamydiae Clade III) within the order *Anoxychlamydiales*, that includes the two families *Endochlamydiaceae* and *Anoxychlamydiaceae*. A maximum likelihood phylogenetic tree based on 43 concatenated conserved marker proteins in 227 chlamydial genomes is shown. Support values based on 1000 ultrafast bootstrap replicates and 1000 replicates of the SH-like approximate likelihood ratio test are indicated on the branches. Scale bar represents 0.1 amino acid substitutions per site. Bars indicate size (left) and percent GC and AT content (right) of *Endochlamydiaceae* genomes.

Genome reduction in *E. hydrae*

The *E. hydrae* genome size of 2.3 Mbp supersedes that of other family members and with 2542 predicted coding sequences, possesses a high coding density of 89.4%. In addition, we identified a large number of pseudogenes (n=464), representing about 18% of the total number of predicted coding sequences (Figure 6A). These genes fall into a wide spectrum of functional categories according to clusters of orthologous gene (COG) classification (Figure 6B, Table S8). Although studies regarding pseudogenes in chlamydiae are still scarce, reported numbers have been low to none [49].

We next focused on transposase genes as potential causative agents for pseudogene formation and genome reduction. Transposases are indicative of transposable elements (TEs), that in the simplest form of insertion sequences (ISs) consist of only a transposase gene flanked by inverted repeats. They can spread through a genome, disrupting and rearranging genomic content [50]. In endosymbionts they have been shown as mediating genome size reduction under relaxed selection, leading to accumulation of mutations and inactivation of genes [51–54]. A comparison of two closely related *Rhabdochlamydia* species in presumed early and late stages of host transition, has found a high number of transposase genes and pseudogenes in the more recently transitioned species [4]. In the genome of *E. hydrae*, we find a high number of IS elements (n=257) similarly spread across the entire genome (Figure 6). They are dominated by three distinct transposase genes belonging to three different IS element families (Table S9). The most abundant transposase genes belong to the IS1 element family, which make up over 60% of all transposases in the genome and were found to be highly conserved (Table S9). This suggests a relatively recent proliferation of IS1 elements in the *E. hydrae* genome, whereas the other, more degraded IS element families could represent remnants of previous IS element radiation events.

Another striking feature of the *E. hydrae* genome is an unusually large number of predicted genomic islands, i.e. genomic regions that deviate in nucleotide composition and are indicative of horizontal gene transfer events (Figure 6). Based on gene content (e.g. many ribosomal proteins, Table S10) they likely do not represent horizontal transmission events but are rather consequences of genome reorganization and homologous recombination events. This scenario is consistent with the observed pattern of cumulative GC skew, which showed a less pronounced trend in *E. hydrae* (Figure 6) than the characteristic asymmetric pattern usually found in circular chromosomes [55].

Taken together, the high proportion of pseudogenes, a high abundance of highly conserved IS elements and irregular GC skew are indicative of evolutionarily recent or still ongoing genomic rearrangements and genome reduction in *E. hydrae* and point to adaptations to *Hydra* as a relatively new host. In addition *E. hydrae* forms a rather short branch in the *Endochlamydiaceae*, with a relative

evolutionary divergence (RED [56]) of 0.022 to its nearest neighbour and 0.045 to the nearest branch outside the genus (Figure 5, Figure S3).

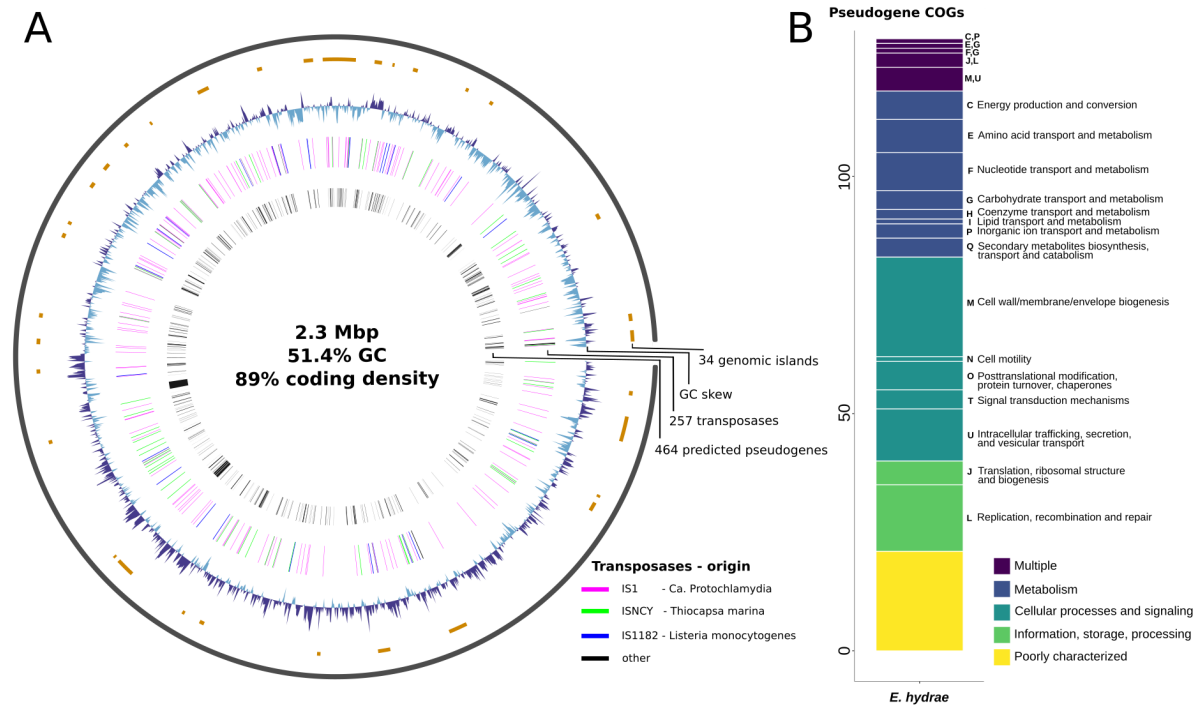


Figure 6. The genome of *E. hydrae* shows signs of many genomic disruptions and rearrangements. (A) Circular plot of the *E. hydrae* genome. The outermost ring shows genomic islands (orange), predicted by IslandPath-DIMOB using islandviewer4. The second ring shows positive (darkblue) and negative (lightblue) GC skew. The third ring represents both putative and functional transposase genes. Transposases are coloured according to sequence similarity to IS families. The innermost ring shows pseudogenes, predicted using pseudofinder. Legend shows transposases listed from highest to lowest abundant sequence together with their closest ISfinder BLAST hit. **(B)** Functional categories of predicted pseudogenes with a COG annotation in *E. hydrae*.

Potential host-symbiont interaction features

Overall, Chlamydiota genomes have a comparatively large core genome, a more limited accessory genome with genes shared by different subgroups of organisms and few unique gene families [42, 38]. We analysed species-specific genes by comparing orthologous groups (OGs) of *E. hydrae* and 225 other chlamydial genomes (Table S5). A total of 254 genes clustered into 19 different OGs were found to be unique to *E. hydrae* (Table S11). 211 of those genes encode transposases, later identified as part of IS elements described above. Other unique genes contain signal peptide and membrane protein domains, none of which could be assigned a specific function (Table S11).

Among the unique genes were also two genes encoding ankyrin repeat domains (ANK). ANK domains are typically found in a class of eukaryotic-like proteins (ELPs) that are widespread in bacteria, mediating protein-protein and protein-nucleic acid interactions [57]. They are regarded to have originated via horizontal gene transfer from eukaryotes to bacteria [58] and are considered to be part of a system ensuring bacterial survival in host cells [59]. Other ELPs encoded in the genome of *E. hydrae* include proteins with tetratricopeptide repeats (TPR, n=12), membrane occupation recognition nexus repeat proteins (MORN, n=4) and WD40 repeat proteins (WDR, n=2). (Table S12).

Taken together, the unique genes and ELPs found in *E. hydrae* could represent small-scale adaptations to the *Hydra* host.

When extending our analysis to the entire *Endochlamydiaceae* family, we were able to identify seven OGs that are unique to the family and are present in at least 15 out of 24 *Endochlamydiaceae* genomes. Two OGs were detected in 23 out of 24 genomes (Table S11). OG0002141 consists of a protein with a signal peptide, the other OG0002142 has no homologs to proteins in the NCBI or Interpro databases. Predictions of the structure using AlphaFold2 [60] did not provide conclusive results about the function of this protein. Similarly only a small number of species or family specific genes have been observed for chlamydiae infecting sponges and corals [5, 42]. Thus, our findings further support the notion that chlamydiae are in general well adapted to infect eukaryotic hosts and only need a limited number of additional adaptations for interacting with non-chordate host organisms.

