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Preface – Voorwoord

"What we know is a drop, what we don't know is an ocean" (Isaac Newton).

Wat Newton blijkbaar toen nog niet wist, is hoeveel leven er zich in één druppel water kan bevinden. Hoewel, zandfilters werden reeds gebruikt ten tijde van de oude Egyptenaren voor het zuiveren van water en deze techniek wordt tot op heden nog heel veel gebruikt. Vandaag de dag is in onze streken (en ver daarbuiten) de toegankelijkheid tot drinkbaar water vanzelfsprekend geworden. Het zuiveren van water (grondwater of oppervlakte water) tot drinkbaar water en het transporteren ervan is echter alles behalve een eenvoudige klus. Doch slagen de verschillende drinkwaterbedrijven in Vlaanderen hierin met als resultaat dat drinkwatergerelateerde ziekten zelden voorkomen in Vlaanderen.

Desalniettemin, bevindt er zich nog leven in ons kraantjeswater. Eén van deze levensvormen zijn bacteriën. Met deze studie hebben we dan ook getracht een beter inzicht te krijgen in hun gemeenschapssamenstelling in de verschillende stappen van het drinkwaterproductie- en distributieproces. Alsook hoe deze gemeenschappen wijzigen doorheen het hele proces. Bijkomend hebben we isolaten van het geslacht *Acinetobacter* verder onderzocht. We hebben voornamelijk gefocust op hun fenotypische eigenschappen die omgevingsisolaten (afkomstig van voornamelijk water) kunnen onderscheiden van klinische isolaten. Daarenboven hebben we ook onderzocht hoe deze fenotypische eigenschappen fylogenetische verwantschappen kunnen beschrijven binnen het *Acinetobacter* geslacht.

Uiteraard spreek ik hierboven in de 'wij'-vorm, hoewel het maken van een doctoraal proefschrift vaak op één iemands schouders rust, toch doe je dit niet alleen. Wat volgt is dan ook een welgemeende "dank u" voor al diegene die hiertoe hebben bijgedragen.

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Ook alle mensen die meegewerkt hebben aan het Toegepast Wetenschappelijk Onderzoek voor Leefmilieu (TWOL) project wil ik bedanken, deze studie was uiteindelijk de 'echte' start van mijn doctoraat.

Bedankt aan alle drinkwaterbedrijven, familie, vrienden en collega's die waterstalen beschikbaar stelden en zo dit doctoraat hebben mogelijk gemaakt.

Zonder OmniLog geen doctoraat, therefore, thank you Barry, Andre and all other employees of Biolog, you were always very generous with wise advice concerning this wonderful technology.

Zonder een uitgebreide *Acinetobacter* collectie geen doctoraat, daarom wil ik de mensen van het Leiden Universitair Medisch Centrum bedanken voor hun hulp. In het bijzonder, bedankt Lenie,

jouw kennis aangaande het *Acinetobacter* geslacht en de uitgebreide collectie die je door de jaren heen hebt verzameld, is indrukwekkend.

Bedankt aan alle coauteurs voor de hulp bij onze publicaties, waarin we onze wetenschappelijke resultaten hebben kunnen tentoonstellen.

Verder woorden van dank aan alle collega's (inclusief KU Leuven, Scientia Terrae, MicroBioMetrix, …) waarmee ik doorheen dit doctoraat heb mogen samenwerken. Bedankt voor jullie actieve (raad en daad in het labo) en/of passieve (koffiepauzes, cafébezoeken, …) bijdrage aan dit werk.

Sam, bedankt voor je hulp bij de data-analyse en zo veel meer alsook voor de weinige cafébezoekjes en korte of lange reizen, veel succes met al wat je nog zal doen.

Sergio, gracias, unfortunately we are no longer colleagues, yet, your valuable help and your impressive knowledge were of key importance to this work. I wish you all the best in your new challenges, remember a new beginning always starts at mile zero and I predict you'll make it all the way.

En tot slot, jaren geleden heb ik jullie verteld dat ik een doctoraatstudie zou uitvoeren, wel dit is nu eindelijk geschreven en een feit. Beste familie en vrienden, ik wil jullie bedanken voor het geduld en de ondersteuning die jullie mij gedurende deze periode hebben getoond. Jullie steun was van groot belang en woorden van dank schieten te kort maar tussen pot en pint zullen we het er nog wel eens over hebben (dixit de witte profeet).

Ado, september 2019

List of abbreviations

Acb	*Acinetobacter calcoaceticus-Acinetobacter baumannii*
AFLP	amplified fragment length polymorphism
AIC	Akaike's information criterion
ANI	average nucleotide identity
ANOSIM	analysis of similarities
ANOVA	analysis of variance
ARDRA	amplified ribosomal DNA restriction analysis
ASV	amplicon sequence variant
AUC	area under the curve
AWCD	average well color development
BLAST	basic local alignment search tool
BUG	Biolog's universal growth
CLSI	clinical and laboratory standards institute
DDH	DNA–DNA hybridization
DO	dissolved oxygen
DWPDS	drinking water production and distribution system
EC	electrical conductivity
ECOFF	epidemiological cut-off
EUCAST	European committee on antimicrobial susceptibility testing
GAC	granular activated carbon filtration
GN	Gram negative
GW	groundwater
gyrB	DNA gyrase subunit B encoding gene
HGT	horizontal gene transfer
HTW	household tap water
IF-A	inoculation fluid A
ISA	indicator species analysis
IQR	inter quartile range
LPSN	list of prokaryotic names with standing in nomenclature
MALDI-TOF MS	matrix-assisted laser desorption-ionization – time of flight mass spectrometry
MIC	minimum inhibitory concentration
MID	multiplex identifier
MLSA	multilocus sequence analysis
MDR	multidrug resistance
NGS	next generation sequencing

NJ	Neighbour-joining
NMDS	non-metric multidimensional scaling
NS	nutrient score
non-WT	non-wild type
OPPP	opportunistic premise plumbing pathogen
OTU	operational taxonomic unit
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PM	phenotype microarray
PW	processed water
qPCR	quantitative real-time PCR
RAPD	random amplified polymorphic DNA
recA	DNA recombinase A encoding gene
RDA	redundancy analysis
RDP	ribosomal database project
REP-PCR	repetitive element sequence-based PCR
rpoB	RNA polymerase beta subunit encoding gene
rRNA	ribosomal RNA
RSF	rapid sand filtration
SSF	slow sand filtration
SW	surface water
TOC	total organic carbon
TS	tolerance score
TSA	tryptic soy agar
WGS	whole genome sequencing
WHO	World Health Organization
WSP	water safety plan
WT	wild type

Samenvatting

De productie en distributie van drinkwater dat onmiddellijk geschikt is voor consumptie is niet vanzelfsprekend. De kwaliteit van drinkwater wordt beïnvloed door zijn chemische en microbiële samenstelling, die op hun beurt kunnen beïnvloed worden door het bronwater en de verschillende processen die worden toegepast in de productie en zuivering van het water. Bacteriën van de genera *Legionella*, *Mycobacterium* en *Acinetobacter* zijn belangrijke water-gerelateerde pathogenen. *Acinetobacter* soorten zijn doorgaans commensale micro-organismen, maar veroorzaken ook sporadisch infecties, vooral bij patiënten met een verminderde weerstand in een ziekenhuisomgeving. Hoewel *Acinetobacter* spp. vaak worden aangetroffen in bodem en aquatische milieus, is er tot nu toe weinig bekend over hun ecologie in drinkwater en drinkwaterproductie- en distributiesystemen. Het hoofddoel van dit doctoraatsonderzoek was om de bacteriële samenstelling van drinkwaterproductie- en distributiesystemen in Vlaanderen (België) te karakteriseren, met een bijzondere nadruk op *Acinetobacter* spp.

In het eerste hoofdstuk (**Hoofdstuk I**) bespreken we het drinkwaterproductieproces, met bijzondere aandacht voor de bacteriële gemeenschappen in drinkwater en een aantal bacteriële soorten met mogelijk gezondheidsrisico's. Verder wordt een uitgebreid literatuuroverzicht gegeven over het genus *Acinetobacter*, inclusief taxonomische geschiedenis, de belangrijkste fenotypische kenmerken, waarom acinetobacters in diverse leefomgevingen kunnen gedijen en hun klinische relevantie. In **Hoofdstuk II** onderzochten we aan de hand van hoge doorvoer sequenering van een deel van het 16S ribosomaal RNA (rRNA) gen de bacteriële diversiteit in waterstalen van dertien drinkwaterproductie- en distributiesystemen in Vlaanderen (België), die oppervlaktewater of grondwater als bronwater gebruiken. Waterstalen werden over twee seizoenen genomen van het bronwater, het afgewerkte drinkwater in de productie-installatie, en van kraantjeswater in het distributienetwerk. De grootste verschillen in bacteriële diversiteit werden gevonden tussen de watertypes (i.e. bronwater, afgewerkt product en kraantjeswater) van drinkwaterbedrijven die oppervlaktewater gebruiken als bronwater. Voor bedrijven die grondwater gebruiken als bronwater waren deze verschillen minder uitgesproken. Het meest voorkomende fylum was *Proteobacteria*. Verder waren verscheidene fyla waaronder *Actinobacteria* significant meer aanwezig in oppervlaktewater, terwijl *Cyanobacteria* voornamelijk aanwezig waren in oppervlaktewater en het afgewerkt product dat afkomstig was van oppervlaktewater. *Gallionella*, *Acinetobacter* en *Pseudomonas* waren de drie meest voorkomende bacteriële geslachten. Leden van het *Acinetobacter* genus werden zelfs in bepaalde stalen van het afgewerkt water aangetroffen aan een relatieve abundantie tot 47.5 %. Om verder te onderzoeken hoe bacteriële gemeenschappen worden gevormd tijdens drinkwaterproductie en -distributie en om na te gaan welke *Acinetobacter* soorten in dit ecosysteem voorkomen, werd een vervolgstudie uitgevoerd bij één welbepaalde drinkwaterproductiefaciliteit in Antwerpen (België) (**Hoofdstuk III**). Het doel van deze studie was om de veranderingen in de bacteriële gemeenschapssamenstelling tijdens de productie en distributie van

drinkwater te onderzoeken, uitgaande van een parallel productiesysteem waarin verschillende behandelingen werden toegepast, en waarbij hetzelfde bronwater (oppervlaktewater) werd gebruikt. Verder hebben we de aanwezigheid en abundantie van *Acinetobacter* in elke stap van de productielijn opgevolgd met behulp van klassieke uitplatingen en een cultuuronafhankelijke methode (kwantitatieve real-time PCR (qPCR)). Ten slotte onderzochten we het belang van de fysicochemische eigenschappen van het drinkwater op de samenstelling van de bacteriële gemeenschap in het afgeleverde product (huishoudelijk kraantjeswater). Kwantificering van het totaal aantal bacteriën onthulde dat voor beide productielijnen de hoeveelheid bacteriën afnam wanneer een behandeling werd toegepast, en dat de laagste hoeveelheid bacteriën werd gedetecteerd na chlorering. Er was een duidelijk verschil waarneembaar tussen de samenstelling van de bacteriële gemeenschap in beide productielijnen. In de eerste productielijn (lijn A; toegepaste behandelingen (in volgorde): snelle zandfiltratie, langzame zandfiltratie, actieve koolfiltratie, UV behandeling en chlorering) werd na langzame zandfiltratie een aanzienlijke verschuiving in de bacteriële gemeenschap waargenomen, wat resulteerde in een aanzienlijke toename van het aantal operationele taxonomische eenheden (OTUs; i.e. een operationele definitie om groepen van nauw verwante individuen te classificeren) gebaseerd op een standaard cutoff van 97 % 16S rRNA gensequentie-identiteit. In de tweede productielijn (lijn B; toegepaste behandelingen: flotatie, dubbellaagfiltratie, actieve koolfiltratie, UV behandeling en chlorering) nam het aantal OTUs geleidelijk toe na elke behandelingsstap. Voor beide lijnen nam het aantal OTUs af na chlorering. Uit de taxonomische classificatie van de OTUs bleek dat *Proteobacteria* opnieuw het meest voorkomende fylum was, hoewel er wel veranderingen in de relatieve abundantie van de fyla tussen de verschillende behandelingsstappen werden waargenomen. Opvallend was dat de samenstelling van de bacteriële gemeenschap van het water op het einde van lijn A (kraantjeswater) en in het distributienetwerk van lijn B (opslagtank) sterk op elkaar leken. Seizoenseffecten bleken van geringe invloed te zijn op de samenstelling van de bacteriële gemeenschap. In tegenstelling tot Hoofdstuk II kwam *Acinetobacter* in vrij lage relatieve abundantie voor, met een maximale relatieve abundantie van 2.8 %. Absolute abundantie van *Acinetobacter* was het laagste na chlorering voor beide lijnen. Toch werd voor lijn A een significante toename van *Acinetobacter* waargenomen in het leidingwater van de huishoudens t.o.v. het afgewerkt product in de drinkwaterproductiefaciliteit. Door middel van uitplatingen op een voedingsagar werden er in totaal 14 verschillende soorten *Acinetobacter* waargenomen (bepaald aan de hand van *rpoB* (RNA polymerase beta-subeenheid) gensequentie-similariteit), waaronder drie potentiële ziekteverwekkers die werden gevonden in het afgewerkt drinkwater of het huishoudelijk kraantjeswater (i.e. *A. guillouiae*, *A. johnsonii* en *A. lwoffii*). Chemische parameters vertoonden een seizoensgebonden trend, met uitzondering van trihalomethanen. De concentratie aan trihalomethanen was lager in het kraantjeswater van één van de onderzochte locaties die drinkwater verkregen van lijn A. Interessant genoeg vertoonde de bacteriële gemeenschap in het drinkwater op deze locatie een hogere relatieve abundantie van *Methylophilus* soorten, waarvan geweten is dat ze gechloreerde methanen kunnen gebruiken. Vervolgens onderzochten we in **Hoofdstuk IV** de

genetische en fenotypische verwantschap tussen *Acinetobacter* isolaten uit omgevingsstalen (vnl. (drink)water) en klinische omgevingen. Hiervoor werden 58 isolaten die behoren tot vier *Acinetobacter* soorten geassocieerd met menselijke infecties geselecteerd (*A. calcoaceticus, A. guillouiae, A. johnsonii* en *A. lwoffii*). Isolaten werden gefenotypeerd met behulp van de GENIII identificatiemicrotiterplaat van Biolog, die de metabolische activiteit van een micro-organisme analyseert in 94 fenotypische testen, waaronder 71 koolstofbronnen en 23 chemische gevoeligheidstesten. Een Spearman rangcorrelatie analyse werd uitgevoerd om te bepalen of het vermogen om verschillende koolstofbronnen te gebruiken co-varieerde tussen de verschillende isolaten. Bovendien werden antibiotica-gevoeligheidstesten uitgevoerd voor een totaal van 15 antibiotica. Verder werden de isolaten gegenotypeerd door een gedeeltelijke sequentiebepaling van het *rpoB* gen, en werd een Mantel test uitgevoerd tussen het fenotype en de *rpoB* gensequentie-similariteit. Significante verschillen werden gevonden voor het gebruik van zes koolstofbronnen wanneer de isolaten werden gegroepeerd op basis van hun herkomst (i.e. aquatisch/milieu vs. klinisch/veterinair) (ongeacht de soortclassificatie). Daartegenover waren 34 koolstofbronnen en 14 chemische gevoeligheidstesten significant verschillend wanneer de isolaten werden gegroepeerd per soort, wat suggereert dat fenotypische kenmerken meer soort- dan habitatafhankelijk zijn. De Spearman rangcorrelatietest toonde aan dat verschillende koolstofbronnen co-variëren tussen de verschillende isolaten. Sterke significante correlaties werden gevonden voor suikers, en zijn meestal soort afhankelijk. De antibiotica-gevoeligheidstesten toonden aan dat vooral isolaten afkomstig van het afgewerkt drinkwater (i.e. na chlorering) resistentie vertoonden tegen één of meerdere antibiotica. Een significante relatie tussen de paarsgewijze fylogenetische afstand en de kenmerkende fenotypische verschillen tussen isolaten werd gevonden voor vier koolstofbronnen (i.e. L-arginine, L-histidine, citroenzuur en γ-amino-boterzuur) en één chemische stressor (i.e. natriumbromaat). Ten slotte hebben we in **Hoofdstuk V** onderzocht of bacteriële fylogenie moleculaire functies en fenotypische kenmerken kan weerspiegelen met *Acinetobacter* als model. Een totaal van 133 stammen van 33 erkende *Acinetobacter* soorten en acht genoomsoorten werd geanalyseerd met behulp van de GENIII technologie van Biolog. De sterkte en de betekenis van het fylogenetisch signaal werd geschat voor elk fenotype aan de hand van fylogenetische reconstructies op basis van *rpoB* gensequenties en volledige genoomsequenties. Verder werd door een Mantel test nagegaan of fylogenetische afstanden kunnen aangewend worden om fenotypische verschillen te voorspellen. Ten slotte werd met behulp van evolutionaire modellen onderzocht of de fenotypische eigenschappen overeenkwamen met de fylogenetische positie van de isolaten of dat dat eerder willekeurig was. Uit onze gegevens bleek dat enkele belangrijke fenotypische kenmerken gerelateerd aan substraatgebruik en chemische gevoeligheid gekoppeld zijn aan de fylogenetische plaatsing van *Acinetobacter* soorten. De sterkste fylogenetische signalen die werden gevonden, waren voor het gebruik van koolstofbronnen, zoals organische zuren, aminozuren en suikers, wat erop wijst dat bij de diversificatie van acinetobacters de assimilatie van koolstofbronnen een relevante rol heeft gespeeld.