A model for studying adaptations to host innate immune response

Hydra belongs to one of the basal metazoan lineages and possesses only a basic innate immune system with TLR-like and NLR-like pattern recognition systems that are linked to a basic NF- κ B pathway and the activation of antimicrobial peptide production to control bacteria [19–21]. The symbiosis with *E. hydrae* offers a bridge between chlamydial hosts without an immune system - such as protists - towards chordates with innate and adaptive immune responses. Of the various genes characterized in chordate-infecting chlamydiae that interfere with the host innate immune system only the tail-specific protease (Tsp, CT441) is encoded in *E. hydrae* (HYCHLAM_01890, HYCHLAM_12045, Table S5). Tsp blocks I κ B α degradation and thus the activation of NF- κ B [61, 62] and as a consequence antibacterial response. Tsp containing the PDZ domain responsible for this activity is one of the chlamydial core genes present in all members of the phylum that might be able to interfere with the basic innate immune system of *Hydra*.

Conclusion

In this study we characterized the first cultivated isolate of a member of the family *Endochlamydiaceae* as an endosymbiont in the endoderm of the freshwater polyp *H. oligactis*. An in-depth characterization of the *E. hydrae* genome revealed that it encodes all the hallmark genes characteristic for the chlamydial intracellular lifestyle and showed only a small set of unique genes, some of which potentially play a role in host interaction and may represent specific adaptations to *Hydra* as a host. Similar to *E. hydrae*, other chlamydial families prevalent in marine cnidarians, were also described as having little genomic specialization, suggesting a lack of host specificity among cnidarian-associated chlamydiae [5]. We have instead found evidence for ongoing genome reduction and interpreted this to be the result of a more recent host switch and ongoing host adaptations.

Hydra represents yet another early eukaryotic lineage serving as natural host for chlamydiae, which further underscores the versatility and success of this bacterial phylum to infect an immensely diverse range of eukaryotes. The availability of *Hydra* to culture a representative of a distinct and phylogenetically diverse chlamydial family offers the potential to study the evolution of host interaction and adaptation, as well as adaptations to the host immune response. In the future this might contribute to a better understanding of the evolution of important human and animal pathogens in the phylum.

Materials and Methods

Maintenance of *Hydra* populations in the lab

Hydra species and strains were derived from a culture collected in April 2021 and provided by Frédéric Thomas [22]. These include *Hydra oligactis* strains SpB (St. Petersburg), X11/14, C2/7, Montaud and *Hydra vulgaris* [22]. The animals were maintained at 18°C in 200 ml glass beakers, containing sterilised Volvic® water, with a photoperiod of 12 hours. They were fed 2-3 times per week with *Artemia salina* nauplii. Nauplii were obtained according to the methodology described in [63], using 400 ml of seawater (Classic Sea Salt, Tropic Marin, at 32-33 ppm). Each feeding of *Hydra* cultures was followed by a water change 4-8 hours post-feeding.

DNA isolation, PCR and symbiont quantification

To check for chlamydial presence, 68 polyps were tested from *Hydra oligactis* strains SpB (St. Petersburg), X11/14, C2/7, Montaud and *Hydra vulgaris*. For DNA extraction from individuals or culture supernatant the DNeasy Blood and Tissue Kit for DNA Isolation (Qiagen) or the InnuPREP DNA mini kit (IST Innuprep) were used according to the manufacturer protocols (Table S1). For DNA isolation from culture supernatant 30 ml of supernatant was collected from seven *Hydra oligactis* Montaud cultures (Table S2).

The presence of chlamydiae in individual animals was first determined with PCR using the chlamydiae-specific primer pair SigF2 (=Chl40F) (5' CRG CGT GGA TGA GGC AT 3'; [64]) and Chl523R (5' CCY YMC GTA TTA CCG CAG CT 3') targeting the 16S rRNA gene in a T100 Thermo Cycler (BioRad) under the following cycling conditions: Initial denaturation at 95°C for 3 min, 35 cycles with 95°C for 30 sec, 64.5°C for 30 sec, 72°C for 1 min, and final elongation at 72°C for 5 min, using the DreamTaq Green

PCR Master Mix (Thermo Scientific). 25 individuals were sequenced with Sanger sequencing at Microsynth AG (Austria). To quantify chlamydiae digital PCR (dPCR) was performed using the QIAcuity EvaGreen PCR kit (Qiagen) and the chlamydiae-specific primer pair described above on a QIAcuity One digital PCR device (Qiagen) as recommended by the manufacturer. The cycling conditions were 95°C for 2 min; 40 cycles of 95°C for 30 sec, 64.5°C for 45 sec, 72°C for 1 min; 40°C for 5 min with imaging at an exposure time of 350 ms and a gain of 3. Direct quantification data were acquired with the QIAcuity Software Suite 2.5.0.1 (Qiagen). Chlamydial 16S rRNA copy numbers per hydra or ml supernatant, respectively, were calculated based on these data and divided by two to obtain genome copy numbers. Cell numbers above 100 chlamydiae per *Hydra* were considered to be positive.

Histological sample processing

Four individuals of symbiotic *H. oligactis* Montaud polyps were incubated in a 3% (w/v) formaldehyde solution in Volvic® water for four hours at 4°C. The liquid was carefully removed and animals were washed 3x with PBS (40g*l⁻¹ NaCl (Roth), 9g*l⁻¹ Na₂HPO₄*2H₂O (ROTH), 1.2g*l⁻¹ KH₂PO₄ (Sigma), 1g KCl (Millipore)). Fixed animals were stored in PBS for no longer than a day at 4°C. Animals were embedded in paraffin and cut into 4 µm thin sections at the Vienna Biocenter Core Facility Histology. Dewaxing of the sections on the slide was performed directly before processing the slides for fluorescence *in situ* hybridization (described below). Slides were incubated three times for 10 minutes in Roti® Histol (Carl Roth GmbH), followed by two times incubation for 10 minutes in LiChrosolv® ethanol (Merck, KGaA) and then three times for 5 minutes in PBS.

Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization (FISH) was performed on previously fixed and embedded tissue sections. The fixed sections were covered with hybridization buffer (900 mM NaCl, 20 mM Tris-HCl, 0.01% SDS, 25% formamide) which was premixed with 4 different probes (each at 5 pM) in a 1:10 (probe to buffer) ratio and hybridized at 46°C for 2 hours. Probes used in the hybridization buffer mix included Chls-523 (5'-Cy3 CTTCCGTATTACCGCAGC -3') [65], Chls-282 (5'-Cy3 CTCAATCCGCCTAGACGT -3') [66], EUB338 I - DOPE (5'-6-FAM GCTGCCTCCCGTAGGAGT 6-FAM-3'), EUB338 II - DOPE (5'- 6-FAM GCAGCCACCCGTAGGTGT 6-FAM-3'), EUB338 III - DOPE (5'-6-FAM GCTGCCACCCGTAGGTGT 6-FAM-3') [67] and the NonEUB probe (5'-Cy3 ACTCCTACGGGAGGCAGC -3') [68]. Slides were washed for 15 minutes in washing buffer (20 mM Tris-HCl, 0.25 mM EDTA and 0.149 M NaCl) at 48°C and briefly washed in ice-cold deionized water. Slides were then stained with 4',6-diamidino-2-phenylindole (DAPI) for 7 minutes and washed in 99% ethanol for 1 minute. Imaging was done on a TCS SP8 X confocal laser scanning microscope (Leica Microsystems) equipped with a 93x glycerol objective and the Thunder Imager Live Cell and 3D Imager (Leica Microsystems) with a 63x glycerol-immersion objective and a 100x oil objective. Images were analyzed using the Leica application suite X software v3.7.6 (Leica Microsystems).

Transmission electron microscopy

Two individuals of symbiotic *Hydra oligactis* Montaud polyps, were maintained without food for two days. Then individuals were transferred into high-pressure freezing type A carriers (3 mm diameter, 200 µm depth) and covered with 10% bovine serum albumin and type B carriers (Leica Microsystems). Before sample application the carriers were coated with 1-hexadecene. After closing, the samples were frozen at approximately 2000 bar, using an HPM100 high-pressure freezer (Leica

Microsystems). Frozen samples were freeze substituted in an automated freeze substitution system AFS2 (Leica Microsystems). For this, carriers containing high-pressure frozen samples were placed in -90°C 1% OsO₄ in acetone in 2 ml cryotubes. Freeze substitution was performed under agitation following this temperature program: 2 h from -105 to -95°C, 40 h at -95°C, 2 h from -95°C to -90°C, 5 h at -90°C, 2 h from -90°C to -60°C, 2 h at -60°C, 4 h from -60°C to 20°C. Then samples were washed with acetone and infiltrated with epoxy resin Epon (Agar100 Scientific Ltd.) at room temperature. Polymerization of the resin was conducted at 63°C for 72 hours. Ultrathin sections of 70 nm thickness were prepared using a diamond knife (Diatome) with a Leica ultramicrotome EM UC7 (Leica Microsystems) and mounted on 200 mesh copper grids. Contrasting was done using 4% Neodymium(III) acetate (Thermo Fisher Scientific) hydrate for 50 minutes and 3% lead citrate for 8 minutes. The stained ultrathin sections were examined with a Libra 120 transmission electron microscope (Zeiss) with a Bottom mount camera Sharp:eye TRS (2 × 2 k).

Isolation and cultivation in insect cells

Insect cells (*Spodoptera frugiperda* Sf9 cell line) were cultured in Grace's insect medium (Thermo Fischer Scientific) supplemented with 10% fetal bovine serum (Thermo Fischer Scientific) at 28°C. Chlamydial EBs were harvested by transferring >200 *Hydra* polyps into a 1 ml Wheaton® dounce tissue homogenizer and douncing 40 times with the loose pestle and 40 times with the tight pestle. Sufficient lysis of the cells was ensured using epifluorescence microscopy on DAPI stained lysates, using a Zeiss Axio imager A1 microscope. Bacterial cells were then filtered using a 1.2 µm filter. EB density was assessed with the QUANTOM Tx™ Microbial Cell Counter (Logos biosystems). Insect cells were seeded in a 6-well plate (Thermo Fischer) at a density of 1 × 10⁵ cells per ml and infected with EBs at a multiplicity of infection (MOI) of 100 or higher. To prevent contamination 10 µl/ml of 100x antibiotic, antimycotic solution (Sigma-Aldrich) were added. To enhance infection probability the plate was centrifuged at 500 × g for 15 minutes and incubated at 28°C. Insect cells were grown until confluence and transferred into 75 cm² cell culture flasks (Nunc™ EasYFlask™). Infection success was monitored monthly with PCR of 1.2 µm filtered culture supernatant, using the chlamydiae-specific primer pair mentioned above and subsequent Sanger sequencing (Microsynth AG). After five months of co-cultivation, FISH was performed as described above.

16S rRNA gene-based diversity and environmental distribution of *E. hydrae*

The IMNGS database, which is a collection of pre-clustered NCBI SRA sequencing data [34], was queried on November 25, 2024 for 16S rRNA genes with at least 95% identity to the reference 16S rRNA gene sequence of *E. hydrae*. The categories provided by the SRA were manually curated so

that sequences were assigned to one of the following categories: freshwater, freshwater sediment, groundwater, marine, marine sediment, soil and wastewater (Table S3).

Sampling, sequencing and analysis of wild *Hydra*-associated chlamydiae

Three distinct *Hydra oligactis* populations were sampled in Hungary over a period of two years (between June 2021 and June 2023). Each population was sampled once every season. At each sampling three hydra polyps and one water sample were collected from three distinct locations along shorelines and brought to the laboratory on the day of collection. Polyps were washed with sterile-filtered water to remove debris, and water was sterile-filtered to obtain DNA samples from bacterioplankton (no water samples were available for the first sampling event in June 2021). DNA extraction was performed with a phenol-chloroform extraction method as described in [69] and extracted DNA was sent to Novogene (Cambridge) for amplicon sequencing of the V4 region. *uparse* v7.0.1001 was used to cluster OTUs based on the SILVA database with a 97% similarity threshold. Detailed methods for sampling and sequencing can be found in Tökölyi *et al.* (2025), in revision.

OTUs belonging to the phylum Chlamydia were extracted using the R package phyloseq [70]. Relative abundances between chlamydiae in each sample were calculated and visualized in a heatmap using R version 4.4.2 and the package pheatmap. Chlamydial sequences were further processed by calculating 16S rRNA gene phylogeny with other members of the Chlamydia (see below).

16S rRNA gene phylogeny of lab and wild derived strains

A dataset of 481 nearly full length (>1200 nt) 16S rRNA gene sequences was generated. This contained chlamydial sequences derived from a dataset by (Schulz *et al.*, 2017, n=55), genomes available on NCBI on April 11th 2024 (n=42), as well as 245 chlamydial and 139 outgroup sequences belonging to the Planctomycetota, Omnitrophota, Verrucomicrobiota, and LD1-PA32 phyla [72]. Full length sequences not yet aligned to the SILVA alignment, were aligned with MAFFT v7.490 (--add --keeplength) [73]. The alignment was trimmed with trimAl v1.5.0 (--gappyout) [74]. A reference tree containing the full length 16S rRNA gene sequences was generated with IQ-TREE v2.3.5 using the SYM+R10 model with 1000 ultrafast bootstraps and the SH-like approximate likelihood ratio test [75]. Short 16S rRNA gene sequences from the environmental screening were added to this alignment with MAFFT v7.490 (--addfragments --keeplength) [73] and subsequently added to the full length reference tree with EPA-ng v0.3.8 (-m GTR+G) [76] and visualized in iTOL v7.0 [77].

Sequencing and assembly of the genome

200 or 115 individuals of the symbiotic *Hydra oligactis* strain Montaud were maintained without food for two days for high or low molecular weight DNA isolation, respectively. Then symbiont cells were harvested as described above. The remaining cell lysate was filtered consecutively through 5 µm and 1.2 µm filters (Sartorius). The filtrate was analysed for sufficient removal of host cells, using epifluorescence microscopy (as described above). Bacteria were harvested by centrifugation at 15,500 x g for 15 minutes at 4°C. The remaining cell pellet was resuspended in PBS and immediately used for extraction of genomic DNA.

Extraction of high molecular weight DNA was carried out using the Wizard® HMW Extraction kit (Promega) according to the instructor's manual, with few deviations. In brief, all centrifugation steps were performed at 15,500 x g, drying of the DNA pellet was done at 60°C for 5 minutes and the subsequent rehydration of the DNA was carried out overnight at room temperature in 25 µl of rehydration solution. Library preparation was performed at the Vienna Bio Center Core Facilities using the Multiplex Ligation Sequencing Kit and the Native Barcoding Kit (Oxford Nanopore Technologies). Long-read sequencing was performed on an ONT Flongle system. Extraction of low molecular weight DNA was done using the DNeasy Blood & Tissue Kit (Qiagen). Libraries for short read sequencing were prepared at the Joint Microbiome Facility (University of Vienna and Medical University of Vienna) using the NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina (New England Biolabs), pooled equimolarly, and sequenced on an Illumina MiSeq instrument (2x 300bp, 600 cycles). Short reads were trimmed using BBDuk (part of BBMap v39.01 [78]) to a minimum of 50 base pairs in length to avoid adapter contamination and low quality sequences. Long reads were trimmed using Porechop 0.2.4 [79]. In total we obtained 1156262 short and 112117 long reads of which roughly 43% and 11% respectively were assembled into a circular genome using Unicycler v0.5.0. [80]. The result was a single chlamydial contig and the origin of replication was determined using Ori-finder 2022 [81].

Phylogenetic analysis

Average nucleotide identity (ANI) was calculated using FastANI v1.34 [82] against a set of 227 chlamydial genomes (Table S6), resulting in no hits against *E. hydrae*. Then a concatenated alignment (Data S2) of 43 CheckM marker proteins (Data S3) from 227 chlamydial genomes and 89 Planctomycetota-Verrucomicrobiota outgroup representatives was generated using CheckM v1.2.3 [83]. This alignment was then used to infer a maximum likelihood phylogenetic tree using IQ-TREE v2.2.6. [75] with the model LG+C20+R4 as selected by Modelfinder plus [84]. The final tree was calculated using 1000 ultrafast bootstraps repeats [85] and 1000 replicates of the SH-like approximate likelihood ratio test [86], it was rooted using the outgroup and visualized with iTOL v.7.0 [77].

Annotation and comparative genomics

Gene calling and annotation were performed using bakta v1.9.3 [87] and eggNOG-mapper v2.1.11 [88]. We analysed metabolic pathways, transporters and secretion systems using KEGG [89], in combination with InterProScan 5 [90]. The presence of specific genes of interest (anaerobic pathway genes, virulence genes, T3SS) were additionally investigated using BLAST and known sequences from the complete, high-quality genomes of either *Simkania negevensis* [91] or *Chlamydia trachomatis* [92]. Secondary metabolites were predicted using antiSMASH v8.0.4 [43].

A comprehensive dataset of all encoded proteins in 225 closed chlamydial genomes and high-quality MAGs (Table S6) were clustered into orthologous groups (OGs) using OrthoFinder v2.5.5 [93] under default parameters. Selected OGs that did not show any sequence similarity to known proteins in public databases were used for structure prediction using AlphaFold v.2.3.2 [60] and compared with structures in the RCSB protein data bank (PDB) [94].