Dit doctoraatsonderzoek heeft nieuwe inzichten opgeleverd over de samenstelling van de bacteriële gemeenschap van drinkwaterproductie- en distributiesystemen die grondwater of oppervlaktewater als bronwater gebruiken. Verder hebben we onderzocht hoe veranderingen in de samenstelling van bacteriële gemeenschappen kunnen worden toegeschreven aan verschillende behandelingsstappen en welke *Acinetobacter* soorten aanwezig zijn in de verschillende stappen van het drinkwaterproductie- en drinkwaterdistributieproces. Daarnaast bieden we nieuwe inzichten in de fenotypische eigenschappen van *Acinetobacter* soorten die gedeeltelijk kunnen worden beïnvloed door hun leefomgeving. En tenslotte hebben we met *Acinetobacter* als studieobject aangetoond dat de fylogenie van bacteriën (bepaalde) moleculaire functies en fenotypische kenmerken kan weerspiegelen.

Summary

The production and delivery of safe drinking water is challenging. The quality of drinking water is influenced by its chemical and microbial composition which in turn may be affected by the source water and the different processes applied in the production of drinking water. Important bacterial waterborne pathogens include members of the genera *Legionella*, *Mycobacterium* and *Acinetobacter*. *Acinetobacter* species are usually commensal organisms, but they occasionally cause infections, predominantly in susceptible patients in hospitals. Whereas *Acinetobacter* spp. are frequently found in soil and aquatic environments, so far only little is known about their ecology in drinking water and drinking water production and distribution facilities. The major aim of this PhD study was to assess the bacterial community composition of drinking water production and distribution systems in Flanders (Belgium), emphasizing *Acinetobacter* spp.

In the first chapter (**Chapter I**), we discuss the drinking water production process, with a particular focus on drinking water bacterial communities as well as a number of bacteria of potential health concern. Furthermore, a comprehensive literature overview is given on the genus *Acinetobacter,* including taxonomic history, its main phenotypic traits, how *Acinetobacter* is adapted to thrive in diverse environments, and their clinical relevance. In **Chapter II**, using high-throughput sequencing of partial 16S ribosomal RNA (rRNA) gene amplicons, we investigated the bacterial diversity in different water samples from the production and distribution chain of thirteen drinking water production and distribution systems from Flanders (Belgium) that use surface water or groundwater as source water. Water samples were collected over two seasons from the source water, the processed drinking water within the production facility, and out of the tap in houses along its distribution network. Strong differences in bacterial community composition were found between processed drinking water originating from companies that use surface water as source water. Differences were less pronounced for companies that use groundwater as source water. *Proteobacteria* was the most abundant phylum in all samples. Yet, several phyla including *Actinobacteria* were significantly more abundant in surface water, while *Cyanobacteria* were more abundant in surface water and processed water originating from surface water. *Gallionella*, *Acinetobacter* and *Pseudomonas* were the three most abundant genera detected. Members of the *Acinetobacter* genus were even found at a relative read abundance of up to 47.5 % in processed water samples. To further investigate how bacterial communities are shaped during drinking water production and distribution as well as to further examine which *Acinetobacter* species occur in this ecosystem, a follow-up study was performed at the site of one particular drinking water production facility in Antwerp (Belgium) (**Chapter III**). More particularly, the goal of this study was to investigate the bacterial community shifts during production and distribution of drinking water, by studying a full-scale drinking water production and distribution facility that uses two series of multi-step treatment processes starting from the same source water (surface water). Furthermore, we investigated the presence and abundance of *Acinetobacter* at each step of the treatment chain using both

culture-dependent and culture-independent methods (i.e. isolation after enrichment and quantitative real-time PCR (qPCR)). Finally, we studied the importance of the physicochemical characteristics of the drinking water on the bacterial community composition in the water delivered to the end user (household tap water). Quantification of total bacteria revealed that for both production lines the amount of bacteria decreased when a treatment was applied, and the lowest amount of bacteria were detected after chlorination. There was a clear difference between the bacterial community composition in both production lines. In the first line (line A; applied treatments: rapid sand filtration, slow sand filtration, activated carbon filtration, UV treatment and chlorination), a substantial community shift was observed after slow sand filtration, resulting in a large increase in richness in operational taxonomic units (OTUs; an operational definition used to classify groups of closely related individuals) defined by a standard cut-off of 97 % 16S rRNA gene sequence identity. In the second production line (line B; applied treatments: flotation, double layer filtration, activated carbon filtration, UV treatment and chlorination), OTU richness gradually increased after every treatment step. For both lines, OTU richness decreased after chlorination. Taxonomy assignment of the OTUs revealed that *Proteobacteria* was again the most abundant phylum, although changes in the relative abundance of phyla was observed between treatment steps. Strikingly, the bacterial community composition of the waters sampled at the end of production line A (tap water) and in the distribution network of line B (storage tank) were highly similar. Seasonal effects showed to be of minor influence in shaping the bacterial community composition. In contrast to Chapter II, *Acinetobacter* was found at low relative abundance in the water samples investigated in this study, reaching a maximum relative abundance of 2.8 %. Absolute abundance of *Acinetobacter* was the lowest after chlorination for both lines. Yet, for line A, a significant increase of *Acinetobacter* was observed in the household tap water in comparison to the finished product within the drinking water facility. Plating of the water samples on agar media revealed a total of 14 different species (based on *rpoB* (RNA polymerase beta subunit) gene similarity), among which three potential pathogenic *Acinetobacter* species (i.e. *A. guillouiae*, *A. johnsonii* and *A. lwoffii*) were found in finished drinking water or household tap water. Water chemical parameters were more significantly different between seasons than between sampling locations, with the exception of trihalomethanes. Trihalomethanes concentrations were lower in the tap water samples taken at one of the locations that received drinking water from line A. Interestingly, the bacterial community in the drinking water sampled at this location was enriched in *Methylophilus* species which are capable of utilizing chlorinated methanes. Next, in **Chapter IV** we determined the level of genetic and phenotypic relatedness between *Acinetobacter* isolates from environmental (mainly (drinking) water) and clinical environments using 58 isolates belonging to four *Acinetobacter* species that are associated with human infections (*A. calcoaceticus, A. guillouiae, A. johnsonii* en *A. lwoffii*). Isolates were phenotyped using Biolog's GENIII identification microplate, which analyzes the performance of a microorganism in 94 phenotypic tests, including 71 carbon source utilization assays and 23 chemical sensitivity assays. A Spearman rank correlation analysis was performed to determine whether the ability to use different carbon sources co-varied

between the different isolates. Additionally, antibiotic susceptibility testing was performed using 15 antibiotic compounds. Further, isolates were genotyped by partial sequencing of the *rpoB* gene, and a Mantel test was performed to assess correlations between phenotypic and *rpoB* gene sequence similarity. When results obtained for the different carbon sources were evaluated based on the origin of the isolates (i.e. aquatic/environmental vs. clinical/veterinary) (irrespective of species classification), significant differences were obtained for six carbon sources. On the other hand, 34 carbon sources and 14 chemical sensitivity assays were significantly different based on grouping by species classification, suggesting that phenotypic traits are more species- than habitat-dependent. The Spearman rank correlation test showed that several carbon sources co-varied between the different isolates. Strong significant correlations were found for sugars and tend to be species-dependent. Antibiotic susceptibility testing revealed that especially isolates from finished drinking water (i.e. after chlorination) displayed resistance to one or more antibiotics. A significant relationship between the pairwise phylogenetic distance and trait differentiation among isolates was found for four carbon source assays (i.e. L-arginine, L-histidine, citric acid and γ-amino-butyric acid) and one chemical stressor (i.e. sodium bromate). Finally, in **Chapter V** we tested the hypothesis whether bacterial phylogeny reflects molecular functions and phenotypic characteristicsfor a large set of *Acinetobacter* strains. A total of 133 strains belonging to 33 *Acinetobacter* species with validly published names and eight genomic species were analyzed using the GENIII technology of Biolog. We estimated the strength and significance of the phylogenetic signal of each trait across phylogenetic reconstructions based on partial *rpoB* and core genome sequences. Secondly, we tested whether phylogenetic distance was a good predictor of trait differentiation by a Mantel test. And finally, evolutionary model fitting was used to determine if the data for each phenotypic character was consistent with a phylogenetic or an essentially random model of trait distribution. Results revealed that some key phenotypic traits related to substrate assimilation and chemical sensitivity are linked to the phylogenetic placement of *Acinetobacter* species. The strongest phylogenetic signals found were for utilization of carbon sources such as some organic acids, amino acids and sugars, thus suggesting that in the diversification of acinetobacters carbon source assimilation has had a relevant role.

In conclusion, this PhD study has provided new insights on the bacterial community composition in drinking water production and distribution systems which use groundwater or surface water as source water. Furthermore, we studied how bacterial community composition shifts can be attributed to different treatment steps and which *Acinetobacter* species are present at the different stages of drinking water production and distribution. Additionally, we provide new insights in the phenotypic traits of different *Acinetobacter* species which can partially be influenced by the habitat of isolation. And finally, using *Acinetobacter* as a study object, we showed that the phylogeny of bacteria can reflect phenotypic characteristics.

Table of content

Chapter I: Introduction

1.1 Drinking water

Drinking water, also known as potable water, is water that is safe to drink and to use for food preparation, without risk of health problems. Additionally, the water should be safe for hygiene and recreational use. According to the World Health Organization (WHO), 91 % of the world's population has access to safe drinking water, among which 58 % of the population receives their drinking water from piped systems (WHO, 2015). The source water which is used to produce drinking water is usually retrieved from surface water or groundwater. In order to make water potable, drinking water treatment is (in most circumstances) necessary and of utmost importance. Water treatment is typically based on a multistep process which depends on the source of the water (Fig. 1.1). In case of surface water and depending on its quality, water treatment usually involves a pre-oxidation step (most likely ozonation), rapid sand filtration (RSF), slow sand filtration (SSF), and granular activated carbon filtration (GAC) (Fig. 1.1 A). When groundwater is used as source water, the water is typically first aerated to remove unwanted gasses such as CO_2 and H_2S as well as to oxidize Fe^{2+} to Fe^{3+}, and is then followed by a softening and flocculation step to produce and remove iron(III)oxides. Subsequently, the water undergoes a filtration step which is either based on a double layer filtration (GAC and sand layer) or just contains a sand layer (Fig. 1.1 B). In both cases, a disinfection step is performed, e.g. by UV treatment followed by a residual disinfection step (chlorination or chloramination). Some drinking water production facilities in Europe do not apply a residual disinfection step (Hammes et al. 2008) and control microbial regrowth by managing the amount of growth limiting substrates (van der Kooij, 2002). After the water leaves the treatment plant, it is stored in reservoirs before further distribution.

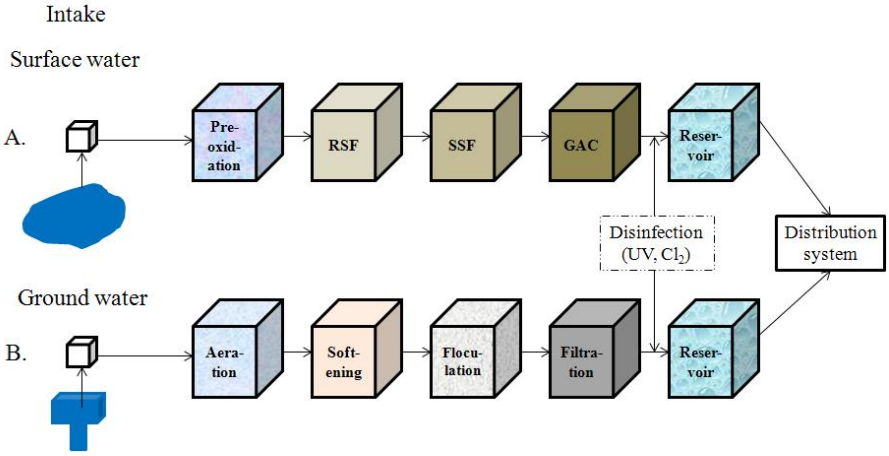


Figure 1.1: Configuration of a drinking water production system using surface water (**A**) or groundwater (**B**) as source. At the top (**A**) a typical series of treatment steps for surface water as source water are shown, including a pre-oxidation step (usually ozonation), rapid sand filtration (RSF), slow sand filtration (SSF) and granular activated carbon filter (GAC). At the bottom (**B**) a configuration of treatment steps is shown when groundwater is used as source water. Depending on the quality of the groundwater, the different steps can include an aeration step to remove undesired volatiles, a softening step, a flocculation and coagulation step and a filtration step (usually a sand or double layer filter). In most cases, at the end of both production systems a residual disinfection is performed which can exist of a UV treatment and chlorination step before it is stored and further distributed.

In Europe the quality of drinking water is regulated by the European Directive 98/83/EG. This directive defines essential quality standards for water intended for human consumption, and clearly states that drinking water should be free from microorganisms and parasites and from any substances which, in numbers or concentrations, constitute a potential danger to human health. It requires Member States to regularly monitor the quality of water intended for human consumption by using a 'sampling points' method and evaluate the quality according to a list of microbial and physicochemical parameters. Conform the directive fecal indicator bacteria like *Escherichia coli*, coliforms and/or enterococci are used to assess the microbiological quality of tap water. Although these bacteria are not typically disease causing, they are associated with fecal contamination and the possible presence of fecal pathogens. Further, monitoring of specific bacteria like *Clostridium perfringens* has proven useful for the assessment of the quality of water resources, and to check the stages of water treatment to evaluate the treatment-works performance. *Clostridium perfringens* is a Gram-positive anaerobic bacterium that forms spores and is commonly found the environment (e.g. in soil and sewage) and in human and animal intestines (Rood and Cole, 1991). The spores of *C. perfringens* survive in water for months, much longer than vegetative fecal indicator bacteria and consequently their presence may indicate remote or intermittent fecal pollution. Additionally, research has demonstrated that *C. perfringens* is a better indicator for viral and parasitic (cysts and oocysts) contamination (Payment and Franco, 1993). According to the European directive, *E. coli*, coliforms, enterococci and *C. perfringens* should be absent in 100 mL water sample. Further, total aerobic colony counts are performed to assess the biological stability of the water during distribution. The biological stability is considered adequate if no substantial changes are observed in time. Although excessive monitoring of the drinking water quality indicates that the majority of the delivered water is of acceptable quality, the European directive 98/83/EG was amended in 2015 with the concept of "water safety plans" (WSPs) as recommended by the WHO as the most effective means of consistently ensuring the safety and acceptability of a drinking water supply. Water safety plans make use of a comprehensive risk assessment and risk management approach that encompass all steps in the water supply from catchment to consumer, with a focus on high priority risks (Davison et al. 2005). Effective risk management requires the identification of potential hazards and hazardous events and an assessment of the level of risk presented by each. Where risks cannot be immediately addressed, the WSP approach allows for incremental improvements to be implemented systematically over time (Bartram et al. 2009). As such, the presence of potential bacterial pathogens is an important focal point in assessing the microbiological quality of drinking water.

Although the classical plating methods mentioned above have greatly helped evaluating the microbiological quality of drinking water (Lee et al. 2003; Martiny et al. 2005; September et al. 2007; Inomata et al. 2009), not all microorganisms are culturable under standard laboratory conditions (Byrd et al. 1991), by which important species may be overlooked, including potential pathogens (Liu et al. 2008). Using flow-cytometric total bacterial cell counts, it is estimated that roughly 10 % of the microorganisms occurring in (drinking) water can be cultivated *in vitro* (Hammes et al. 2008). To

overcome the limitations of culture-dependent techniques as well as to perform in depth analyses of entire microbial communities without resorting to culturing, culture-independent DNA-based methods such as high-throughput sequencing of ribosomal RNA (rRNA) genes (Margulies et al. 2005; Caporaso et al. 2012) are increasingly used to study the drinking water microbial community composition and associated biofilms (e.g., Hong et al. 2010; Navarro-Noya et al. 2013; Prest et al. 2014; Roeselers et al. 2015; Wu et al. 2015).

It is known that within drinking water production and distribution systems many different lifeforms can survive or proliferate, including viruses, bacteria, fungi, protozoa and nematodes (Douterelo et al. 2014). Although all these different lifeforms have been detected in drinking water distribution systems, bacteria are dominantly present and most of them shelter within biofilms. Such biofilms can serve multiple functions such as degradation of disinfection residuals, transformation of metals involved in corrosion, and harboring and supporting the survival of pathogens (Wingender and Flemming, 2011; Douterelo et al. 2018). Furthermore, it is known that bacterial pathogens such as non-tuberculous mycobacteria and *Legionella* species are of concern in drinking water distribution networks (Ashbolt, 2015). Therefore, the focus of this study will be on the bacterial community composition of the drinking water production and distribution systems.