To predict genomic islands, we used islandviewer4 [95]. GC skew was calculated using a custom R script (window size = 1000). Transposases were identified in two parts, by extracting all sequences annotated as transposases in the bakta annotation file (Table S5) together with sequences from two Orthofinder OGs (OG0001333, OG0000802) identified as insertion sequences. To identify larger insertion sequences around the transposon sequences, they were aligned using Aliview [96], grouped based on similarity and further aligned with the *E. hydrae* genome using Unipro UGENE [97]. To identify genes under degradation in the genome of *E. hydrae* we used pseudofinder (v1.0) [98]. This was done by comparing the *E. hydrae* genome against a database of 227 chlamydial genomes using a threshold of 0.3 to distinguish between pseudogenes (>0.3) and genes under purifying selection (≤ 0.3). GC skew, genomic islands, IS sequences and pseudogenes were visualised using the R package circlize [99].

Data availability

The sequencing data for the field collected *Hydra*-associated chlamydiae is available under PRJNA1119407 and the assembled genome sequence and sequencing data of *E. hydrae* are available under PRJEB86254.

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Supplementary Information

Supplementary Figures

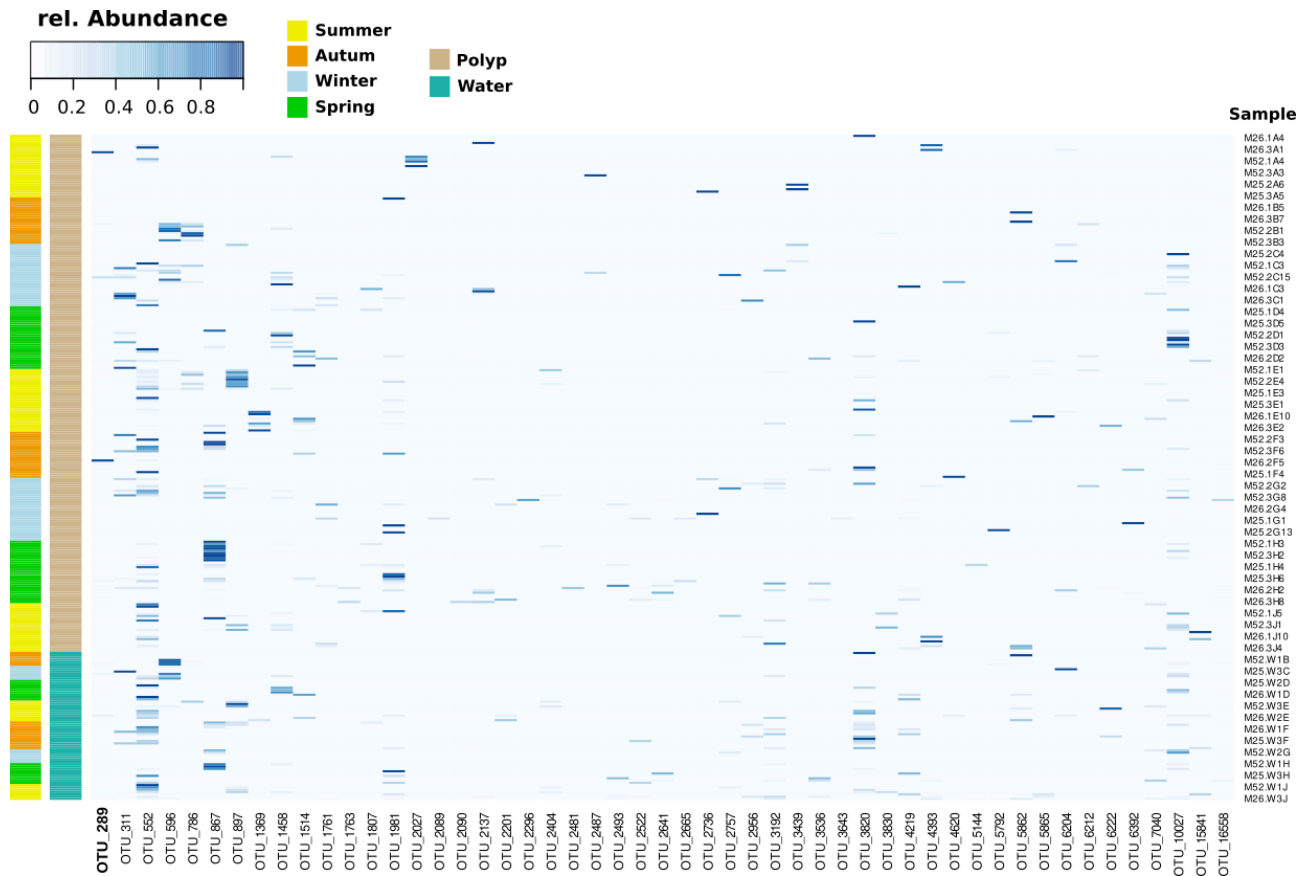


Figure S1. Relative abundance within chlamydiae in wild-derived *Hydra* polyps and water samples. Heatmap (using hierarchical clustering based on Euclidean distances) shows the abundance of each OTU relative to all chlamydial sequences, detected by sequencing of the V4 region of the 16S rRNA gene. In total 64 water and 287 polyp samples were collected from 3 distinct populations in Hungary from 2021 to 2023 in all four seasons. OTU 289 (bold) has >99% sequence identity to the *E. hydrae* 16S rRNA gene.

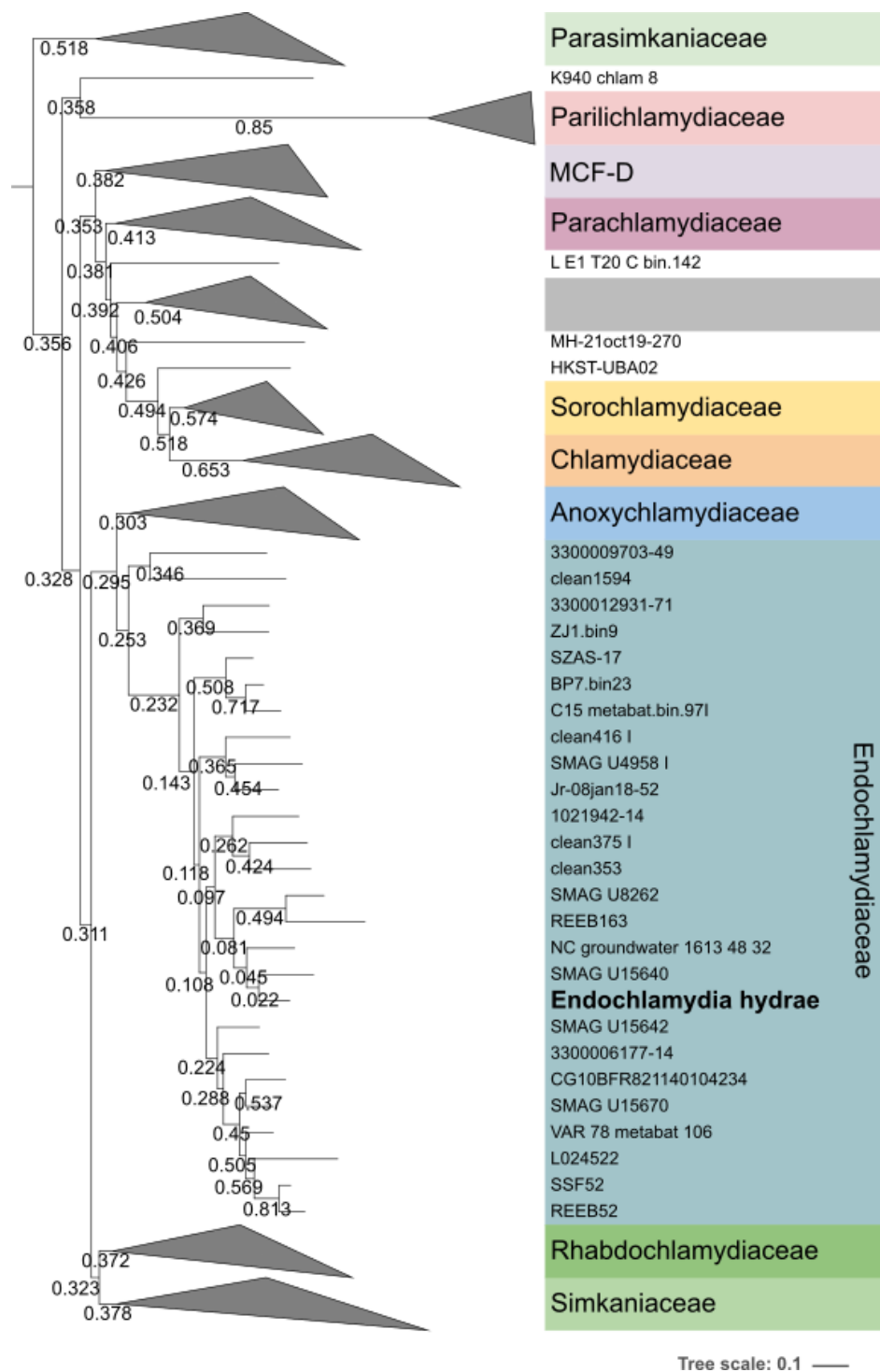


Figure S3. Phylogenetic tree showing the relative evolutionary divergences (REDs) of each tree node. Maximum likelihood phylogenetic tree based on 43 conserved marker proteins in 227 chlamydial genomes is shown. Support values based on 1000 ultrafast bootstrap replicates and 1000 replicates of the SH-like approximate likelihood ratio test are indicated on the branches. RED values are shown below each node. Scale bar represents 1 amino acid substitutions per site.