1.1.1 Bacterial community composition of drinking water

In recent years, the bacterial community composition of drinking water and its production and distribution systems has been studied in detail by deep sequencing of 16S rRNA gene amplicons. Briefly, following polymerase chain reaction (PCR) amplification of (part of) a phylogenetic marker such as the 16S rRNA gene, a large amount of sequences are generated using high-throughput sequencing platforms such as the Roche 454 pyrosequencing system and the Illumina MiSeq sequencer. These so-called "second generation sequencers" generate huge amounts of sequences at a cost-effective price per base/sequence without resorting to cloning. As a downside, obtained sequences are typically short (often up to ~250 bp), limiting taxonomic resolution. Subsequently, obtained sequences are grouped in operational taxonomic units (OTUs), often based on 97 % sequence similarity as a proxy for species, giving a deeper insight in the structure of the microbial community. While such 97 % rRNA gene sequence identity cut-off found its origin in previous studies using the entire 16S rRNA gene (ca. 1500 bp) (Stackebrandt and Goebel, 1994), this threshold has been commonly implemented in microbial community profiling based on partial 16S rRNA gene sequences (Shokralla et al. 2012; Vetrovsky and Baldrian, 2013). Nevertheless, it has to be noted that organisms belonging to closely related yet distinct species may be grouped under the same OTU (Vetrovsky and Baldrian, 2013). The recovered OTUs are assigned taxonomic classification based on publicly available databases such as the Ribosomal Database Project (RDP) database, the SILVA database or GenBank. Previous studies using such technologies revealed that often members of *Proteobacteria* dominate, especially in the distribution system and

household tap water (Bautista-de los Santos et al. 2016). Members of the phylum *Proteobacteria* have a wide metabolic versatility which gives them a selective advantage to compete for the available nutrients and/or are more resistant against disinfection treatments than bacteria from other phyla (Rosenberg et al. 2014; Becerra-Castro et al. 2016), thereby promoting their growth. Although the majority of the bacteria found in the bulk water (planktonic cells) in the distribution system have been shown to originate from the planktonic bacterial community leaving the production facility (Liu et al. 2018a), several factors influence the bacterial community composition. For example, bacterial regrowth after disinfection is influenced by the hydraulic regime, temperature, plumbing materials, available carbon sources, presence of corrosion products, and water residence time (LeChevallier et al. 1996; Berry et al. 2006; Camper et al. 2013; Liu et al. 2013; Proctor and Hammes, 2015; and Bautista-de los Santos et al. 2016). Additionally, within buildings the pipe diameter of the premise plumbing has an influence on the bacterial community composition (Lautenschlager et al. 2010; Ji et al. 2015). It has been schown that a smaller pipe diameter results in stronger changes of the bacterial community composition. Furthermore, it has been shown that the source of the water has an important impact on the drinking water bacterial community composition (Gomez-Alvarez et al. 2015; Ji et al. 2015). Surface water has a more diverse bacterial community and contains more distantly related species in comparison to groundwater (Gomez-Alvarez et al. 2015). On the other hand, it has been shown that the bacterial community composition in drinking water production facilities using different surface water may be different (Ji et al. 2015). Besides the source of the water, several treatment steps applied in the drinking water production influence the bacterial community composition in the water (Pinto et al. 2012; Ma et al. 2017; Xu et al. 2017). Substantial changes in bacterial community composition have been observed following filtration steps such as slow sand filtration, double layer (dual layer) filtration and granular activated carbon filters (Pinto et al. 2012; Lautenschlager et al. 2014; Ma et al. 2017; Xu et al. 2017; Hou et al. 2018). By contrast, treatments like pre-oxidation, coagulation and rapid sand filtration seem to have a lower impact on the bacterial community structure (Pinto et al. 2012; Xu et al. 2017; Liu et al. 2018a). Furthermore, the type of disinfection applied may have a strong impact on the bacterial community composition. For example, the increase of chlorine concentrations or the alternation between the use of chlorination and chloramination cause a different bacterial community composition in the tap water (Hwang et al. 2012; Wang et al. 2014; Stanish et al. 2016). Microbial community analysis revealed that among major core populations, *Cyanobacteria*, *Methylobacteriaceae*, *Sphingomonadaceae*, and *Xanthomonadaceae* were more abundant in chlorinated water, and *Methylophilaceae*, *Methylococcaceae*, and *Pseudomonadaceae* were more abundant in chloraminated water (Hwang et al. 2012). Stanish et al. (2016) reported that within chloramine-treated systems there is an increase of microbes associated with nitrification and iron-cycling. Furthermore, they also observed that with an increase of residual chlorine there is a shift from *Alphaproteobacteria* and *Betaproteobacteria* to *Firmicutes* and *Gammaproteobacteria*. Within the phylum of *Proteobacteria*, *Gammaproteobacteria* are known to be more resistant to chlorine (Mathieu et al. 2009). Nevertheless, in a meta-analysis of a

large amount of bacterial community composition studies, there were less differences in community composition between chlorinated and chloraminated systems in comparison with disinfected residual-free systems (Bautista-de los Santos et al. 2016). Furthermore, when ozone is applied in the production of drinking water from surface water it has been shown that there is an increase of mycobacteria, most likely because ozone degrades the organic matter, resulting in a higher concentration of assimilable organic matter (Torvinen et al. 2004). Additionally, it has also been documented that the microbial community of drinking water changes with the different seasons, particularly due to temperature effects (Pinto et al. 2014; Prest et al. 2016; Potgieter et al. 2018).

1.1.2 Drinking water: a bacterial health risk?

Occurrence of opportunistic pathogens in drinking water systems is a major public concern, not only because they can cause disease in hosts experiencing atypical environmental stressors or having impaired immune function, but also because they can be easily transported through the drinking water distribution system (Bartram et al. 2003). Waterborne pathogens typically include genera such as *Shigella, Salmonella* and other enteric bacteria which have a fecal-oral route of infection. Their presence in drinking water systems is usually because of a contamination event, yet they generally do not multiply in water supplies (Falkinham III et al. 2015a). On the other hand, the so-called "opportunistic premise plumbing pathogens" (OPPP) are native to the premise plumbing environment and ideally adapted to survival, growth, and persistence in drinking water distribution systems and premise plumbing (Falkinham III et al. 2015b). Most OPPP infections lead to pulmonary and blood stream infections, and it is estimated that the hospital costs of infections caused by OPPP are about 0.6 billion USD per year in the USA, mainly for elderly and immunocompromised patients (Naumova et al. 2016). Due to the high observed prevalence of *Legionella pneumophila*, *Mycobacterium avium* and *Pseudomonas aeruginosa* in drinking water, these species are model pathogens for OPPP. Yet, several other bacteria belonging to genera such as *Acinetobacter*, *Aeromonas* and *Stenotrophomonas,* and other members of the *Mycobacterium* genus should not be overlooked (Falkinham III et al. 2015b). Non-tuberculous *Mycobacterium* species (i.e. *M. avium*, *M. fortuitum*, *M. gordonae*, *M. mucogenicum* and *M. peregrinum*) are increasingly isolated in tap waters and are correlated based on a chemometric method with isolates from clinical samples (Donohue et al. 2015; Dovriki et al. 2016). Further, studies within intensive care units have revealed that infection with *P. aeruginosa* may originate from hospital tap water (Trautmann et al. 2006; Crivaro et al. 2009). According to a recent surveillance report for waterborne disease outbreaks associated with drinking water, there were 42 drinking water-associated outbreaks in the U.S.A in the period 2013-2014 (Benedict et al. 2017). Among these, 24 cases were caused by *L. pneumophila*, five by *Cryptosporidium*, three by *Giardia duodenalis*, two by Norovirus, one by *Clostridium*/*E. coli* and one by *Campylobacter*. Other cases were due to chemical toxins or had an unknow cause.

In addition, several other bacteria are known to be spread via water and cause waterborne diseases in humans. These mainly include members of cyanobacteria, the genera *Acinetobacter*, *Aeromonas*, *Arcobacter*, *Flavobacterium*, *Klebsiella*, *Salmonella*, *Serratia*, *Shigella*, *Staphylococcus*, *Stenotrophomonas* and *Yersinia*, and the species *E. coli*, *Helicobacter pylori* and *Vibrio cholerae* (Percival et al. 2004). Yet, while members of these bacteria are rarely reported as the cause of a waterborne outbreaks (Benedict et al. 2017), some members are known to cause hospital acquired infections and have sometimes been linked to the tap water of the hospital. Among these, the *Acinetobacter* genus represents an important taxon, which has been frequently found and isolated from tap water (LeChevallier et al. 1980; Bifulco et al. 1989).

1.2 The genus *Acinetobacter*

The genus *Acinetobacter* comprises a large group of species that are known to flourish in diverse natural ecosystems as well as many man-made environments (Doughari et al. 2011; Touchon et al. 2014). *Acinetobacter* species frequently occur in soil, (drinking) water, insect guts and plant related environments such as floral nectar and tree bark (e.g. Anandham et al. 2010; Vaz-Moreira et al. 2011; Álvarez-Pérez et al. 2013; Choi et al. 2013; Kim et al. 2014; Narciso-da-Rocha et al. 2013; Krizova et al. 2014; Li et al. 2015). Especially their versatile metabolism, biofilm formation ability and resistance to disinfectants make them ideally suited to survive drinking water treatment and additionally thrive and persist in the drinking water distribution system (Rodríguez-Baño et al. 2008; Bhargava et al. 2010; Chaves Simões et al. 2010; Peleg et al. 2012). Furthermore, *Acinetobacter* is well adapted to different human body sites, and may cause opportunistic infections (Dijkshoorn et al. 2007). The most common manifestations of *Acinetobacter* are pneumonia and catheter-associated bacteremia, but it can also cause wound infections and urinary tract infections (Wong et al. 2017).

1.2.1 Taxonomy / taxonomic history

Acinetobacter is a genus of Gram-negative bacteria belonging to the class *Gammaproteobacteria*, the order *Pseudomonadales* and the family *Moraxellaceae*. Currently the genus consists of 58 species with a validly published name, and a number of genomic species which are still awaiting formal species description (Table 1.1) (http://www.bacterio.net/acinetobacter.html, last accessed July 2019; http://apps.szu.cz/anemec/Classification.pdf). Identification of *Acinetobacter* species has been complicated by the lack of standard identification techniques. Initially, species identification was based on phenotypic characteristics such as growth temperature, colony morphology, growth medium, assimilation of carbon sources, gelatin hydrolysis, glucose fermentation, and several others (Baumann, 1968; Bouvet and Grimont, 1987; Dijkshoorn et al. 1990; and Gerner-Smidt et al. 1991). *Acinetobacter* taxonomy greatly benefitted from the introduction of DNA fingerprinting methods such as random

amplified ribosomal polymorphic DNA (RAPD), amplified ribosomal DNA restriction analysis (ARDRA), amplified fragment length polymorphism (AFLP) and pulsed-field gel electrophoresis (PFGE), providing more discriminatory power in addition to the phenotype analysis (Vos et al. 1995; Vila et al. 1996). Next, following reductions in DNA sequencing costs, DNA sequencing of housekeeping genes (e.g. 16S rRNA gene, *gyrB* gene encoding the DNA gyrase subunit B and *rpoB* gene encoding the RNA polymerase subunit B) has been increasingly used (Rainey et al. 1994; Yamamoto et al. 1999; La Scola et al. 2006). Nowadays, an increasing number of species is described based on a polyphasic approach of phenotypic assays, multilocus sequence analysis (MLSA) of housekeeping genes, and whole genome information (i.e. average nucleotide identity (ANI)) (Bartual et al. 2005; Diancourt et al. 2010; Chan et al. 2012; Nemec et al. 2019).

Table 1.1: Overview of currently described *Acinetobacter* species

Validly published species name	Main source of isolation	Reference
A. albensis	Soil, water	Krizova et al. 2015a
A. apis	Honey bee intestine	Kim et al. 2014
A. baumannii	Human, warm-blooded animals	Bouvet and Grimont 1986
A. baylyi	Activated sludge, soil	Carr et al. 2003
A. beijerinckii	Human, animals, soil, water	Nemec et al. 2009
A. bereziniae	Human	Bouvet and Grimont 1986; Nemec et al. 2010
A. bohemicus	Soil, water	Krizova et al. 2014
A. boissieri	Floral nectar	Álvarez-Pérez et al. 2013
A. bouvetii	Activated sludge	Carr et al. 2003
A. brisouii	Peat	Anandham et al. 2010
A. calcoaceticus	Soil, water, human	Bouvet and Grimont 1986
A. celticus	Soil, water	Radolfová-Křížová et al. 2016a
A. colistiniresistens	Human	Bouvet and Jeanjean 1989; Nemec et al. 2017
A. courvalinii	Human, animals	Bouvet and Jeanjean 1989; Nemec et al. 2016
A. cumulans	Hospital sewage	Qin et al. 2019
A. defluvii	Hospital sewage	Hu et al. 2017
A. dijkshoorniae (= *A. lactucae*)	Human, lettuce	Cosgaya et al. 2016; Dunlap and Rooney 2017
A. dispersus	Soil, water, human	Bouvet and Jeanjean 1989; Nemec et al. 2016
A. equi	Horse	Poppel et al. 2016
A. gandensis	Horse, cattle, water	Smet et al. 2014
A. gerneri	Activated sludge	Carr et al. 2003
A. grimontii (= *A. junii*)	Activated sludge	Carr et al. 2003; Vaneechoutte et al. 2008
A. guangdongensis (= *A. indicus*)	Lead-zinc ore	Feng et al. 2014; Nemec and Radolfova 2017
A. guillouiae	Soil, water, human	Bouvet and Jeanjean 1989; Nemec et al. 2010
A. gyllenbergii	Human	Nemec et al. 2009
A. haemolyticus	Human	Bouvet and Grimont 1986
A. halotolerans	Soil	Dahal et al. 2017
A. harbinensis	Water	Li et al. 2014a
A. indicus	Soil	Malhotra et al. 2012
A. johnsonii	Soil, water, human, animals	Bouvet and Grimont 1986
A. junii	Human, animals, water, soil	Bouvet and Grimont 1986
A. kookii	Soil, water	Choi et al. 2013
A. lactucae	Lettuce	Rooney et al. 2016
A. larvae	Moth larval gut	Liu et al. 2017a
A. lwoffii	Human, animals, soil, water	Bouvet and Grimont 1986; Nemec et al. 2019
A. modestus	Human, water	Touchon et al. 2014; Nemec et al. 2016
A. nectaris	Floral nectar	Álvarez-Pérez et al. 2013
A. nosocomialis	Human	Tjernberg and Ursing 1989; Nemec et al. 2011
A. pakistanensis (= *A. bohemicus*)	Wastewater	Abbas et al. 2014; Nemec and Radolfova-Krizova 2016
A. parvus	Human, animals	Nemec et al. 2003
A. piscicola	Fish	Liu et al. 2018b
A. pittii	Human, soil, water	Bouvet and Grimont 1986; Nemec et al. 2011
A. populi	Populus bark	Li et al. 2015

A. pragensis	Soil, water	Radolfová-Křížová et al. 2016b
A. proteolyticus	Human	Touchon et al. 2014; Nemec et al. 2016
A. pseudolwoffii	Human, animals, soil, water	Bouvet and Grimont 1986; Nemec et al. 2019
A. puyangensis	Populus bark	Li et al. 2013
A. qingfengensis	Populus bark	Li et al. 2014b
A. radioresistens	Human, soil, cotton	Bouvet and Grimont 1986; Nishimura et al. 1988
A. rudis	Raw milk, wastewater	Vaz-Moreira et al. 2011
A. schindleri	Human, animals	Nemec et al. 2001
A. seifertii	Human	Gerner-Smidt and Tjernberg 1993; Nemec et al. 2015
A. sichuanensis	Hospital sewage	Qin et al. 2018
A. soli	Human, soil	Kim et al. 2008
A. tandoii	Activated sludge, water, soil	Carr et al. 2003
A. tjernbergiae	Activated sludge	Carr et al. 2003
A. towneri	Activated sludge, water, soil	Carr et al. 2003
A. ursingii	Human	Nemec et al. 2001
A. variabilis	Human, animals, soil	Krizova et al. 2015b
A. venetianus	Salt water	Di Cello et al. 1997; Vaneechoutte et al. 2009
A. vivianii	Human, soil, water	Touchon et al. 2014; Nemec et al. 2016
A. wuhouensis	Hospital sewage	Hu et al. 2018

Since the description of the *Acinetobacter* genus by Brisou and Prévot in 1954 and after the reorganization of the oxidase-negative moraxellas by Baumann and colleagues in 1968, the genus consisted officially of two validly named species, i.e. *Acinetobacter calcoaceticus* and *Acinetobacter lwoffii* (Lessel, 1971). Nevertheless, the phenotypic traits of the strains investigated indicated a possible further subdivision of the genus. Indeed, further investigations using DNA-DNA hybridization experiments and transformation assays resulted in several additional groups which were in agreement with the phenotypic differences observed (Bauman et al. 1968; Johnson et al. 1970; Juni et al. 1972), yet no new validly species were named. It was only in 1986 when the first additional species were described. More particularly, Bouvet and Grimont (1986) defined twelve genomic groups which they referred to as *Acinetobacter* genomic species based on DNA-DNA hybridization using the S1 nuclease method. This resulted in an emended description of the species *A. calcoaceticus* and *A. lwoffii*, but also led to the description of four novel species (i.e. *Acinetobacter baumannii*, *Acinetobacter haemolyticus*, *Acinetobacter johnsonii* and *Acinetobacter junii*). Using DNA-DNA hybridization also Bouvet/Jeanjean (BJ) (1989) and Tjernberg/Ursing (TU) (1989) identified five and three new genomic species, respectively. These genomic species are referred to as 13 through 17 BJ and 13 through 15 TU, respectively. In later studies genomic species 13BJ was linked to genomic species 14TU (Gerner-Smidt et al., 1991). Additionally, the type strain of *Acinetobacter radioresistens* which was described by Nishimura et al. (1988) appeared to be a member of genomic species group 12 of Bouvet and Grimont's study in 1986 (Tjernberg and Ursing, 1989). Although most strains used in the study of Bouvet/Jeanjean and Tjernberg/Ursing were of clinical origin, the strains used in the description of *A. radioresistens* were environmental isolates. Furthermore, due to the higher DNA relatedness and the lack of discriminatory phenotypic data, Tjernberg and Ursing (1989) suggested to group *A. calcoaceticus* (genomic species 1), *A. baumannii* (genomic species 2) and genomic species 3 and 13TU into one species. These four genomic species are referred to as the *Acinetobacter calcoaceticus-Acinetobacter baumannii* (Acb) complex. Further molecular studies on the Acb complex revealed two additional genomic species, i.e.