Text S1

Description of *Endochlamydia hydrae*, gen. nov., sp. nov.

Based on the complete genome sequence (PRJEB86254) and the intracellular location within *Hydra oligactis* endodermal epithelial cells detected with fluorescence microscopy and transmission electron microscopy, we refer to the chlamydial endosymbiont described in this study as ‘Endochlamydia hydrae’ [‘Endo-’ is a scientific Latin prefix derived from ancient greek ἐνδο- (endo-) meaning ‘within’; Chlamydia, taxonomic name of a bacterial genus; hydrae, referring to the host organism of this particular isolate]. ‘Endochlamydia hydrae’ is a strictly intracellular symbiont within inclusions inside the endodermal epithelial cells of the freshwater predator *Hydra oligactis*. It was discovered in *H. oligactis* polyps isolated from a freshwater pond (Montaud, France). The symbionts are non-motile and are not cultivable outside eukaryotic host cells but have been found successfully replicating in *Spodoptera frugiperda* (Sf9) insect cell lines. The morphology of ‘Endochlamydia hydrae’ resembles that of most chlamydiae, appearing in two distinct morphological forms the replicating reticulate body, which are round and about 500-800 nm in diameter and the infectious non-replicating elementary body, which are elongated and about 300-500 nm in length. Based on phylogenetic analysis of marker genes and the 16S rDNA gene sequence, ‘Endochlamydia hydrae’ forms a genus with the two previously described MAGs ‘NC_groundwater_1613_48_32’ and ‘SMAG_U15640’ within the Chlamydiae Clade III family, order Anoxychlamydiales, phylum Chlamydia. The currently closest cultured representative is *Simkania negevensis* Z (48% amino acid identity and 87.91% 16S rRNA gene identity).

Supplementary Tables

Table S1. Presence of chlamydiae in hydra individuals determined by PCR and digital PCR.

Nr	ID	Lab-origin	Hydra-strain	Individuals	DNA isolation	DNA total volume	PCR	Sanger sequencing	dPCR chlamydiae/hydra
1	C2_7-SA 10_1 NT 9.16	Hungary	C2_7	1	Qiagen Blood & Tissue kit	30 µl	pos	yes	1090098200
2	C2_7-SA 12_5 NT 9.16	Hungary	C2_7	1	Qiagen Blood & Tissue kit	30 µl	neg		nd
3	C2_7-SA 5_1 NT 9.20	Hungary	C2_7	1	Qiagen Blood & Tissue kit	30 µl	pos	yes	419955850
4	C2_7-SA 5_2 NT 9.16	Hungary	C2_7	1	Qiagen Blood & Tissue kit	30 µl	neg		22
5	C2_7-SA 7_6 NT 9.20	Hungary	C2_7	1	Qiagen Blood & Tissue kit	30 µl	pos	yes	8500715
6	C2_7-SA-3_5 NT 9.23	Hungary	C2_7	1	Qiagen Blood & Tissue kit	30 µl	pos	yes	1106405
7	C2_7-SA 3_6 T 8.26	Hungary	C2_7	1	Qiagen Blood & Tissue kit	30 µl	pos	yes	124041825
8	C2_7-SA 8_3 T 9.13	Hungary	C2_7	1	Qiagen Blood & Tissue kit	30 µl	neg		nd
9	C2_7-STR 2_2 T 8.26	Hungary	C2_7	1	Qiagen Blood & Tissue kit	30 µl	pos	yes	3077993700
10	C2_7-STR 3_6 T 8.26	Hungary	C2_7	1	Qiagen Blood & Tissue kit	30 µl	pos		56445
11	C2_7-STR 6_1 T 8.26	Hungary	C2_7	1	Qiagen Blood & Tissue kit	30 µl	pos		1401243350
12	C2_7-STR 9_3 T 8.24	Hungary	C2_7	1	Qiagen Blood & Tissue kit	30 µl	pos		1663144800
13	X 11_14 OSA 19_1 NT 4.22	Hungary	X11_14	1	Qiagen Blood & Tissue kit	30 µl	pos	yes	1625189300
14	X 11_14 OSA 21_4 NT 4.30	Hungary	X11_14	1	Qiagen Blood & Tissue kit	30 µl	pos	yes	1308880
15	X 11_14 OSA 4_3 NT 4.29	Hungary	X11_14	1	Qiagen Blood & Tissue kit	30 µl	pos		1560120350
16	X 11_14 OSA 5_2 NT 4.27	Hungary	X11_14	1	Qiagen Blood & Tissue kit	30 µl	neg		44
17	X 11_14 SA 1_3 NT 8.30	Hungary	X11_14	1	Qiagen Blood & Tissue kit	30 µl	pos		1063938850
18	X 11_14 SA 1_6 NT 9.6	Hungary	X11_14	1	Qiagen Blood & Tissue kit	30 µl	pos		818059550
19	X 11_14 OSA 10_5 T 4.23	Hungary	X11_14	1	Qiagen Blood & Tissue kit	30 µl	pos	yes	10489060
20	X 11_14 OSA 14_5 T 4.27	Hungary	X11_14	1	Qiagen Blood & Tissue kit	30 µl	pos		621788950
21	X 11_14 OSA 19_5 T 4.22	Hungary	X11_14	1	Qiagen Blood & Tissue kit	30 µl	pos		1244
22	X 11_14 SA 10_5 T 8.30	Hungary	X11_14	1	Qiagen Blood & Tissue kit	30 µl	pos	yes	2193413
23	X 11_14 SA 11_4 T 9.16	Hungary	X11_14	1	Qiagen Blood & Tissue kit	30 µl	pos		1003730000
24	X 11_14 SA 11_6 T 9.17	Hungary	X11_14	1	Qiagen Blood & Tissue kit	30 µl	pos		307543450
25	Mont saine 27	France	Montaud	1	Qiagen Blood & Tissue kit	30 µl	neg		0
26	Mont saine 28	France	Montaud	1	Qiagen Blood & Tissue kit	30 µl	neg		0
27	Mont saine 29	France	Montaud	1	Qiagen Blood & Tissue kit	30 µl	pos		221325
28	Mont saine 30	France	Montaud	1	Qiagen Blood & Tissue kit	30 µl	pos		1255105

Nr	ID	Lab-origin	Hydra-strain	Individuals	DNA isolation	DNA total volume	PCR	Sanger sequencing	dPCR chlamydiae/hydra
29	Mont saine 31	France	Montaud	1	Qiagen Blood & Tissue kit	30 µl	pos	yes	292325
30	Mont saine 32	France	Montaud	1	Qiagen Blood & Tissue kit	30 µl	pos	yes	291625
31	Mont T3	France	Montaud	1	Qiagen Blood & Tissue kit	30 µl	neg		9937
32	Mont T	France	Montaud	1	Qiagen Blood & Tissue kit	30 µl	pos	yes	100905
33	Mont T	France	Montaud	1	Qiagen Blood & Tissue kit	30 µl	pos	yes	126402
34	Mont T	France	Montaud	1	Qiagen Blood & Tissue kit	30 µl	pos		684470
35	Mont T2	France	Montaud	1	Qiagen Blood & Tissue kit	30 µl	pos		2847350
36	Mont T7	France	Montaud	1	Qiagen Blood & Tissue kit	30 µl	pos	yes	1112710
37	ADN OliC	France	Spb	1	Qiagen Blood & Tissue kit	30 µl	neg		0
38	ADN Oli ctrl 1	France	Spb	1	Qiagen Blood & Tissue kit	30 µl	neg		0
39	ADN ctrl 1.1	France	Spb	1	Qiagen Blood & Tissue kit	30 µl	neg		0
40	ADN ctrl ?	France	Spb	1	Qiagen Blood & Tissue kit	30 µl	neg		0
41	Oli ctrl 68	France	Spb	1	Qiagen Blood & Tissue kit	30 µl	neg		0
42	Oli ctrl 67	France	Spb	1	Qiagen Blood & Tissue kit	30 µl	pos		86945
43	Oli T14	France	Spb	1	Qiagen Blood & Tissue kit	30 µl	neg		0
44	Oli T14	France	Spb	1	Qiagen Blood & Tissue kit	30 µl	neg		0
45	Oli T2	France	Spb	1	Qiagen Blood & Tissue kit	30 µl	neg		67
46	Oli tum 73	France	Spb	1	Qiagen Blood & Tissue kit	30 µl	pos	yes	2060220
47	Oli tum 74	France	Spb	1	Qiagen Blood & Tissue kit	30 µl	neg		2045
48	Olit tum 75	France	Spb	1	Qiagen Blood & Tissue kit	30 µl	pos	yes	106304000
49	Viln 1	France	Vilneuvette	1	Qiagen Blood & Tissue kit	30 µl	neg		nd
50	Viln 2	France	Vilneuvette	1	Qiagen Blood & Tissue kit	30 µl	neg		nd
51	Viln 3	France	Vilneuvette	1	Qiagen Blood & Tissue kit	30 µl	neg		nd
52	Viln 4	France	Vilneuvette	1	Qiagen Blood & Tissue kit	30 µl	neg		0
53	Viln 5	France	Vilneuvette	1	Qiagen Blood & Tissue kit	30 µl	pos		766
54	Vul	France	Vulgaris	1	Qiagen Blood & Tissue kit	30 µl	neg		0
101	7.C27T	Hungary	C2_7	1	InnuPREP DNA mini kit	40 µl	pos		3008
102	SpBTK	France	SpB	1	InnuPREP DNA mini kit	40 µl	neg		91
103	SpBT	France	SpB	1	InnuPREP DNA mini kit	40 µl	pos		10483293
104	SpBNT	France	SpB	1	InnuPREP DNA mini kit	40 µl	pos	yes	171533
106	MTD1	France	Montaud	1	InnuPREP DNA mini kit	40 µl	pos	yes	733763533
107	MTD2	France	Montaud	1	InnuPREP DNA mini kit	40 µl	pos	yes	225192200