"between 1 and 3" and "close to 13TU", which are also members of the Acb complex (Gerner-Smidt and Tjernberg, 1993). Molecular techniques such as DNA fingerprinting (i.e. ARDRA, AFLP, etc.) and gene sequencing (16S rRNA and *rpoB* genes) as well as multilocus sequence typing (MLST), whole-genome comparison and matrix-assisted laser desorption-ionization – time of flight mass spectrometry (MALDI-TOF MS) enabled the description of genomic species 3, 10, 11, 13TU, close to 13TU, 15TU, 14BJ, 17, 13BJ/14TU and 8 as the following *Acinetobacter* species, respectively: *A. pittii*, *A. bereziniae*, *A. guillouiae*, *A. nosocomialis*, *A. seifertii*, *A. variabilis*, *A. courvalinii*, *A. dispersus*, *A. colistiniresistens* and *A. pseudolwoffii* (Krizova et al. 2015b; Nemec et al. 2010; 2011; 2015; 2016; 2017; 2019). Most of the strains belonging to these species were isolated either from clinical and/or environmental habitats such as soil, water and animals. Other genomic species remain unnamed.

Additional important *Acinetobacter* species with clinical strains and often also environmental strains have been described as *A. beijerinckii*, *A. dijkshoorniae*, *A. gyllenbergii*, *A. parvus*, *A. proteolyticus*, *A. schindleri*, *A. ursingii*, and *A. vivianii* (Nemec et al. 2001; 2003; 2009; 2016; Cosgaya et al. 2016). *Acinetobacter dijkshoorniae* is a heterotypic synonym (i.e. a taxonomic synonym, a synonym that comes into being when a taxon is reduced in status ("reduced to synonymy") and becomes part of a different taxon.) of *Acinetobacter lactucae*, which was isolated from lettuce (Rooney et al. 2016; Dunlap and Rooney. 2017). Furthermore, several species have exclusively been found in environmental habitats. In 2003, Carr and colleagues validly named seven different *Acinetobacter* species isolated from activated sludge plants (i.e. *A. baylyi*, *A. bouvetii*, *A. gerneri*, *A. grimontii* (heterotypic synonym for *A. junii* (Vaneechoutte et al. 2003)), *A. tandoii*, *A. tjernbergiae* and *A. towneri*). Further, *Acinetobacter celticus*, *Acinetobacter halotolerans*, *Acinetobacter pragensis* and *Acinetobacter soli* were isolated from soil and/or water samples (Kim et al. 2008; Radolfová-Křížová et al. 2016b; 2016b; Dahal et al. 2017). *Acinetobacter soli* was later also recognized to cause human infections (Endo et al. 2014). Other *Acinetobacter* species that are not yet mentioned above and that are related with soil or water are *A. albensis*, *A. bohemicus*, *A. brisouii*, and *A. kookii*, (Anandham et al. 2010; Choi et al. 2013; Krizova et al. 2014; Krizova et al. 2015a). Several other *Acinetobacter* species have also been isolated from aquatic environments, i.e. *A. gandensis*, *A. harbinensis*, *A. pakistanensis*, *A. rudis* and *A. venetianus* (Abbas et al. 2014; Li et al. 2014a; Smet et al. 2014; Vaneechoutte et al. 2009; Vaz-Moreira et al. 2011). *Acinetobacter rudis* was also found in raw milk and *A. gandensis* contains a significant amount of strains isolated from veterinary samples. *Acinetobacter venetianus* was isolated from seawater and is currently used to produce a commercial bioemulsifier. *Acinetobacter pakistanensis* was found in a textile dye wastewater treatment plant and was identified as a heterotypic synonym of *A. bohemicus* (Nemec and Radolfová-Křížová, 2016). Other *Acinetobacter* species which were isolated from contaminated environments are *A. indicus*, originally isolated in a hexachlorocyclohexane dump site, and *A. guangdongensis*, which was found in a lead-zinc ore (Malhotra et al. 2012; Feng et al. 2014). Later, both species were found to be heterotypic synonyms (Nemec and Radolfová-Křížová, 2017). So far, five *Acinetobacter* species have been isolated from plant

related environments. *Acinetobacter populi*, *A. puyangensis* and *A. qingfengensis* originated from populus bark (Li et al. 2013; 2014a; 2015), and *A. boissieri* and *A. nectaris* have been recognized as important floral nectar bacterial community members (Álvarez-Pérez et al. 2013). *Acinetobacter apis* and *A. larvae* were isolated from the digestive track of a honey bee and a larval moth, respectively (Kim et al. 2014; Liu et al. 2017a). Besides insects, acinetobacters are known to be hosted by several animals, for example *A. equi* and *A. piscicola* were isolated from horses and fish, respectively (Poppel et al. 2016; Liu et al. 2018b). Lately, several *Acinetobacter* species (i.e. *A. cumulans*; *A. defluvii*, *A. sichuanensis* and *A. wuhouensis*) have been found in hospital sewage (Hu et al. 2017; 2018; Qin et al. 2018, 2019). Figure 1.2 represents a phylogenetic tree based on partial *rpoB* gene sequences for all known *Acinetobacter* species as well as some genomic species, illustrating their phylogenetic relationships.

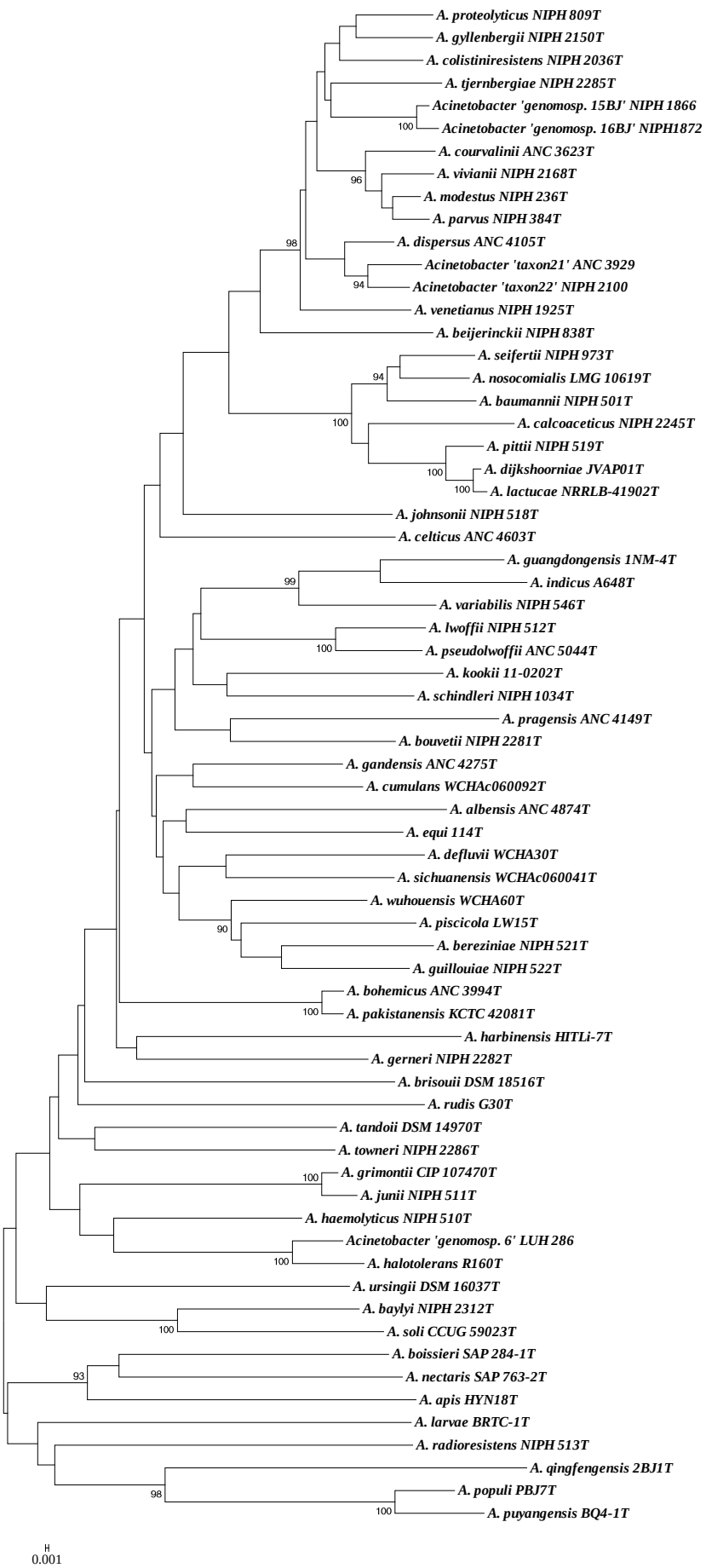


Figure 1.2: Neighbour-joining (NJ) tree, based on partial *rpoB* gene sequences, showing the phylogenetic relationships of all known *Acinetobacter* species (type strains (T)) and some genomic species. Evolutionary distances were computed using the Maximum Composite Likelihood method and the unit is number of base substitutions per site. There were a total of 844 positions in the final dataset. All positions containing gaps and missing data were eliminated. Node support values (bootstrap percentages, based on 1000 simulations) ≥ 90% are shown next to the branches.

1.2.2 Phenotypic characterization of Acinetobacter *species*

Phenotypic characterization has always been an important aspect of species classification within the *Acinetobacter* genus. *Acinetobacter* species are described as Gram-negative, strictly aerobic, oxidase-negative, and catalase-positive coccobacilli typically occurring in pairs. Furthermore, they are non-motile or exhibit twitching motility, are positive for the transformation assay of Juni, and are capable to grow in defined media based on a single carbon source and ammonia as the sole nitrogen source (Baumann et al. 1968; Juni, 1972, 1984; Lautrop, 1974). Additionally, they are able to grow in a temperature range from 1 to 44 °C, although only a few species are able to grow at 44 °C. These include *A. baumannii*, *A. dijkshoorniae* (= *A. lactucae*), *A. nosocomialis*, *A. pittii*, *A. seifertii* and *A. variabilis* (Radolfova-Krizova et al. 2015b; Hu et al. 2018). Originally, *Acinetobacter* species were described based on phenotypic traits only. The phenotypic tests used at that time comprised a total of 16 compounds which are testing the growth on a single carbon source (Baumann, 1968; Bouvet and Grimont, 1986). Nowadays, the phenotypic characterization scheme for delineation of *Acinetobacter* species consists of 36 carbon sources, which include mostly organic and amino acids as well as some sugars and amines (Nemec et al. 2009; Krizova et al. 2015b). Furthermore, it also includes growth at different temperatures, acidification of D-glucose, haemolysis of sheep blood and liquefaction of gelatine. Although these assays are very useful, they are typically labour intensive and prone to errors, e.g. due to mistakes made during media preparation. Therefore, more recently, studies are increasingly being performed using commercially available phenotype assays such as Biolog's identification technology or Biolog's Phenotype MicroArrays (PM) (Bernards et al. 1995; Mara et al. 2012; Peleg et al. 2012), which allow for high-throughput standardized phenotyping. Typically, using these assays strains are tested in 96-well plates pre-coated with metabolic substrates or inhibitory chemicals (Fig. 1.3 A). The redox chemistry of the technology contains a colourless tetrazolium dye which is reduced to purple/violet coloured formazan due to cell's respiration. Plates can be read for endpoint testing or at various time points by the so-called OmniLog instrument which also provides information on the respiration kinetics (Fig. 1.3 B). Currently, PM plates are available for testing nearly 2000 phenotypes, and include assays for carbon, nitrogen, phosphor and sulphur source utilization, nutrient stimulation, pH and osmotic stresses, and chemical sensitivities with 240 inhibitory chemicals (Bochner, 2001 and 2009).

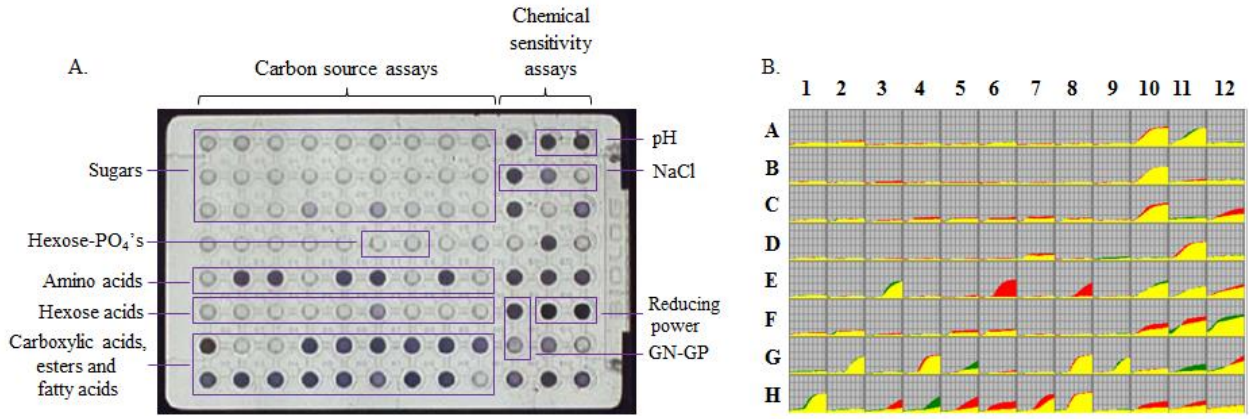


Figure 1.3: A. Live image of a GENIII microplate inoculated with an *Acinetobacter* isolate after 36 hours of incubation at 33 °C. **B.** GENIII kinetic profile comparison of *Acinetobacter* isolates from clinical (red) versus environmental (green) origin. The overlay of two curves is shown in yellow.

Using Biolog's Gram negative (GN) microplates, which are designed for identification of Gram-negative bacteria and contain carbon substrates appropriate for this group of microorganisms, Bernards and colleagues (1995) performed a phenotypic characterization of 127 *Acinetobacter* strains representing the genomic species 1 to 14 *sensu* Bouvet and Grimont (1989) (*A. calcoaceticus*, *A. baumannii*, *A. pittii*, *A. haemolyticus*, *A. junii*, *A. johnsonii*, *A. lwoffii*, *A. bereziniae*, *A. guillouiae*, *A. radioresistens* and *A. nosocomialis*). Out of the 95 carbon sources, 34 organic substrates were found to be useful for species differentiation. The majority of these carbon sources were organic acids, amino acids and a few sugars. Tween 40 and Tween 80 were used by all *Acinetobacter* strains tested and most strains were able to use methylpyruvate and acetic acid as a sole carbon source. On the other hand, 16 carbon sources could not be used by any of the strains tested. These carbon sources included several sugars, sugar amino acid derivatives and sugar alcohols. The study was performed by endpoint measurements using Biolog's microplate reader. Using Biolog's PM technology Mara and colleagues (2012) tested a set of *A. venetianus* strains for utilization of a variety of carbon and nitrogen sources (PM1-PM3), and found that they were unable to use carbohydrates (except for dextrin), but consumed organic acids and amino acids.. Additionally, they showed that *A. venetianus* uses inorganic nitrogen and several amino acids as a nitrogen source, but that they did not use dipeptides. Using the full set of PM microplates (PM1-PM20), Peleg and colleagues (2012) concluded that *A. baumannii* is metabolically more active using peptide nitrogen sources and is more tolerant to pH stress than *A. calcoaceticus*, *A. nosocomialis* and *A. pittii*. On the other hand, *A. baumannii* and *A. pittii* had a reduced ability to utilise most of the phosphorus and sulphur sources (Peleg et al. 2012). In a study of different *Acinetobacter* strains isolated from mosquitoes, it was shown that although reference strains were able to utilize a broader range of carbon sources, some carbon sources (i.e. α-keto valeric acid and glycine, which are blood related compounds) were only used by *A. baumannii* isolates from mosquitoes. Additionally, it was found that 4-hydroxybenzoic acid and xylose (which are common plant related

compounds) were only used by *A johnsonii* isolates from mosquitoes (Minard et al. 2013). Although further research is needed using more strains, these findings may suggest that carbon source preferences within *Acinetobacter* species may be driven by habitat-related factors, and that subpopulations have been evolved that are specifically adapted to thrive in certain habitats (Minard et al. 2013). Additionally, it is clear from the studies mentioned above that these phenotypic assays not only contributed in describing novel species, but also demonstrated that *Acinetobacter* spp. are highly metabolically versatile and are able to use a wide variety of substrates, which may help them thriving in diverse habitats.