Nr	ID	Lab-origin	Hydra-strain	Individuals	DNA isolation	DNA total volume	PCR	Sanger sequencing	dPCR chlamydiae/hydra
108	MTD3	France	Montaud	1	InnuPREP DNA mini kit	40 µl	pos		277982733
109	MTT	France	Montaud	1	InnuPREP DNA mini kit	40 µl	pos		412487400
110	MTNT	France	Montaud	1	InnuPREP DNA mini kit	40 µl	pos	yes	1499346133
111	VilTR1	France	Vilneuvette	1	InnuPREP DNA mini kit	40 µl	pos	yes	359018267
112	VilTR2	France	Vilneuvette	1	InnuPREP DNA mini kit	40 µl	pos	yes	197970067
113	VilTR3	France	Vilneuvette	1	InnuPREP DNA mini kit	40 µl	pos		85513
114	VilTRT	France	Vilneuvette	1	InnuPREP DNA mini kit	40 µl	pos	yes	1608876333
115	VilTRNT	France	Vilneuvette	1	InnuPREP DNA mini kit	40 µl	pos	yes	4398182533

Table S2. Presence of chlamydiae in the supernatant of Hydra cultures determined with digital PCR.

Nr	ID	Lab_origin	Hydra_strain	Volume	DNA isolation	DNA total volume	dPCR chlamydiae/ml supernatant
G1	Hvul	France	Hydra vulgaris	30 ml	innuPREP DNA/RNA minikit	50 µl	1
G2	MonCtrl	France	Montaud	30 ml	innuPREP DNA/RNA minikit	50 µl	771261
G3	WildMTHydra	France	Montaud	30 ml	innuPREP DNA/RNA minikit	50 µl	4949
G4	Hor_drousm.Hydra	France	Montaud	30 ml	innuPREP DNA/RNA minikit	50 µl	1752231
G5	Hor_drousm.Hydra.tum	France	Montaud	30 ml	innuPREP DNA/RNA minikit	50 µl	187225
G6	Mon.tum	France	Montaud	30 ml	innuPREP DNA/RNA minikit	50 µl	11853
G7	Mon.tum_plates	France	Montaud	30 ml	innuPREP DNA/RNA minikit	50 µl	6940
G8	specialMon.tum	France	Montaud	30 ml	innuPREP DNA/RNA minikit	50 µl	22303

Table S7. Biosynthetic gene clusters in E. hydrae predicted with antiSMASH

Region	LocusTag	Product	NT	AA	Function
Terpene precursor	HYCHLAM_01370	Geranylgeranyl pyrophosphate synthase, chloroplastic/chromoplastic	861	286	biosynthetic
NRPS-like	HYCHLAM_05540	Beta-lactamase	1869	622	biosynthetic-additional
NRPS-like	HYCHLAM_05565	Pseudouridine synthase	840	279	biosynthetic-additional
NRPS-like	HYCHLAM_05590	Phospholipid/glycerol acyltransferase domain-containing protein	2712	903	biosynthetic
NRPS-like	HYCHLAM_05705	Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha	1002	333	biosynthetic-additional

Region	LocusTag	Product	NT	AA	Function
NRPS-like	HYCHLAM_05710	Putative Lipid A export ATP-binding/permease protein	1914	637	transport
Terpene precursor	HYCHLAM_06090	HAD family phosphatase	606	201	biosynthetic-additional
Terpene precursor	HYCHLAM_06100	Inositol-1-monophosphatase	804	267	biosynthetic-additional
Terpene precursor	HYCHLAM_06110	UDP-glucose 4-epimerase	1014	337	biosynthetic-additional
Terpene precursor	HYCHLAM_06120	Geranyl transferase	879	292	biosynthetic
Terpene precursor	HYCHLAM_06125	UDP-glucose 6-dehydrogenase	1335	444	biosynthetic-additional
Terpene precursor	HYCHLAM_06130	Sugar transferases involved in lipopolysaccharide synthesis	606	201	biosynthetic-additional
Terpene precursor	HYCHLAM_06140	Lipoprotein-releasing system ATP-binding protein LolD	648	215	transport
Proteusin	HYCHLAM_07150	hypothetical protein	417	138	biosynthetic
T3PKS	HYCHLAM_07960	ABC transporter domain-containing protein	678	225	transport
T3PKS	HYCHLAM_07970	hypothetical protein	2148	715	transport
T3PKS	HYCHLAM_07985	Na(+)/H(+)-K(+) antiporter GerN	1185	394	transport
T3PKS	HYCHLAM_08000	3,5-dihydroxyphenylacetyl-CoA synthase	1128	375	biosynthetic
T3PKS	HYCHLAM_08010	Bifunctional 2-polyprenyl-6-hydroxyphenol methylase/3-demethylubiquinol 3-O-methyltransferase UbiG	750	249	biosynthetic-additional
T3PKS	HYCHLAM_08055	Major facilitator superfamily (MFS) profile domain-containing protein	1263	420	transport
T3PKS	HYCHLAM_08060	EamA domain-containing protein	963	320	transport
T3PKS	HYCHLAM_08075	CPBP family intramembrane metalloprotease	864	287	biosynthetic-additional
T3PKS	HYCHLAM_08085	Aminotransferase class V domain-containing protein	1119	372	biosynthetic-additional

Table S9. Transposases encoded in the *E. hydrae* genome

Group	occurrences	basepairs	Locus_Tag	Phylogeny	Name	Sequence
1	156	216	HYCHLAM_00175	Chlamydiales bacterium, Waddlia chondrophila WSU 86-1044, Waddlia chondrophila WSU 86-1044	IS1 transposase	FNFLVVLNMCQCKKFLRPPYLASFIMESF MECKCPSCASLLVKKNGHIHNGKQNHRC LACQRQFVVEPDQKIIDDHTKVLVRRTLLE RVSLEGVCRIENVSMWPWLLGFIDEVATLP EDLNAQVVKENDDIEVLLADEQWSYVG SKDNPQWLWLVMSKTRQIVAMQVGAR NKQTAERLFAKLPEPLKKKPATIVTTSMTCTKRSPLSNTDQSGKSLERHLTLRDLTALG SVVADWVGKHSRSPKNWQTTLESNTSYAT IISAGEHYMFRTTN

Group	occurrences	basepairs	Locus_Tag	Phylogeny	Name	Sequence
2	35	957	HYCHLA_M_02270	Bdellovibrio sp._CG11_big_fil_rev, Chlamydia bacterium, Nitrosopumilus sp.	Transposase IS4-like domain-containing protein	MVQQVSGQLDFADSFVANNPKLNQRLDK IDRLVNWGAFDLLLSSSYSSHTGRPSYPLL LLFKCLLVQQWYAASDYLLLEMLDDRSLF RRFVGLSLSQKAPDHSTICRFREQILPFKE QLFLELDRQLDRLGLMIKKGTLVDATVIEA AVSKPPKNEDGSSGKSPLDPEAGWAIKN GCFSYGYKAHLGVDQESELLRRCEMTAA NVHDSRMLGAVITGEEKWVFADKAYDSQ ANGDLLREKQINNGILHSRKKKPTKAAGR CNRILSKLRVPIERIFGTLKRSYRYARGRYI GLEKNSLQMMMLMCFAYNLRRMEVICR
3	1	363	HYCHLA_M_02675	Chlamydia bacterium, Parachlamydiales bacterium, Verrucomicrobia bacterium	Transposase	MSQYEPLTDLQWQLEPLFPNPVKRTRG KPHSAWRAVINSIFYVLTGLKWGSLPKD PAFATKSAHRWFVLWEKSGFLKEIVDTY QSMTQMASGIKLPRRRNRLPAKRVPVE TAIAAG
4	5	276	HYCHLA_M_07050	Chlamydia bacterium, Chlamydiales bacterium, Chlamydiales bacterium	Transposase	MAILQWCQEQGIRWDYIQPGKPYQNGYIE SFNGQLRDECLNENIFVNLQEAKRLVEAW REEYNKRRPHSSLNGKTPEEVAQEEELT GTSN
5	169	735	HYCHLA_M_08535	Chlamydiales bacterium, Bdellovibrionales bacterium, Ca. Protochlamydia naegleriophila	IS1 transposase	MECKCPSCASLLVKKNGHIHNGKQNHRC LACQRQFVVEPDQKIIDDHTKVLVRRTLLE RVSLEGVCRIFNVSMPWLLGFIDEVVATLP EDLNAQVVKENDDIEVVLLEADEQWSYVG SKDNPQWLWLVMSKTRQIVAMQVGAR NKQTAERLFAKLPEPLKKKARYYTDYFNV YYETIPSEQHRPVGKESGKTSYIERFNCTL RQRCSRLVRKTLFSKLANHIGMIKYFVC DYNLRRRALHV
6	158	267	HYCHLA_M_08735	Chlamydiales bacterium, Ca. Protochlamydia naegleriophila, Waddlia chondrophila	IS1 transposase	MYETIPSEQHRPVGKESGKTSYIERFNC TLRQRCSRLVRKTLFSKLANHIGMIKYF VCDYNLRRRAYMFRTTTTLRIFAFISIFCS
7	5	333	HYCHLA_M_12565	Chlamydia bacterium, Chlamydiales bacterium, Afipia sp.	Transposase	MLTVVDDFTRESIKIAVDMSLNGRRVCEE LEQVIEARGKPDRILSDNGTEFTSMALQW CQEQGIRWDYIQPGKPYQNGYIESFNGKL RDECLNENIFVNLQEAKRLVEA
total	529					

conservation_group1
Entire sequence:
45 sequences align 100 percent
121 align 99%
150 align 76%
158 align 52%
Only transposase sequence:
146 align 100% (putative transposase)
152 align 100% (additional gene)
Only repeats:
156 align 100%

Table S12. Proteins with eukaryotic-like domains in *E. hydrae*

ELP	LocusTag	Gene	Product	max_annot	COG	Description	PFAMs
Ankyrin							
132	HYCHLAM_00660		hypothetical protein	4751 Fungi	S	Ankyrin repeats (3 copies)	Ank_2
498	HYCHLAM_02490		hypothetical protein	4751 Fungi	M	Ankyrin repeat	Ank_2,Ank_4
1325	HYCHLAM_06625		hypothetical protein	33208 Metazoa	T	Receptor-interacting serine-threonine kinase 4	Ank_2,Ank_3,Ank_4,Ank_5,Pkinase,Pkinase_Tyr
1326	HYCHLAM_06630		hypothetical protein	33208 Metazoa	S	Ankyrin repeat family A	Ank,Ank_2,Ank_3,Ank_4,Ank_5
2091	HYCHLAM_10455		hypothetical protein	NA	NA	NA	NA
2065	HYCHLAM_10325		hypothetical protein	NA	NA	NA	NA

ELP	LocusTag	Gene	Product	max_annot	COG	Description	PFAMs
TPR							
135	HYCHLAM_00675		Nucleotidyl transferase domain-containing protein	2 Bacteria	M	ADP-heptose-lipopolysaccharide heptosyltransferase activity	Glyco_transf_9,TPR_2
405	HYCHLAM_02025		Outer membrane lipoprotein BamD-like domain-containing protein	204428 Chlamydiae	S	Tetratricopeptide repeat	TPR_16,TPR_6
519	HYCHLAM_02595	bamD	Outer membrane protein assembly factor BamD	2 Bacteria	S	protein trimerization	TPR_16,TPR_6,TolA_bind_tri,YfiO
704	HYCHLAM_03520		Tetratricopeptide repeat protein	204428 Chlamydiae	S	Tetratricopeptide repeat	TPR_19
1053	HYCHLAM_05265		hypothetical protein	204428 Chlamydiae	S	Type III secretion	TPR_3
1091	HYCHLAM_05455		hypothetical protein	1117 Cyanobacteria	M	tetratricopeptide repeat	TPR_1,TPR_10,TPR_11,TPR_16,TPR_2,TPR_8
1189	HYCHLAM_05945	sunS	SPBc2 prophage-derived glycosyltransferase SunS	909932 Negativicutes	M	Glycosyl transferase family 2	Glycos_transf_2,TPR_8
1353	HYCHLAM_06765		hypothetical protein	33208 Metazoa	O	chaperone cofactor-dependent protein refolding	DnaJ,TPR_1,TPR_16,TPR_2,TPR_8
1867	HYCHLAM_09335		TPR-like protein	204428 Chlamydiae	S	Tetratricopeptide repeat	TPR_16,TPR_8
1969	HYCHLAM_09845		DUF268 domain-containing protein	121069 Pythiales	S	TPR domain protein. Source PGD	DUF268

ELP	LocusTag	Gene	Product	max_annot	COG	Description	PFAMs
2081	HYCHLAM_10405		hypothetical protein	28221 Deltaproteobacteria	O	repeat-containing protein	Glyco_transf_41,TPR_1,TPR_10,TPR_11,TPR_16,TPR_2,TPR_7,TPR_8
2143	HYCHLAM_10715		Beta-barrel assembly-enhancing protease	204428 Chlamydiae	S	Transglutaminase-like superfamily	TPR_8,Transglut_core2
2488	HYCHLAM_12440		hypothetical protein	28221 Deltaproteobacteria	O	repeat-containing protein	Glyco_transf_41,TPR_1,TPR_10,TPR_11,TPR_16,TPR_2,TPR_7,TPR_8

LRR							
none							

MORN							
181	HYCHLAM_00905		Membrane-binding protein	204428 Chlamydiae	S	MORN repeat variant	MORN_2
287	HYCHLAM_01435	ywqK	Toxin-antitoxin system YwqK family antitoxin	204428 Chlamydiae	S	MORN repeat variant	MORN_2
605	HYCHLAM_03025	ywqK	Toxin-antitoxin system YwqK family antitoxin	204428 Chlamydiae	S	repeat protein	MORN_2
2227	HYCHLAM_11135		hypothetical protein	28216 Betaproteobacteria	S	MORN repeat variant	MORN_2

Sel-1							
none							

WD40							
881	HYCHLAM_04405		hypothetical protein	1117 Cyanobacteria	E	WD40-like Beta Propeller Repeat	PD40,Peptidase_S9
998	HYCHLAM_04990		hypothetical protein	4751 Fungi	O	Cyclophilin type peptidyl-prolyl cis-trans isomerase/CLD	Pro_isomerase,WD40

Chapter IV

Discussion

Discussion

Endosymbiosis allows novel forms of energy-sharing, which can confer fitness advantages and access to unexplored ecological niches. Just as varied as the symbioses we find, are the studies we perform to unravel their functions. For the chlamydiae a mix of phylogenetic, metagenomic and laboratory studies on the limited number of isolates, has revealed their ancient association with eukaryotic hosts, their distribution in virtually every environment, importance as human and animal pathogens and importance as contributors to gene exchange with other endosymbionts. Their obligate intracellular lifestyle and wide distribution among many eukaryotic clades is both a fascinating, but also challenging aspect of their biology, as the majority of eukaryotic clades are hard to obtain as stable isolates for laboratory work.

Over the course of this thesis I have taken two different approaches to analyse the impact chlamydial symbionts have on their hosts in the context of controlled laboratory conditions as well as under more complex environmental settings, utilising both well studied chlamydial species and a relatively new isolate.

Manuscript I consists of a study that utilised two chlamydial symbionts with different levels of parasitism towards a common soil amoeba host to measure the impact they have on their immediate symbiont-host and broader host-environment systems. Previous studies had revealed protists as primary consumers of bacterial biomass and drivers of the microbial loop, stimulating microbial respiration and biomass turnover and releasing plant-available nutrients by predating on microbial cells (Adl and Gupta, 2006; Bonkowski and Clarholm, 2012; Clarholm, 1985; Geisen and Bonkowski, 2018). Chlamydial symbionts on the other hand, had previously shown potential in affecting their hosts' growth rate as a function of their level of parasitism (Herrera et al., 2020) and showed attenuated infectivity from exposure to environmental changes (Herrera et al., 2023). Some chlamydial symbionts' reported protective properties against amoeba pathogens (Arthofer et al., 2022; Ishida et al., 2014; König et al., 2019) were especially interesting aspects to consider in a complex environment like soil.