1.2.3 Adaptation to thrive in diverse environments

Acinetobacter species are found in diverse natural and man-made environments. Their success of survival in these environment can be attributed to different features. First, *Acinetobacter* spp. are metabolically versatile and can degrade a wide variety of organic compounds or detoxify heavy metals as discussed above. Further, they can grow across a wide range of pH (4 - 10) and temperatures (1 - 44 °C) and are able to survive on dry surfaces for extended periods (Bergogne-Bérézin and Towner, 1996; Jawad et al. 1996). Additionally, they are capable of phosphate accumulation as polyphosphates which serve as a phosphorus and energy reserve and attributes to their responses to stresses and stringencies and has also been linked to the virulence of pathogens and the precipitation of metals (Boswell et al. 2001; Lloyd and Lovley, 2001; Rao et al. 2009). In addition, they have the ability to withstand osmotic pressure induced by salts (including heavy metals) as well as sugar-rich environments (Yavankar et al. 2007; Akbulut et al. 2014; Álvarez-Pérez et al. 2019). Finally, and importantly for this study, they have several features that attribute to their survival and persistence in drinking water. For example, they are capable of tolerating chlorine levels typically applied during drinking water production (Karumathil et al. 2014). Additionally, they are able to produce the siderophore acinetobactin which are high-affinity iron chelators and aid in the survival in iron limiting habitats (Lee et al. 2017). They are also known to produce biofilms in which they can survive for a long time even on dry surfaces and their resistance to disinfection increases in case of multispecies biofilms (Chaves Simões et al. 2010; Espinal et al. 2012; Biswas and Mettlach, 2019). It has been shown that biofilm formation of *Acinetobacter calcoaceticus* was not only autoaggregated but also coaggregation with other genera (*Burkholderia cepacia*, *Mycobacterium mucogenicum*, *Sphingomonas capsulata*, and *Staphylococcus* sp.) occurred (Chaves Simões et al. 2008). Finally, there is a strong interest in the genus, due to the ease with which clinically relevant *Acinetobacter* spp. have developed resistance to antibiotics. Globally it has been noticed that *Acinetobacter* strains have increasingly emerged with resistance to multiple classes of antibiotics, thereby representing an additional public health risk (Van Looveren et al. 2004; Zhang et al. 2009; Roca et al. 2012, Harding et al. 2018). Antibiotic resistance has been reported for β-lactams, aminoglycosides,

quinolones and other antibiotics such as tetracycline, colistin, chloramphenicol and trimethoprim–sulphamethoxazole (Henwood et al. 2002; Van Looveren et al. 2004; Qureshi et al. 2015).

1.2.4 Clinical relevance of Acinetobacter *species*

Acinetobacter species can cause both community acquired and hospital acquired infections and have therefore attracted lots of interest from both the scientific community and governmental organisations. Community acquired infections are increasingly reported in tropical countries with a hot and humid climate (Joly-Guillou, 2005), while hospital acquired infections are globally a major concern (Peleg et al. 2008; Visca et al. 2011). Of high importance is its multidrug resistance (MDR), for which WHO has ranked *Acinetobacter baumannii* as one of the bacteria with the highest priority for new development of antibiotics (WHO, 2017). Likewise, *A. baumannii* is considered a very serious threat according to the Center of Disease Control and Prevention (CDC, https://www.cdc.gov/drugresistance/biggest_threats.html#aci). *Acinetobacter baumannii* is the most common *Acinetobacter* species to cause infections, followed by other members of the Acb complex and *A. lwoffii* (Cosgaya et al. 2016; Wong et al. 2017). Yet, several other *Acinetobacter* species are also known to cause or contribute to infections (see Table 1.1, several species have been isolated from humans). In most developed non-tropical countries these species are opportunistic pathogens in hospital environments such as intensive care units or are able to infect immunocompromised humans. In an eight year survey in a Dutch university hospital a total of 359 *Acinetobacter* strains were obtained from clinical samples and identified to the species level by AFLP analysis (van den Broek et al. 2009). *Acinetobacter baumannii* was the most frequently isolated encountered species (27 % of the strains), followed by *A. pittii* (26 %), *A. lwoffii* (11 %), *A. johnsonii* (4 %), *A. ursingii* (4 %), *A. junii* (3 %), *A. nosocomialis* (3 %), *A. calcoaceticus* (2 %), *A. beijerinckii* (1 %), *A. bereziniae* (1 %), *A. guillouiae* (1 %) and *A. radioresistens* (1 %). Other species encompassed less than 1 % of the strains isolated, and included *A. courvalinii*, *A. gyllenbergii*, *A. haemolyticus*, *A. parvus*, *A. seifertii*, *A. variabilis*, 'genomic species 16' and 'between 1 and 3' as well as 12 unidentified strains. Furthermore, in a large study in the United Kingdom and the Republic of Ireland a total of 690 non-*baumannii* acinetobacters were collected in a 20 month period from about 135 hospitals and were identified based on their *rpoB* gene sequence and PFGE fingerprints (Turton et al. 2010). The most frequently isolated non-*baumannii* species were *A. lwoffii* (8.8 %), followed by *A. ursingii* (4.0 %), *A. pittii* (1.7 %), *A. johnsonii* (1.6 %), *A. parvus* (1.3 %), *A. colistiniresistens* (0.9 %), *A. radioresistens* (0.6 %), *A. guillouiae* (0.4 %), *A. calcoaceticus* (0.4 %), *A. nosocomialis* (0.3 %), *A. haemolyticus* (0.3 %), *A. junii* (0.3 %), *A. beijerinckii* (0.1 %), *A. bereziniae* (0.1 %), *A. gyllenbergii* (0.1 %), *A. schindleri* (0.1 %) and 'genomic species 16' (0.1 %) as well as a three unassigned isolates. Additionally, in a burn wound centre of the Belgian military hospital 157 *Acinetobacter* isolates were collected during a 52 month period, among which the majority was identified as *A. baumannii* (83.4 %), followed by *A. pittii* (9.6 %) and *A. nosocomialis* (4.5 %) (De Vos

et al. 2016). Other isolates were identified as *A. calcoaceticus*, *A. courvalinii* and *A. dijkshoorniae* (= *A. lactucae*). Although the majority of clinical studies indicate that *A. baumannii* is the most frequently isolated species, recently several studies have indicated that non-*baumannii* acinetobacters are increasingly found in clinical settings (Karah et al. 2011; Pailhoriès et al. 2018). The increased prevalence of non-*baumannii* acinetobacters has been attributed to better identification methods (molecular tools) and the spread of antibiotic resistance genes among acinetobacters (Park et al. 2010; Cayô et al. 2018), by which they have become resistant against commonly used antibiotics.

1.3 Thesis objectives and outline

Previous studies have shown the importance of understanding the bacterial community composition in drinking water production and distribution systems for the delivery of clean, safe drinking water. However, several topics, including, amongst others, the impact of the source water and the impact of different treatment steps on the delivered product are still poorly understood. Further, as mentioned above, *Acinetobacter* species have been commonly encountered within these systems. Yet, there is a lack of knowledge which species are of importance and whether they display different phenotypic traits in comparison to clinical isolates of the same species. Moreover, it remains unknown if these phenotypic traits can predict the phylogenetic distance between *Acinetobacter* species. Therefore, the main goal of this thesis was to assess the bacterial community composition of drinking water production and distribution systems, emphasizing *Acinetobacter* spp. More specifically, we aimed to:

(i) Investigate the bacterial diversity in different water samples from the production and distribution chain of several drinking water companies using surface water or groundwater (**Chapter II**);

(ii) Investigate and compare the bacterial community shifts in two parallel multi-step drinking water treatment processes using the same source of water (**Chapter III**). We hypothesized that bacterial community composition was strongly determined by the different treatment steps;

(iii) Assess inter- and intraspecific genotypic and phenotypic variation within *Acinetobacter* species isolated from environmental samples (particularly water) and their counterparts isolated from clinical samples (**Chapter IV**);

(iv) Assess the presence of a phylogenetic signal in phenotypic traits related to carbon source in assimilation and chemical sensitivity in *Acinetobacter* species (**Chapter V**).

As such, whereas the first part of this PhD study primarily focused on molecular/microbial ecology (study of microbial communities in drinking water), the second part was more microbiological, using *Acinetobacter* spp. as model organisms for addressing relevant ecological/evolutionary questions. In **Chapter II**, using high-throughput sequencing of partial 16S rRNA gene amplicons the bacterial community composition was investigated in water samples from the production and distribution chain

of thirteen drinking water production and distribution systems from Flanders (Belgium) that use surface water or groundwater as source water. Water samples were collected over two seasons from the source water, the processed drinking water within the production facility and out of the tap in houses along its distribution network. Observed taxa were related with the type of water and season using an indicator species analysis (ISA). For the next chapter (**Chapter III**), we selected a drinking water production system consisting of a parallel treatment process starting from the same source of water (surface water) to investigate and compare the bacterial community shifts (measured by 16S rRNA gene amplicon sequencing) caused by different water treatments. Further, we investigated the presence and abundance of *Acinetobacter* at each step of the treatment chain using both classical plating and culture-independent quantitative real-time PCR. Finally, we studied the importance of the physicochemical characteristics of the drinking water on the bacterial community composition in the water delivered to the end user (household tap water). Next, *Acinetobacter* isolates obtained in Chapter III belonging to clinically relevant species were selected for genotypic and phenotypic comparison with a set of *Acinetobacter* strains belonging to the same species but isolated from humans or a clinical setting (**Chapter IV**). Isolates were phenotyped using Biolog's GENIII identification microplate, which analyzes a microorganism in 94 phenotypic tests, including 71 carbon source utilization assays and 23 chemical sensitivity assays, and antibiotic resistance profiles. Further, isolates were genotyped by partial sequencing of the *rpoB* gene. A Mantel test was performed to assess correlations between phenotypes and *rpoB* gene sequence similarity. Finally, in a last experimental chapter (**Chapter V**) we assessed whether phylogenetic distance of *Acinetobacter* species is related to variation in carbon source assimilation and chemical sensitivity as determined by Biolog GENIII phenotyping. To perform the study, a comprehensive collection of isolates from *Acinetobacter* species with validly published names, genomic species and of an as-yet unknown taxonomic status was used. Initially, we estimated the strength and significance of the phylogenetic signal of each trait across phylogenetic reconstructions based on partial *rpoB* sequences and the core genome of diverse *Acinetobacter* species. Furthermore, we tested whether phylogenetic distance was a good predictor of trait differentiation for this bacterial group by Mantel test analysis. Finally, evolutionary model fitting was used to determine if the data for each phenotypic character was consistent with a phylogenetic or an essentially random model of trait distribution. In **Chapter VI**, the main conclusions of this PhD are summarized and an outlook is given for future research.

Chapter II: Characterization of the bacterial community composition in water of drinking water production and distribution systems in Flanders, Belgium[1]

[1] This chapter is based on the following publication:
Van Assche, A., Crauwels, S., De Brabanter, J., Willems, K.A. and B. Lievens. 2018. Characterization of the bacterial community composition in water of drinking water production and distribution systems in Flanders, Belgium. MicrobiologyOpen. 8:e726.

2.1 Introduction

The delivery of safe, clean drinking water is important for public health. The quality of the final drinking water is influenced by its chemical and microbial composition. Especially microbial growth in drinking water can be problematic as it may result in the multiplication and rapid spread of opportunistic pathogens (van der Kooij et al. 1982; LeChevallier et al. 1996). Additionally, it may lead to aesthetic problems such as deteriorated taste and odor, and technical problems such as corrosion of the pipe material (Hoehn, 1988; Christensen et al. 2011; Camper, 2013).

Traditionally, microbiological characterization of drinking water is specified in national and international norms and rely on culture-based detection methods such as heterotrophic plate counts and counts of fecal indicator bacteria (i.e. *Escherichia coli*, coliforms and enterococci) (European Directive 98/83/EG). Although these classical plating methods have greatly helped evaluating the microbial quality of drinking water (Lee et al. 2003; Martiny et al. 2005; September et al. 2007; Inomata et al. 2009), not all microorganisms are culturable under standard laboratory conditions (Byrd et al. 1991), by which important species may be overlooked (Liu et al. 2008). Culture-independent DNA-based methods such as 454 pyrosequencing or Illumina MiSeq sequencing of ribosomal RNA genes overcome these limitations and allow in-depth analysis of entire microbial community composition with an unprecedented level of resolution (Margulies et al. 2005; Caporaso et al. 2012). Therefore, these technologies are increasingly used to study drinking water microbial community composition and associated biofilms, and have greatly contributed to our understanding of the true diversity of these bacterial community compositions (e.g., Hong et al. 2010; Navarro-Noya et al. 2013; Prest et al. 2014; Roeselers et al. 2015; Wu et al. 2015).

Several recent studies have focused on how the microbial community composition in drinking water is shaped by different drinking water production steps and found a significant impact of the treatment method (Pinto et al. 2012; Lautenschlager et al. 2014; Shaw et al. 2015; Li et al. 2017; Ma et al. 2017; Xu et al. 2017; and Oh et al. 2018). Further, recent studies have investigated the spatial and/or long-term temporal variation in bacterial community composition from source water to tap water (Pinto et al. 2014; Roeselers et al. 2015; Hull et al. 2017). Some studies indicated a major impact of seasonal effects on the bacterial community composition (Pinto et al, 2014), while others found the treatment method(s) as most important factor (Pinto et al. 2012; Roeselers et al. 2015; Ma et al. 2017). Nevertheless, still little is known about the impact of the source water on the bacterial community composition in the final drinking water. Therefore, the goal of this study was to assess the bacterial diversity of different water samples from the production and distribution chain of a number of drinking water production and distribution systems (DWPDS) from Flanders (Belgium) that use either surface water or groundwater as source water. Additionally, we explored potential differences in the bacterial community composition between two different seasons. Concomitantly, we also identified the key taxa depending on the type of water and season using an indicator species analysis.

2.2 Material and methods

2.2.1 Study samples

In total 41 water samples were collected from 13 DWPDS distributed across Flanders (Belgium). Among these, six DWPDS use surface water (SW) as their source water, while seven use groundwater (GW). Six DWPDS were sampled in April 2013 (two using SW; four using GW), six in November 2013 (three using SW; three using GW), and one (using SW) in April and November 2013 (Table S2.1, Supporting Information). For each DWPDS the source water, the processed water (PW) (immediately taken after the purification process) and the household tap water (HTW) (water delivered to the consumer) were sampled. As a result, samples represented a diverse collection of different water types, including groundwater; surface water, processed water originating from groundwater (PWg), processed water originating from surface water (PWs), household tap water originating from groundwater (HTWg) and household tap water originating from surface water (HTWs). For DWPDS 'E6' the household tap water was not included as this was also supplied with drinking water from another DWPDS (Table S2.1, Supporting Information). Due to confidentiality reasons, information about the water treatment process steps was not provided by the DWPDS surveyed. At each sampling point, after letting running a few liters of water away, 2 L water was collected under aseptic conditions in a sterile bottle, stored in an ice cooled container for transport and further stored at 4 °C prior to analysis (maximum within 1 day after sampling).

2.2.2 DNA extraction, PCR amplification and 454 amplicon pyrosequencing

Following filtration of 2 L water over a 0.45-µm filter (mixed sterile cellulose ester filter (Millipore, Billerica, Massachusetts, USA)), genomic DNA was extracted using the phenol-chloroform extraction method described in Lievens et al. (2003) using the filter as starting material. Obtained DNA was subjected to PCR amplification and 454 pyrosequencing of the 16S ribosomal RNA (rRNA) gene. More specifically, an amplicon library was created using the primer combination 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACVSGGGTATCTAAT-3'), generating amplicons which cover the prokaryotic (bacterial and archaeal) hypervariable V4 region of the 16S rRNA gene (Bates et al. 2011). This broad spectrum primer combination has been commonly used in diverse metagenomics studies, including water research (Wang et al. 2014; Ng et al. 2015; Wu et al. 2015), and has been recommended by the Earth Microbiome Project to characterize microbial communities (Soergel, et al. 2012). Unfortunately, current limitations in second generation sequencers only allow for sequencing of short fragments (typically ~250 bp), by which only part of the 16S rRNA gene can be exploited as a taxonomic marker. This constraint limits the taxonomic resolution to which

the reads can be classified, typically only to the family- or genus-level. Furthermore, taxonomic resolution varies for different groups of bacteria when using different portions of the 16S rRNA gene (Schloss 2010). For example, whereas V2-V3 have been shown to provide higher resolution for lower rank taxa (genera or species) in some studies (Bukin et al. 2019), other regions including V4 may be better suited for particular bacterial groups (Schloss 2010). Advantageously, the use of a standard primer pair also allows fair comparisons between different studies using the same primers. 'Fusion' primers, required for the 454 pyrosequencing process, were designed according to the guidelines for 454 GS-FLX Titanium Lib-L sequencing and contained the Roche 454 pyrosequencing adapters and a sample-specific multiplex identifier (MID) sequence in between the adapter and the forward primer for sample-specific sequence tracking. A T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) was used for PCR amplification. The total reaction volume was 20 µL and contained 1.0 µL 10x diluted genomic DNA, 1.5 µL dNTP mixture (2 mM stock; Invitrogen, Carlsbad, CA, USA), 0.5 µL of each primer (20 µM stock), 2.0 µL 10x Titanium *Taq* PCR buffer, 0.4 µL Titanium *Taq* DNA polymerase (Clontech Laboratories, Palo Alto, CA, USA) and 14.1 µL nuclease-free water. The following PCR conditions were used: initial denaturation of 2 minutes at 94 °C, followed by 30 cycles of 45 s at 94 °C, 45 s at 59 °C, and 1 min at 72 °C, followed by a final extension phase of 10 min at 72 °C. Following agarose gel electrophoresis, amplicons of the expected size range were excised and extracted from the gel using the QIAquick gel extraction kit, according to the manufacturer's instructions (Qiagen, Hilden, Germany). Purified dsDNA amplicons were quantified using a Qubit 2.0 fluorometer and the high-sensitivity DNA reagent kit (Invitrogen, Carlsbad, CA, USA). Next, all samples were diluted to equimolar concentrations and an amplicon library containing $1.00 * 10^9$ molecules/µL per sample was prepared. A final quality check was done on an Agilent Bioanalyzer 2100 with high-sensitivity chip (Agilent Technologies, Waldbronn, Germany), and the library was sequenced using the Roche GS-FLX instrument with Titanium chemistry according to manufacturer's instructions (Roche Applied Science, Mannheim, Germany).

Pyrosequencing yielded a total of 1,014,047 reads. Sequences were assigned to the appropriate sample based on their barcodes and primer sequences, allowing zero discrepancies, and were subsequently trimmed from the fusion primer sequence using a custom Python script implemented within the USEARCH v.8 analysis pipeline (Edgar 2013) (data deposited in the Sequence Read Archive under BioProject accession PRJNA479747). Subsequently, reads with a total expected error threshold above 0.5 for all bases were discarded, so that the most probable number of errors was zero for all sequences that remained in the dataset. Next, remaining sequences (180,562 out of 230,016, after quality filtering) were trimmed to 250 bp and subsampled (so-called "rarefied") to the least number of sequences per sample obtained (i.e. 850 sequences for each sample). Remaining sequences were then grouped into species-level operational taxonomic units (OTUs) based on a 3 % sequence dissimilarity cut-off while discarding chimeric sequences using the UPARSE greedy algorithm implemented in USEARCH (Edgar 2013) as well as global singletons (i.e. OTUs representing only a single sequence in the entire dataset)

(Rosselló-Mòra, 2011; Waud et al. 2014; Brown et al. 2015). Next, OTUs were assigned taxonomic identities using the "classify.seqs" command in Mothur (v. 1.36.1) (Schloss et al. 2009) using the Silva taxonomy database (Quast et al. 2013). Taxonomic assignments up to the genus level were considered reliable when bootstrap confidence values exceeded 80.

2.2.3 Data analysis

OTU richness, the Ace richness estimator, Pielou's evenness and Shannon diversity were calculated using Mothur (v. 1.36.1) (Schloss et al. 2009), all representing so-called "α diversity parameters". Whereas OTU richness represents the observed number of OTUs in a sample, the Ace richness estimator is an abundance-based coverage estimator predicting the true number of OTUs in the samples (Gotelli and Colwell, 2011). Pielou's evenness refers to how close in numbers each OTU in a sample is, and varies between zero and one. The index will be closer to one if all members are equally present in the investigated sample. Vice verca, it will be closer to zero if the community is dominated by one or a few OTUs. The Shannon diversity index is another index that is commonly used to characterize species diversity and takes into account OTU richness and evenness. Differences in these parameters were assessed using the 'aov' function in R (R Development Core Team, 2015). Similarities between the bacterial community composition of the different water types studied (GW, SW, PWg, PWs, HTWg and HTWs) were quantified using the ANOSIM (ANalysis Of SIMilarities) and ADONIS (i.e. a permutational multivariate analysis of variance using distance matrices) functions of the Vegan package (v. 2.4-1) (Oksanen, 2013). In both cases, the Bray-Curtis distance matrix (abundance data) was used. The same analyses were performed to assess seasonal effects on the bacterial community composition. Additionally, rarefaction curves, a non-metric multidimensional scaling (NMDS) plot and a hierarchically clustered heatmap were created with the Vegan (v. 2.4-1) and ggplot2 (v. 2.1.1) packages in R. Boxplots were generated using the boxplot function in R. Additionally, an indicator species analysis was performed for each type of water and season using the Indicspecies package (v. 1.7-1) in R (De Cáceres, 2013; R Development Core Team, 2015). For all samples originating from the same type of source water core bacteria were determined, i.e. OTUs that occurred in at least one sample of the source water, processed water and tap water. Venn diagrams showing the distribution of the different OTUs over different subgroups were constructed using the VennDiagram package (v. 1.6.19) for R (Chen and Boutros, 2011). Finally, given the fact that a relatively huge proportion of sequences was identified as *Acinetobacter* and that the 16S rRNA gene is known to not vary greatly between *Acinetobacter* species (La Scola et al. 2006), OTUs corresponding to the genus *Acinetobacter* were further analyzed in order to improve identification. More specifically, all unique sequences belonging to the *Acinetobacter* OTUs were blasted against a custom database containing the 16S rRNA gene sequences of the type strains of all *Acinetobacter* species with validly published names (at the time of analysis 50 species) and a number of *Acinetobacter* genomic species, i.e. species that have yet to receive

a Latin binomial name but that are genetically different from the formerly described *Acinetobacter* species (Bouvet and Grimont, 1986; Tjernberg and Ursing, 1989). Additionally, to visualize phylogenetic relationships a maximum likelihood phylogenetic tree was constructed based on these sequences using MEGA 5.10 (Kumar et al. 2008).

2.3 Results

Following rarefying of all samples to 850 sequences per sample, a total of 1,570 OTUs were recovered, ranging from a minimum of 58 OTUs per sample to a maximum of 235 OTUs per sample (Table S2.1, Supporting Information). Based on the Ace estimator, the mean sampling coverage was 69.4 % (range between 50.0 % and 100.0 %) (Table S2.1, Supporting Information), suggesting that the most abundant bacterial community members were covered, as can also be observed from the rarefaction curves (Fig. S2.1, Supporting Information). No significant differences ($p < 0.05$) could be observed between the number of OTUs per sample between the different water types (groundwater, surface water, processed water originated from groundwater or surface water, and household tap water originated from groundwater or surface water) (Fig. 2.1; Table S2.2, Supporting Information). Likewise, no significant differences were found in the calculated diversity indices (Fig. 2.1; Table S2.2, Supporting Information). By contrast, significant differences in OTU richness, Ace and Shannon diversity were observed between the two sampling periods (i.e. April and November; Fig. 2.1; Table S2.2, Supporting Information), but not for the evenness ($p > 0.05$). Significant differences ($p < 0.05$) were also found when the communities of the different water types were analyzed using ANOSIM and ADONIS (Table 2.1). Greatest differences were observed between the microbial community composition from surface versus groundwater ($p < 0.001$ for both ANOSIM and ADONIS), and the least differences were observed between the bacterial community composition of household tap water originating from groundwater versus household tap water originating from surface water ($p = 0.069$ and 0.040 for ANOSIM and ADONIS, respectively; Table 2.1). When seasonal effects were evaluated, no substantial differences were observed within the different water types (p value ranging from 0.109 to 0.811 for ANOSIM, and from 0.069 to 0.500 for ADONIS; Table 2.1), except for the surface water and the processed water originating from surface water ($p \leq 0.05$; Table 2.1).

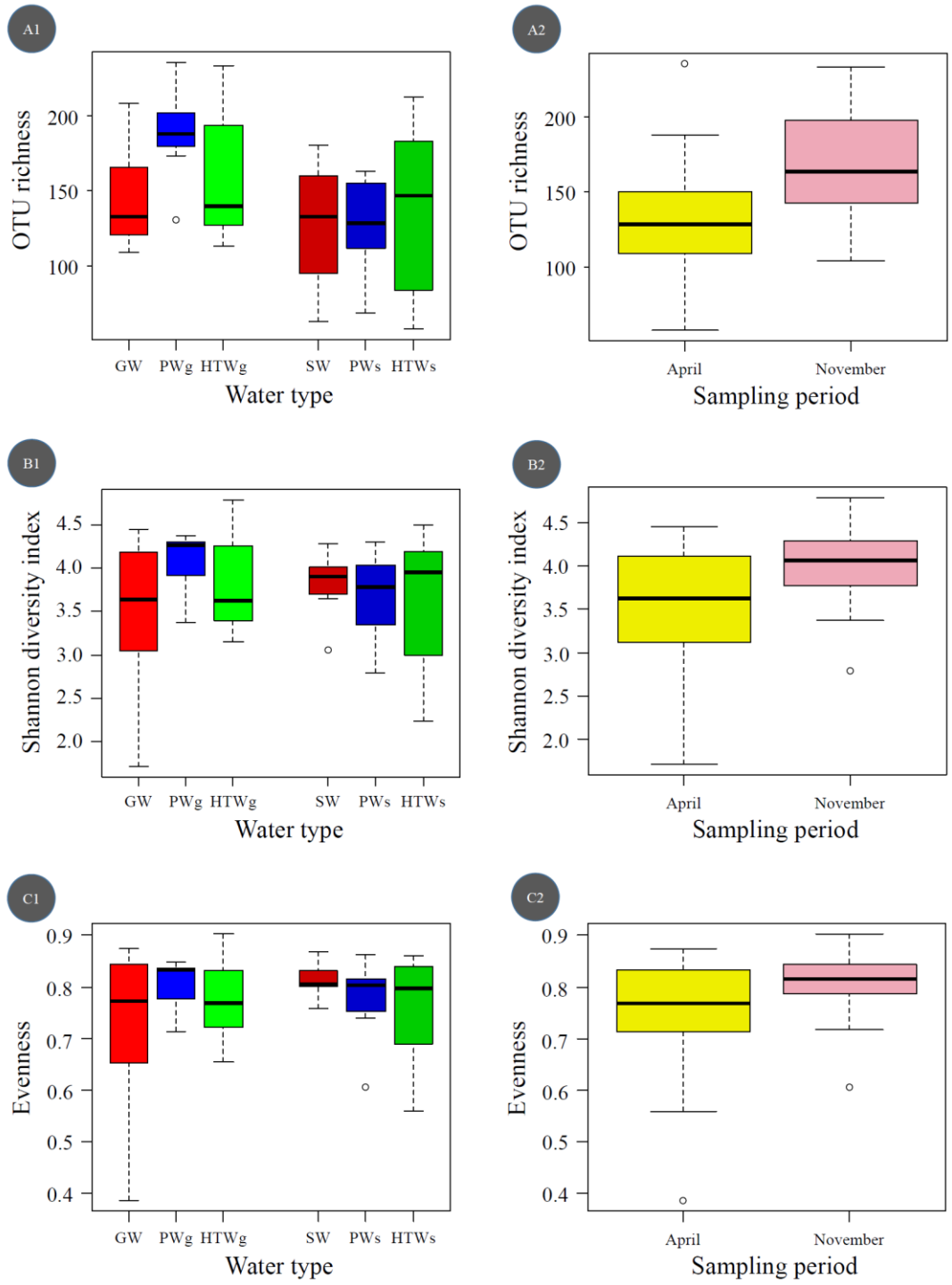


Figure 2.1: Boxplot representation of OTU richness (**A**), Shannon diversity (**B**) and Pielou's evenness (**C**) of the bacterial community composition in the water samples investigated in this study. Water samples were grouped based on water type (A1, B1 and C1) and sampling period (A2, B2 and C2). The boxplots show the upper and lower quartiles; the whiskers indicate variability outside the upper and lower quartiles which is no more than 1.5 times the interquartile range. Further, the median is plotted as a thick black line. GW, groundwater (n = 7); PWg, processed water produced from groundwater (n = 7); HTWg, household tap water processed from groundwater (n = 7); SW, surface water (n = 7); PWs, processed water produced from surface water (n = 7); HTWs, household tap water processed from surface water (n = 6); April (n = 21); November (n = 20).

Table 2.1: Values and significance scores of the ANOSIM and ADONIS functions.

Grouping of samples	ANOSIM			ADONIS		
	R	*p* value[b]		F	*p* value	
Overall comparison based on the different water types[a] (i.e. GW, SW, PW [g & s] and HTW [g & s])	0.356	0.001	***	1.970	0.001	***
Comparison of the different water types based on the source of the source water (i.e. groundwater versus surface water)						
Source water	0.643	0.001	***	3.589	0.001	***
Processed water	0.390	0.002	**	1.734	0.036	*
Household tap water	0.177	0.069	.	1.419	0.040	*
Comparison of the different sampling periods (i.e. April versus November)						
Groundwater	0.232	0.109		1.292	0.100	.
Surface water	0.694	0.026	*	1.994	0.036	*
Processed water (produced from GW)	0.185	0.144		1.255	0.069	.
Processed water (produced from SW)	0.676	0.050	*	2.053	0.032	*
Household tap water (produced from GW)	-0.157	0.811		1.062	0.335	
Household tap water (produced from SW)	-0.037	0.500		0.917	0.500	

[a] Different water types: GW, groundwater; SW, surface water; PW, processed water; and HTW, household tap water; g or s, originating from groundwater or surface water, respectively.
[b] Asterisks: *p* value 0 < *** < 0.001 < ** 0.01 < * < 0.1

Taxonomic assignment of the OTUs revealed the presence of 28 bacterial and archaeal phyla and 253 genera (Table S2.3, Supporting Information) with an officially published scientific name. *Proteobacteria* was the most abundant phylum detected (52.1 % of the total number of sequences), followed by *Actinobacteria* (12.6 %), and *Firmicutes* (6.9 %). Based on water type, analysis of variance indicated a significantly higher relative abundance of the phyla *Actinobacteria*, *Bacteroidetes* and *Verrucomicrobia* in the surface water ($p < 0.05$). Further, members of the phylum *Cyanobacteria* were more abundantly present in surface water and processed water originating from surface water (Fig. 2.2). Furthermore, relative abundance of the phyla *Firmicutes* and *Gemmatimonadetes* was higher in November than in April ($p < 0.05$). Analysis of variance also indicated a higher relative abundance of *Nitrospirae* in water samples from facilities using groundwater ($p < 0.05$) (Fig. S2.2, Supporting Information). Indeed, highest number of *Nitrospirae* sequences were observed in two production systems located in the province of Antwerp using groundwater (A1 and A2).

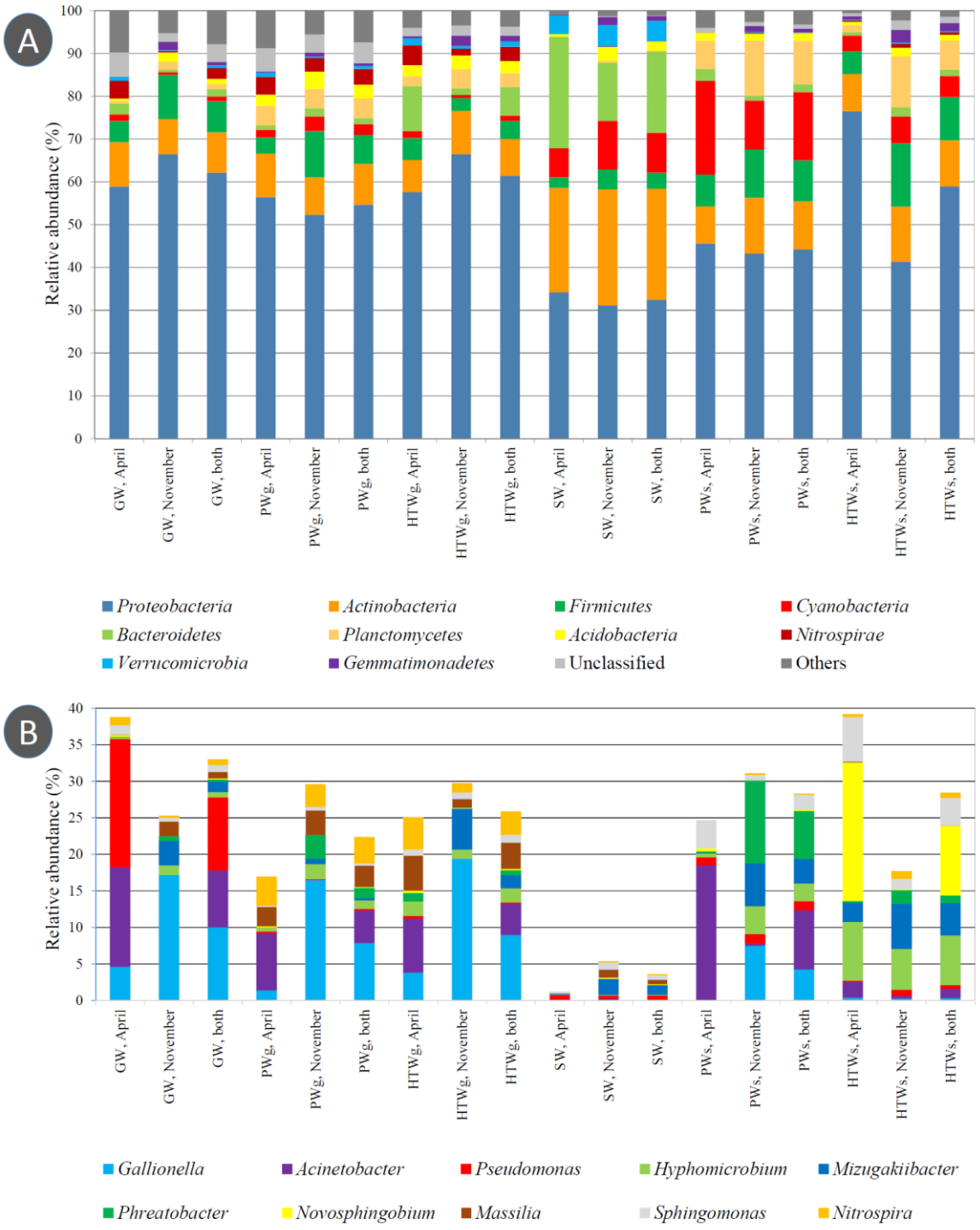


Figure 2.2: A: Relative abundance of bacterial phyla in the different water samples collected in April, November, and both sampling periods combined. Phyla representing less than 5 % of the sequences (in total) are grouped together as 'Others'. **B:** Relative abundance of the ten most abundant genera in the different water samples collected in April, November, and both sampling periods combined. Numbers of samples included are reported between brackets. GW, groundwater (n = 7); PWg, processed water produced from groundwater (n = 7); HTWg, household tap water processed from groundwater (n = 7); SW, surface water (n = 7); PWs, processed water produced from surface water (n = 7); HTWs, household tap water processed from surface water (n = 6).