This study was designed to test how the presence of chlamydial symbionts would change known impacts of their protist hosts in a soil ecosystem. It was aimed at addressing the knowledge gap that persists between the many known functions protists have as predators and the impact of their common association with bacterial endosymbionts.

By designing a soil microcosm system to better understand the complex interactions from several different angles, I was able to simultaneously follow host-symbiont growth dynamics, bacterial

community growth and environmental measurements like microbial biomass, microbial respiration and biomass turnover as well as nitrogen mineralization. This was complemented by analysis of the soil bacterial community composition, based on rRNA measurements, which allowed me to follow changes more quickly compared to DNA based measurements and with less noise by extracellular nucleic acids.

The results of this study confirmed the different chlamydial symbionts cause expected impacts on their hosts' survival also under complex conditions. Their presence led to diverging effects on microbial community shifts, compared to what I observed with the aposymbiotic hosts alone, with four taxa (*Mucilaginibacter*, *Chthoniobacter*, *Reyranella* and *Pseudomonas*) especially negatively affected in the symbiont treatments. I observed enhanced microbial turnover, microbial activity and nitrogen mineralization predominantly in the milder of the two chlamydial symbionts, compared to the aposymbiotic hosts' effects. This I argue is achieved both by the more stable association with its host, leading to prolonged enhancement of host predatory activity and might be further increased by host protective traits, conferred by the symbiont, leading to a broader consumption of food bacteria.

I think this project raises interesting questions that could be addressed in future studies. It would be possible for example to use this system to test more host-symbiont systems, or include protist pathogens such as giant viruses to test protective properties of the symbionts in a more natural setting. An investigation of the observed effect of increased nitrogen mineralization, in presence of the milder chlamydial symbiont, could confirm higher nitrogen release is directly caused by predation, or reveal soil nutrient stoichiometry as the underlying reason. Controlled experiments on feeding preferences of the host-symbiont mixtures, could answer whether some of the groups that were heavily affected by the symbiont treatments like the four genera *Mucilaginibacter*, *Chthoniobacter*, *Reyranella* and *Pseudomonas*, are actually predated on, or indirectly affected by other processes.

Overall, the findings of this project, which I immensely enjoyed working on, showed that symbionts are in-fact overlooked players of the soil food-web and very likely of other complex environments too. We are far from understanding the role that symbionts of protists play in their respective environments. Further studies in this direction will certainly create an even more complex spectrum of possibilities, but they will be essential when estimating the impact these predators have in their ecosystems. In addition I hope this will also inspire new approaches for defining the roles that symbionts and hosts have in relation to each other.

Manuscript II started out quite differently. The freshwater predator *Hydra oligactis* in the lab of Frédéric Tomas in Montpellier, showed occurrence of spontaneous tumors in some of his and his PhD student Justine Boutry's laboratory populations. When searching for answers in the microbiome of their animals, they discovered high relative abundances of chlamydial 16S rRNA gene sequences,

which were especially high in animals that had developed tumors (Boutry et al., 2023). These were then brought to Matthias Horn's lab in Vienna and handed over to me for further characterization of the *Hydra*-chlamydia symbiosis. When I started out on this project, there were three main questions I wanted to answer: **1.** Is there a connection of chlamydiae and tumor occurrence in *Hydra*. **2.** After sequencing and analysing the genome of the chlamydial symbiont can I find differences to other described chlamydiae and do they point to specific adaptations to the *Hydra* host. **3.** What is the morphology of the first cnidarian-infecting chlamydial isolate and how widely is it distributed environmentally.

Another question that arose later in the project was whether other chlamydial clades were associated with wild *H. oligactis* populations. This I answered together with the help of Jácint Tökölyi, who luckily was just writing a paper on the microbiome of these wild populations (Tökölyi et al., 2026).

Very early on in the project, it became clear, after quantifying absolute numbers of chlamydial cells per animal, that numbers were actually similar between tumorous and non-tumorous animals. Thus, it was the disappearance of other bacteria in the *H. oligactis* microbiome that had caused the dominance of chlamydiae in relative abundance in the original study, making chlamydial involvement in *Hydra* tumor formation less likely.

At the same time, it became apparent that this isolate might be the first cultured representative of the recently described family Chlamydiae Clade III (CCIII) (Dharamshi et al., 2023), described so far only from metagenomes and sister family to the very unique *Anoxychlamydiaceae*, known for their potentially obligate anaerobic lifestyle and unique genomic repertoires among the chlamydiae (Stairs et al., 2020). Therefore, and because there were no known hosts yet for the CCIII family, analysis of the genome of this isolate was a very interesting prospect. The genome analysis of this symbiont we termed *Endochlamydia hydrae*, revealed it encodes all the hallmark genes characteristic for chlamydial intracellular survival and host communication and only a small set of genes unique to this symbiont. The unique genes, containing autotransporter and eukaryotic-like domains, likely have functions in host-communication and possibly represent specific adaptations to *Hydra* as a host. However, the main adaptations to this host seem to have been on the genome-wide level in the form of genomic rearrangements, a large number of genomic islands and pseudogenes, proliferation of IS elements and uneven GC-skew all pointing to ongoing genomic rearrangements.

An investigation into the distribution of the *E. hydrae* phylotype, showed a broad distribution also in *Hydra*-less environments, indicating a host range that likely extends to a group common as chlamydial hosts: microbial eukaryotes; that could represent an environmental reservoir also for this chlamydial species. A following analysis of wild *H. oligactis* populations revealed that actually many distinct chlamydial families associate closely with *Hydra* polyps. Thus, at least transient symbioses

between *Hydra* and chlamydiae could be a common phenomenon, but possibly lack the stability we see with *E. hydrae*.

The morphological characterization revealed a tissue specific intracellular location in the hosts' endodermal epithelial cells. The colonization throughout the entire body column meant that they are passively transmitted to daughter animals during the budding process. Under laboratory conditions this symbiosis remained stable, with infected animals persisting in our cultures for over two years and showing no gradual loss of the symbionts in our lab populations. In fact, as I had learned shortly after receiving the cultures, these chlamydial symbionts seem to persist when other bacterial symbionts do not. Chlamydiae have several ways of evading host antimicrobial defense systems and apoptosis pathways and thus are well equipped to handle host defense to a level that other microbial taxa might not (Collingro et al., 2011; Stelzner et al., 2023). It is conceivable they could even induce the production of antimicrobial substances, priming the host against other pathogens and serving the stability of the symbiosis. Many also have a repertoire of biosynthetic gene clusters (Dharamshi et al., 2022), as does *E. hydrae*, that could serve to eliminate other co-habitants even further.

There are many possibilities for follow-up studies on this or a similar system. For example, a specific search for other isolates of the *E. hydrae* genotype in the environment would give a much clearer picture of this species' actual host range. As we have found *E. hydrae* to be very stably associated with its hosts, a fitness study, using cured *Hydra* polyps could provide an answer as to the role these chlamydiae play in the fecundity of their host. Additional parameters to be investigated could also include protection against other residents of *Hydra* or the symbionts' reaction to *Hydra*-specific antimicrobial substances like periculin. It would also be interesting to further investigate transmission to *Hydra* offspring during egg production, which could represent a way for the host to inhibit transmission to the next generation, as eggs in *H. oligactis* are derived from ectodermal cells, which is uncolonised by the chlamydial symbiont.

The availability of *Hydra* as host for chlamydiae also offers the potential to study the evolution of chlamydial adaptations to the host immune response in the future and might contribute to a better understanding of the evolution of the important human and animal pathogens in the phylum. Studies showing genomic adaptations that chlamydiae undergo when establishing in a new host are still rare, yet offer important information on the mechanisms involved in adaptation in the phylum. Thus, studies using novel chlamydiae-host systems will contribute to a better understanding of the chlamydiae-way-of-life, with all its flexibility towards hosts and environments.

The diversity of chlamydial families that are predicted to still be undiscovered (Collingro et al., 2020; Lagkouvardos et al., 2014) is matched only by their potential host range. Though some can be cultivated using model protist hosts, natural hosts are still needed to understand their ecological niche and functions they might have in relation to a natural host. Environmental chlamydiae have been shown to harbour a much broader genomic repertoire than their vertebrate-infecting cousins (Collingro et al., 2020; Dharamshi et al., 2023). Metagenomic studies have been an invaluable tool to understand the characteristics of this large group of undescribed species. Yet, other parameters such as secondary metabolite production, protective properties, host range, or environmental impact need experimental validation, especially as they hold important factors for understanding the ecosystems and hosts these bacteria colonize and thrive in.

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