When zooming in at genus level, the 10 most abundant genera encountered in this study encompassed the genera *Gallionella* (5.4 % of all sequences recovered, *Proteobacteria*), *Acinetobacter* (4.4 %, *Proteobacteria*), *Pseudomonas* (2.2 %, *Proteobacteria*), *Hyphomicrobium* (2.1 %, *Proteobacteria*), *Mizugakiibacter* (2.1 %, *Proteobacteria*), *Phreatobacter* (1.7 %, *Proteobacteria*), *Novosphingobium* (1.5 %, *Proteobacteria*), *Massilia* (1.4 %, *Proteobacteria*), *Sphingomonas* (1.4 %, *Proteobacteria*), and *Nitrospira* (1.4 %, *Nitrospirae*) (Fig. 2.2). Whereas these genera were generally found in the different water types investigated, *Gallionella* and *Phreatobacter* species were not detected in any sample from the surface water. The NMDS ordination of the bacterial community composition revealed a clear clustering of the surface water samples, while samples from the other water types appeared scattered on the plot (Fig. 2.3), as can also be observed from the heatmap clustering shown in Fig. S2.3 (Supporting Information). The clustering of the different surface water samples indicates that the bacterial community composition of surface water is more similar to each other than to water samples of another origin, and are characterized by a specific microbial community composition. Indeed, indicator species analysis revealed as much as 63 OTUs as significant indicators for the surface water bacterial community composition (Table S2.4, Supporting Information). Most of these OTUs represented taxa belonging to the phylum of *Actinobacteria* (Table S2.4, Supporting Information). Furthermore, indicator species analysis revealed the presence of a number of unique OTUs within particular DWPDS (i.e. for DWPDS A2, B1, D1 and E5, indicating that these DWPDS are characterized by particular bacterial populations. Indicator species analysis also revealed 18 and 70 indicator OTUs for April and November, respectively (Table S2.5, Supporting Information).

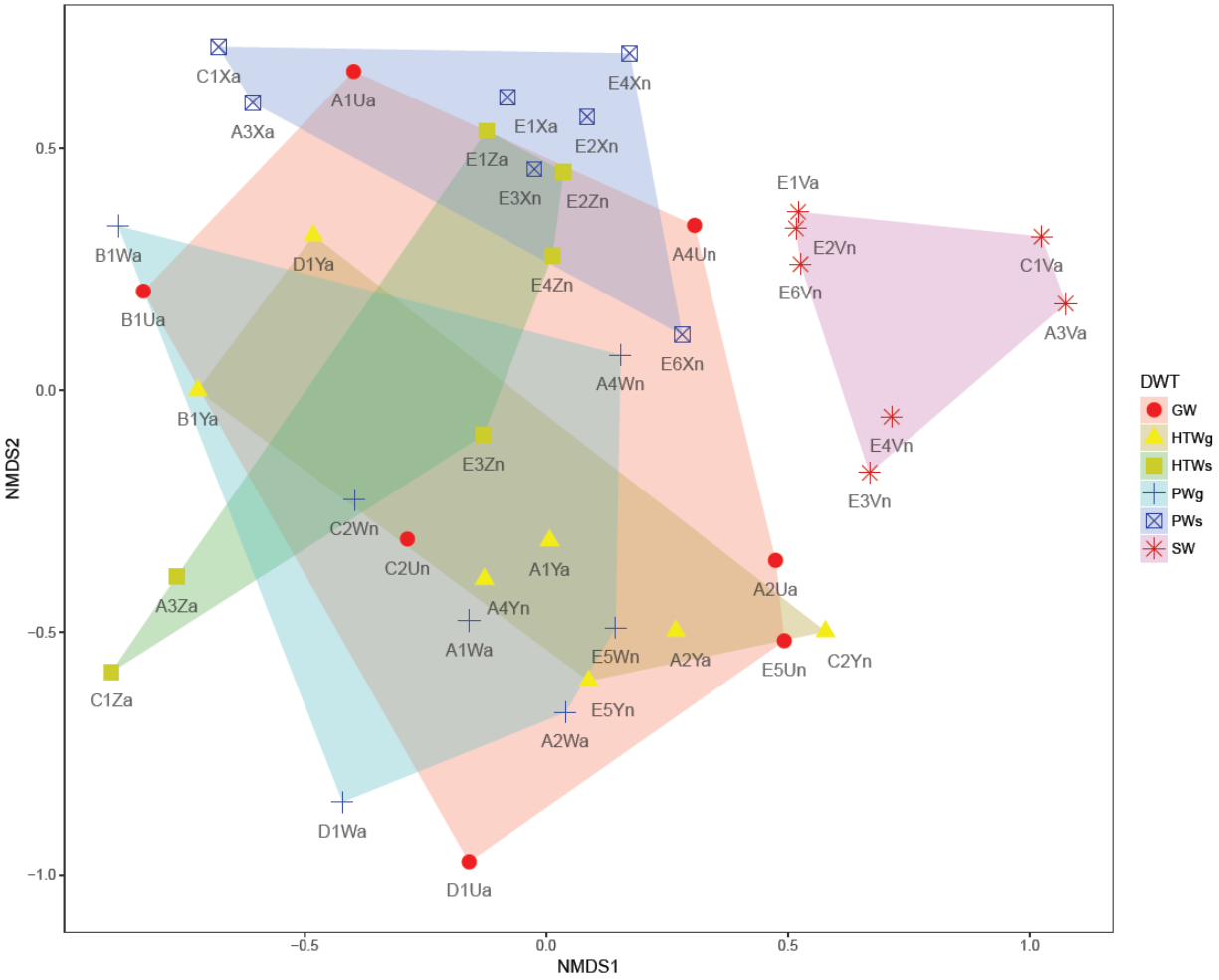


Figure 2.3: Non-metric dimensional scaling (NMDS) ordination plot of the bacterial community composition (stress value 0.242) of all water samples studied (based on Bray-Curtis distance matrix (abundance data)). GW, groundwater; PWg, processed water originating from groundwater; HTWg, household tap water originating from groundwater; SW, surface water; PWs, processed water originating from surface water; HTWs, household tap water originating from surface water. For more information about the studied samples the reader is referred to Table S1 (Supporting Information).

In order to evaluate differences in core OTUs and the OTU distribution between the samples originating from groundwater and those from surface water a Venn diagram was generated (Fig. 2.4). In total 1,244 and 894 OTUs out of the 1,570 OTUs were present in the subgroup containing the groundwater-derived samples and the subgroup containing the surface water-derived samples, respectively. For the first set a core community of 302 bacterial OTUs was observed, representing 24.3 and 70.4 % of the OTUs and sequences, respectively. For the surface water related samples, the core community consisted of 117 OTUs, representing 13.1 and 38.8 % of the OTUs and sequences, respectively. Overall the core community of groundwater-related samples was represented by 18 different bacterial phyla, while the core community of surface water-related samples was represented by 13 phyla. In both cases *Proteobacteria* was the most abundant phylum corresponding to 68.3 and 52.2 % of the core community sequences for groundwater- and surface water-related samples, respectively. Further, the core community of the groundwater-related samples mainly consisted of

Actinobacteria (10.1 %), *Firmicutes* (6.9 %), *Nitrospirae* (3.9 %) and *Acidobacteria* (1.8 %), together with the *Proteobacteria* covering over 90 percent of the core community sequences. For the surface water-related core community, aside from *Proteobacteria* (52.2%), the majority of sequences belonged to *Actinobacteria* (18.9 %), *Firmicutes* (10.4 %), *Cyanobacteria* (4.8 %) and *Bacteroidetes* (4.4 %). Phyla and candidate phyla which were found in groundwater-related samples but not in surface water-related samples were Candidate division OP3, *Omnitrophica*, SHA-109, *Parcubacteria* and *Thaumarchaeota*. The candidate phylum WD272 was present in surface water-related samples but not in groundwater-related samples.

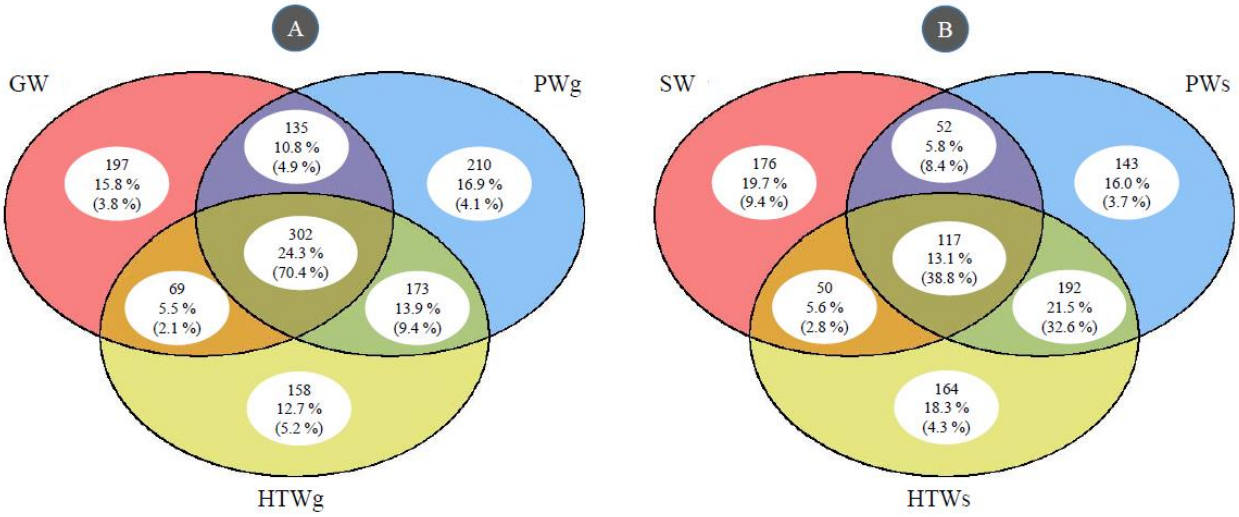


Figure 2.4: Venn diagrams illustrating the OTU distribution over different water types, including water samples related to production systems using groundwater as source water (GW (*n* = 7), PWg (*n* = 7) and HTWg (*n* = 7)) (**A**) and water samples related to production systems using surface water as source water (SW (*n* = 7), PWs (*n* = 7) and HTWs (*n* = 6)) (**B**). When an OTU occurred in at least one sample of each of the subgroups of water types, it was put in the intersection of the groups. The numbers within the Venn diagrams represent: top, number of OTUs within the subset; middle: percentage of OTUs representing the number of OTUs within the subgroup; and bottom: percentage of sequences representing the OTUs within the subgroup. GW, groundwater; PWg, processed water originating from groundwater; HTWg, household tap water originating from groundwater; SW, surface water; PWs, processed water originating from surface water; HTWs, household tap water originating from surface water.

In general, members of the *Acinetobacter* genus were abundantly found in the water samples studied, reaching read abundances of up to 47.5 % for the groundwater sample B1Ua. More particularly, *Acinetobacter* was the most abundant bacterium in several processed water samples taken in April (A3Xa, B1Wa, C1Xa, and E1Xa). Additionally, it was also the most abundant genus in the groundwater sample B1Ua and sample D1Ya, a household tap water sample taken in April (Table S2.3, Supporting Information). Strikingly, whereas *Acinetobacter* was abundantly present in the processed water samples of April, the bacterium was not detected in the corresponding surface water samples (Fig 2.2; Table S2.3, Supporting Information). In total, three OTUs were associated with *Acinetobacter* (OTU 1, 293, and 1434; Table S3, Supporting Information). OTU 1, which represented the most abundant OTU in this study (4.34 % of all sequences studied) was found in all water types investigated with the exception

of surface water (Table S2.3, Supporting Information). In contrast, OTU 293 was not detected in processed and household tap water originating from groundwater and OTU 1434 was not present in surface water neither in groundwater. When comparing and positioning the unique sequences of each of these OTUs in a phylogenetic tree containing the 16S rRNA gene sequences of the type strains of all known *Acinetobacter* species as well as a number of *Acinetobacter* genomic species, most OTU 1 sequences showed highest homology with *A. calcoaceticus*, *A. pitti*, *A. nosocomialis*, *A. seifertii*, *A. dijkshoorniae*, and the genomic species 'between 1 and 3', whereas a few sequences clustered a bit further away (Fig. S2.4, Supporting Information). Most of the unique sequences of OTU 293 and OTU 1434 clustered closely with the type strain of *A. johnsonii*. For OTU 1434, a number of sequences showed highest homology with *A. baumannii*, known as an opportunistic pathogen in humans (Dijkshoorn et al. 2007; Antunes et al. 2014) (Fig. S2.4, Supporting Information). Further, a number of sequences were found clustering together with other *Acinetobacter* species (Fig. S2.4, Supporting Information), suggesting that in total many *Acinetobacter* species were found in the water samples investigated in this study.

2.4 Discussion

In order to support drinking water quality, there is a strong interest in the microbial community composition of drinking water and how the community changes depending on the source water, from the source water to the household tap water, and during the season. Whereas drinking water microbial community compositions have been classically studied using plating techniques (Payment et al. 1988; Kalmbach et al. 1997), here 454 amplicon pyrosequencing was used to investigate these questions.

In line with other studies (Pinto et al. 2012; Prest et al. 2014; Wu et al. 2015) phyla like *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Cyanobacteria*, *Bacteroidetes*, and *Nitrospirae* were commonly found in the water samples investigated. As also observed in this study, several studies have identified *Proteobacteria* as the most abundant phylum in aquatic environments within the drinking water production industry (Liu et al. 2014; El-Chakhtoura et al. 2015; Bautista-de los Santos et al. 2016; Vaz-Moreira et al. 2017; Zanacic et al. 2017). It is clear from our results that the bacterial community composition of surface water strongly differs from those of the other water types studied. Indeed, members of the phyla *Actinobacteria*, *Bacteroidetes* and *Verrumicrobia* were significantly more abundant in the surface water samples. Additionally, members of the phylum *Cyanobacteria* were abundantly present in surface water and processed water originating from surface water. Moreover, a huge number of OTUs could be identified as a robust indicator for surface water bacterial community composition, including (among several others) several OTUs belonging to the *Actinobacteria*. These observations were also confirmed by the core community analysis of groundwater and surface water related subcategories.

Interestingly, whereas significant differences in the bacterial community composition could be observed based on the source of the water at the early stages of the drinking water production and distribution chain, no major differences were found at the stage of the tap, indicating that in general water with a similar microbial composition is delivered irrespective of the water source (Pinto et al. 2012; Henne et al. 2014; Roeselers et al. 2015). A similar conclusion can be drawn when also different sampling periods were taken into account. Further, comparison of the two sampling periods indicated that especially the *Firmicutes* and *Gemmatimondetes* were more abundantly present in water samples of November versus April. Moreover, in total, species richness was found to be higher in November than in April. Nevertheless, significant differences based on the ANOSIM and ADONIS functions were only confirmed for samples from the surface water or processed water originating from surface water, reinforcing that seasonal changes have less impact on the bacterial community composition of water of DWPDS which use groundwater as source water instead of surface water. A main limitation of the current study is that only a limited set of samples was investigated. Therefore, in order to draw strong conclusions on how the bacterial community composition is influenced by the source of the water as well as by seasonal influences, further investigation is needed using more samples from different DWPDS sampled over a longer period of time. Further, it is reasonable to assume that also the different treatment steps applied within the different companies may have influenced the dynamics of the microbial community composition along the distribution system (Shaw et al. 2015; Xu et al. 2017).

Analyses performed at the genus level revealed the common presence of well-known aquatic bacterial genera such as *Gallionella, Acinetobacter, Pseudomonas, Novosphingobium, Nitrospira, Massilia, Sphingomonas,* and *Flavobacterium* (Allen et al. 2004; Berry et al. 2006; Gallego et al. 2006). The relatively newly described genera *Mizugakiibacter* and *Phreatobacter* completed the top 10 of most commonly found genera in this study. *Mizugakiibacter* was recently isolated and described from a sediment sample from a freshwater lake and contains one species to date (i.e. *Muzigakiibacter sediminis*, Kojima et al. 2014). Also *Phreatobacter* has been recently described as a novel genus based on a number of strains isolated from ultrapure water of a Hungarian power plant, and currently one species has been described within the genus (*Phreatobacter oligotrophus*, Tóth et al. 2014). Interestingly, *Acinetobacter* was one of the most abundant taxa encountered in this study, especially in April. In total, three *Acinetobacter* OTUs were identified, among which OTU 1, representing 4.34 % of all sequences recovered, was found to be a good indicator for samples taken in April. *Acinetobacter* are aerobic, non-motile, gram negative bacteria that are ubiquitous in the environment and have been identified in drinking water, sewage water, groundwater, dental lines, rivers, soil, human skin, vegetables, flowers and fruits, ponds and swamps (Baumann, 1968; Barbeau et al.; 1996; Guardabassi et al. 1999; Doughari et al. 2011; Álvarez-Pérez et al. 2013; Van Assche et al. 2017). Although *Acinetobacter* are not generally considered pathogenic, the *A. baumannii - A. calcoaceticus* complex is increasingly associated with nosocomial infections in compromised patients. *Acinetobacter* have been associated with several kind of infections including respiratory infections, wound infections, bacteremia, secondary meningitis, and

urinary infections (Dijkshoorn et al. 2007; Doughari et al. 2011; Visca et al. 2011). In immuno-compromised patients mortality rates can be as high as 64 % (García-Garmendia et al. 2001), especially because many *Acinetobacter* strains are multi-drug resistant (Narciso-da-Rocha et al. 2013). Therefore, the presence of *Acinetobacter* in drinking water requires a high level of alertness (Zhang et al. 2013). Phylogenetic analysis revealed that the *Acinetobacter* sequences retrieved in this study were closely related to multiple *Acinetobacter* spp., including the most clinically important species, i.e. *A. baumannii*. Therefore, future studies should focus on the isolation and further characterization (both genetically and phenotypically) of these drinking water associated acinetobacters, as well as on their clinical relevance in order to better understand the true relevance of this genus for the DWPDS industry.

Chapter III: Characterization of the bacterial community composition in a parallel drinking water production and distribution system, with an emphasis on *Acinetobacter* species

3.1 Introduction

Water treatment and disinfection are of utmost importance to guarantee its biological stability and to protect public health. Nevertheless, despite several advances in drinking water technologies (reviewed in van der Hoek et al. 2014), production of safe drinking water remains an important challenge for several reasons. These include, for example, contamination of the source water (Schuster et al. 2005), prevalence of emerging chemical contaminants (Wang et al. 2016), and occurrence of potential pathogens and increasing antibiotic resistance (Ashbolt et al. 2004; Xi et al. 2009).

Microorganisms play a dual role in drinking water quality and safety. On the one hand, several microorganisms are positive through biologically mediated chemical contaminant removal, most commonly implemented in filtration systems (Albers et al. 2015). On the other hand, microbes can have a negative impact on drinking water quality, e.g. by the production of undesirable tastes and odors (Lin, 1977) or by contributing to infrastructure deterioration (Zhang et al. 2008). Additionally, and even more importantly, drinking water systems can harbor potential pathogens such as *Acinetobacter*, *Campylobacter, Shigella* and *Legionella*, posing health risks to end consumers (Schuster et al. 2005). In Chapter II, we found a remarkable presence of *Acinetobacter* in processed water samples, reaching a relative read abundance in some samples of up to 47.5% (Van Assche et al. 2018). It remains unclear, however, how *Acinetobacter* was able to reach such high relative density in the drinking water and whether observations are consistent. While several *Acinetobacter* species perform important ecological functions such as biogeochemical cycling of nutrients (Jung and Park, 2015), some species can be opportunistic pathogens in humans, affecting people with compromised immune systems (Wong et al. 2017). Therefore, there is a clear need for further investigating the presence and ecology of *Acinetobacter* in drinking water production and distribution systems.

In order to produce and deliver safe drinking water, as well as to meet the stricter drinking water quality standards (van der Hoek et al. 2014), many drinking water production facilities implement multi-step treatment processes with various combinations of pre-oxidation, (e.g. ozonation and chlorination), filtration (e.g. rapid or slow sand filtration, double layer filtration, and granular activated carbon filtration) and disinfection (e.g. UV treatment and chlorination) (Fan et al. 2014; Xu et al. 2017, Hou et al. 2018, Oh et al. 2018, Potgieter et al. 2018) by which undesirable chemicals and microorganisms are more efficiently removed compared to a single treatment step (Shaw et al. 2015). Nevertheless, despite these efforts drinking water (systems) still contain vast numbers of microorganisms (Hammes et al. 2008). Therefore, it is critical to accurately identify the different types of bacteria and to determine how different treatment processes and water quality parameters affect the bacterial community structure.

Previous studies investigating the microbial ecology of drinking water production and distribution systems have mainly focused on determining the bacterial abundance at different locations throughout drinking water systems (Hammes et al. 2008), investigated temporal effects (Revetta et al. 2010; Hull et al. 2017, Potgieter et al. 2018), or tried to identify the origin of the bacteria in the tap water

(Liu et al. 2018a). Although these studies have greatly contributed to our understanding of the microbial ecology of drinking water systems, they generally did not evaluate the role of process operation in shaping the microbial community structure. More recently, however, an increasing number of studies has been performed to elucidate the impact of operational practices on the microbial community composition in drinking water (Poitelon et al. 2010; Ma et al. 2017, Xu et al. 2017, Hou et al. 2018, Liu et al. 2018a). Specifically, treatment processes like disinfection (e.g. ozonation and chlorination) and filtration (e.g. activated carbon, double layer and slow sand filtration) were found to substantially affect the community composition (Pinto et al. 2012, Lautenschlager et al. 2014, Ma et al. 2017, Xu et al. 2017, Hou et al. 2018). In contrast, treatment processes like coagulation, pre-oxidation, and rapid sand filtration had only little effect (Pinto et al. 2012, Xu et al. 2017, Liu et al. 2018a). In general, these studies found a dominance of the phylum *Proteobacteria* in the final drinking water (Bautista-de los Santos et al. 2016). Nevertheless, as most of these studies were performed on different systems having a different configuration or different operational practices and use different source waters, it remains challenging to truly identify the impact of the different treatment steps on the final microbial community composition. This information, however, is crucial for the design and management of cost-effective water treatment plants and the production of high-quality, safe drinking water. Ideally, effects of different treatment processes and their configuration on the drinking water microbial community are studied using a battery of treatment processes that are operated in parallel using the same source water. However, surprisingly, such approach has only been used once up till now (Xu et al. 2017). High-throughput sequencing of 16S ribosomal RNA (rRNA) gene amplicons demonstrated little effect of coagulation/sedimentation and pre-oxidation on the bacterial community composition. In contrast, substantial shifts in bacterial community composition were observed after ozonation, granular activated carbon treatment, sand filtration and disinfection for both series of treatment processes, confirming their strong shaping power of the drinking water bacterial communities (Xu et al. 2017). However, it remains unclear whether the same trends would also be observed when a different drinking water production facility is studied that implements another parallel series of treatment processes.

The goal of this study was to investigate and compare the bacterial community shifts in a full-scale drinking water production and distribution facility in Flanders (Antwerp, Belgium) that uses two series of multi-step treatment processes. Both systems use the same source water, operate in parallel, and use different treatment processes compared to the ones studied before (Xu et al. 2017), thereby providing an excellent system for this study. In contrast to Chapter II, bacterial communities were characterized by Illumina sequencing of 16S rRNA gene amplicons, as Illumina had gained popularity over 454 pyrosequencing due to its lower costs, higher accuracy and higher throughput. Further, emphasis was put on *Acinetobacter* due to its high relative abundance in Flemish drinking water (systems) (Chapter II), and its potential threat to human health. More specifically, we investigated the presence and abundance of *Acinetobacter* at each step of the treatment chain using both plating and isolation and quantitative real-time PCR. Finally, we studied the importance of the physicochemical

characteristics of the drinking water on the bacterial community composition in the water delivered to the end user (household tap water).

3.2 Materials and methods

3.2.1 Study system

A full-scale drinking water production and distribution system (Antwerp, Belgium) consisting of two series of multi-step treatment processes that operate in parallel was investigated in this study. The source water of the production system originated from surface water which was first collected in a reservoir pond before being distributed to each of the two production lines (further referred to as "line A" and "line B"). The main differences between line A and B are in the process selection in the early stages of the production system (Fig. 3.1). In brief, line A uses a serial stepwise process of rapid and slow sand filtration, an activated carbon filter, UV treatment and chlorination, whereas line B applies flotation, double layer filtration, activated carbon filtration, UV treatment and chlorination (Fig. 3.1).

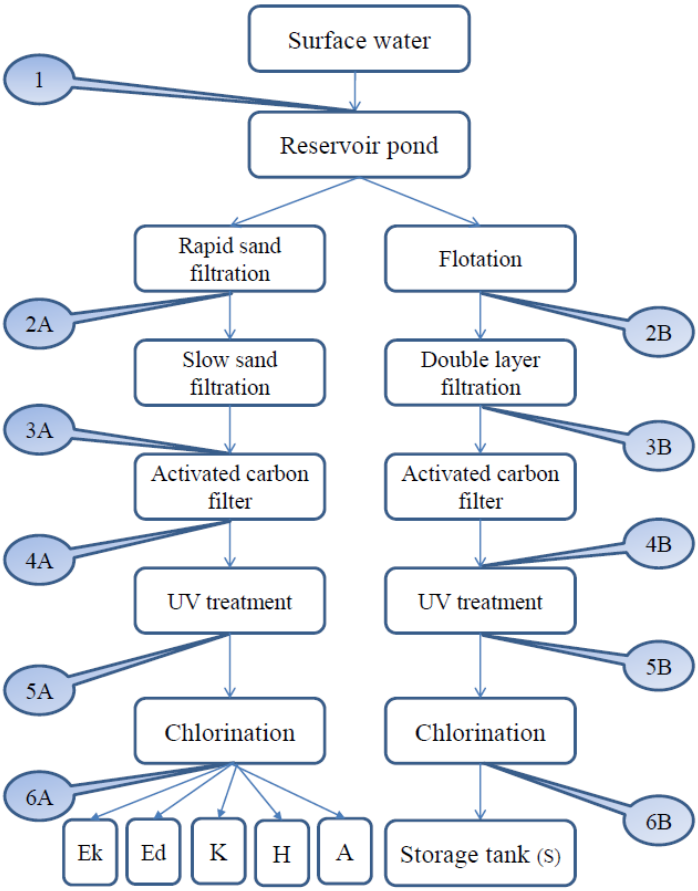


Figure 3.1: Overview of the parallel drinking water production process and distribution system and its sampling locations. The different sampling points are: 1, source water, which is common for line A and B; Line A: 2A, after rapid sand filtration; 3A, after slow sand filtration; 4A, after active carbon filtration; 5A, after UV treatment; 6A, after chlorination; household tap water from line A, i.e. Ek, Ed, K, H, and A; Line B: 2B, after flotation; 3B, after double layer filtration; 4B, after active carbon filtration; 5B, after UV treatment; 6B, after chlorination; and S, storage tank with household tap water from line B.

3.2.2 Sample collection

Water samples (2 L) for microbial analysis were collected at different stages in the production process along both production lines using sterile plastic bottles. More specifically, samples were taken immediately after a treatment unit or before entering the subsequent unit (Fig. 3.1) (6 sampling points for each line). Additionally, for line A, five household tap water samples were collected within its distribution network (i.e. Ek, Ed, K, H and A, located at a distance of 7 up to 23 km from the drinking water production facility). For line B, the water end product was sampled in a storage tank approximately 11 kilometers away from the production facility (sampling point "S"; Fig. 3.1). Samples were taken in each season (i.e. winter (W), spring (Sp), summer (Su), and fall (F)), and at each sampling point two samples were collected with a two week interval, except for the household tap water samples of line A which were only taken once in each season. In total, this resulted in a collection of 116 water samples for microbial analysis (Table 3.1). Further, at the household taps, additional samples were taken for chemical analysis according to a standard method (ISO 5557 and 19458). All samples were transported

in an ice-cooled container to the laboratory, and were subsequently stored at 4 °C prior to further processing (which occurred within 24 hours of sampling).

Table 3.1: Bacterial community diversity indices for the different water samples investigated in this study.

Season	Sampling point	Sample identifier[a]	Sobs[b]	Chao1[c]	Coverage [%][d]	Shannon[e]	Evenness[f]
Winter	1	W_1_a	410	414	99.1	4.18	0.694
Winter	1	W_1_b	415	418	99.2	4.23	0.701
Winter	2A	W_2A_a	308	313	98.5	3.75	0.654
Winter	2A	W_2A_b	397	402	98.7	3.91	0.653
Winter	2B	W_2B_a	323	327	98.7	3.81	0.659
Winter	2B	W_2B_b	393	398	98.6	3.97	0.664
Winter	3A	W_3A_a	990	994	99.6	5.07	0.735
Winter	3A	W_3A_b	1276	1279	99.8	6.01	0.841
Winter	3B	W_3B_a	281	287	97.9	3.72	0.660
Winter	3B	W_3B_b	331	336	98.5	3.73	0.643
Winter	4A	W_4A_a	1196	1199	99.8	5.81	0.820
Winter	4A	W_4A_b	1244	1247	99.8	5.97	0.838
Winter	4B	W_4B_a	527	531	99.2	4.19	0.669
Winter	4B	W_4B_b	824	827	99.7	5.14	0.765
Winter	5A	W_5A_a	1237	1240	99.7	5.79	0.813
Winter	5A	W_5A_b	1138	1142	99.7	5.92	0.841
Winter	5B	W_5B_a	625	629	99.4	4.5	0.700
Winter	5B	W_5B_b	735	737	99.8	5.07	0.768
Winter	6A	W_6A_a	1225	1226	99.9	6.27	0.882
Winter	6A	W_6A_b	1522	1525	99.8	6.51	0.889
Winter	6B	W_6B_a	933	935	99.7	5.32	0.779
Winter	6B	W_6B_b	933	935	99.8	5.7	0.834
Winter	S	W_S_a	282	285	98.9	2.55	0.453
Winter	S	W_S_b	253	257	98.3	2.47	0.446
Winter	Ek	W_Ek	503	506	99.4	4.37	0.702
Winter	Ed	W_Ed	307	310	99.2	3.85	0.672
Winter	K	W_K	272	276	98.6	2.5	0.445
Winter	H	W_H	340	342	99.3	3.1	0.532
Winter	A	W_A	286	291	98.4	3.86	0.683
Spring	1	Sp_1_a	177	188	94.2	3.67	0.709
Spring	1	Sp_1_b	185	188	98.3	3.58	0.685
Spring	2A	Sp_2A_a	307	314	97.7	3.72	0.650
Spring	2A	Sp_2A_b	308	315	97.8	3.84	0.671
Spring	2B	Sp_2B_a	181	189	96.0	3.5	0.674
Spring	2B	Sp_2B_b	268	275	97.6	4.08	0.730
Spring	3A	Sp_3A_a	665	674	98.7	3.95	0.607
Spring	3A	Sp_3A_b	1226	1229	99.7	5.47	0.769
Spring	3B	Sp_3B_a	242	245	98.8	3.36	0.612
Spring	3B	Sp_3B_b	386	394	98.0	4.07	0.684
Spring	4A	Sp_4A_a	1117	1121	99.7	5.54	0.789
Spring	4A	Sp_4A_b	1404	1407	99.8	6.5	0.897
Spring	4B	Sp_4B_a	601	605	99.3	4.23	0.661

Spring	4B	Sp_4B_b	850	853	99.7	5.28	0.782
Spring	5A	Sp_5A_a	1148	1152	99.6	5.59	0.793
Spring	5A	Sp_5A_b	1017	1021	99.6	5.29	0.764
Spring	5B	Sp_5B_a	615	618	99.5	4.19	0.652
Spring	5B	Sp_5B_b	871	875	99.6	5.46	0.806
Spring	6A	Sp_6A_a	1403	1406	99.8	6.3	0.870
Spring	6A	Sp_6A_b	1244	1246	99.9	6.37	0.894
Spring	6B	Sp_6B_a	914	917	99.7	5.51	0.808
Spring	6B	Sp_6B_b	974	977	99.7	5.69	0.827
Spring	S	Sp_S_a	145	149	97.4	1.99	0.399
Spring	S	Sp_S_b	174	183	95.0	2.15	0.417
Spring	Ek	Sp_Ek	465	467	99.5	4.39	0.715
Spring	Ed	Sp_Ed	322	325	99.1	3.87	0.671
Spring	K	Sp_K	270	278	97.1	2.76	0.492
Spring	H	Sp_H	324	327	99.1	3.07	0.530
Spring	A	Sp_A	345	351	98.4	4	0.684
Summer	1	Su_1_a	254	263	96.6	4.3	0.777
Summer	1	Su_1_b	286	291	98.3	4.21	0.745
Summer	2A	Su_2A_a	530	536	98.9	4.45	0.710
Summer	2A	Su_2A_b	344	352	97.8	4.08	0.699
Summer	2B	Su_2B_a	243	249	97.5	3.52	0.640
Summer	2B	Su_2B_b	234	240	97.6	3.7	0.679
Summer	3A	Su_3A_a	1060	1065	99.6	5.25	0.754
Summer	3A	Su_3A_b	1489	1491	99.9	6.5	0.890
Summer	3B	Su_3B_a	370	375	98.6	4.06	0.687
Summer	3B	Su_3B_b	425	432	98.5	4	0.661
Summer	4A	Su_4A_a	928	934	99.4	3.86	0.564
Summer	4A	Su_4A_b	1067	1071	99.6	5.57	0.799
Summer	4B	Su_4B_a	573	577	99.3	3.96	0.624
Summer	4B	Su_4B_b	647	653	99.1	4.46	0.688
Summer	5A	Su_5A_a	1011	1016	99.5	3.99	0.577
Summer	5A	Su_5A_b	1207	1210	99.7	6.01	0.847
Summer	5B	Su_5B_a	615	620	99.2	3.86	0.601
Summer	5B	Su_5B_b	682	687	99.3	4.3	0.659
Summer	6A	Su_6A_a	997	1000	99.7	5.26	0.761
Summer	6A	Su_6A_b	1053	1056	99.7	5.7	0.819
Summer	6B	Su_6B_a	786	789	99.6	5.32	0.798
Summer	6B	Su_6B_b	769	772	99.7	5.08	0.765
Summer	S	Su_S_a	160	172	93.3	2.59	0.511
Summer	S	Su_S_b	129	152	84.8	2.41	0.495
Summer	Ek	Su_Ek	635	637	99.7	4.9	0.759
Summer	Ed	Su_Ed	368	373	98.6	3.64	0.616
Summer	K	Su_K	329	331	99.4	3.59	0.620
Summer	H	Su_H	545	548	99.5	4.24	0.672
Summer	A	Su_A	389	391	99.6	4.57	0.767
Fall	1	F_1_a	242	246	98.3	4.03	0.733
Fall	1	F_1_b	310	314	98.7	4.19	0.730
Fall	2A	F_2A_a	586	589	99.5	4.48	0.704

Fall	2A	F_2A_b	756	759	99.7	4.87	0.735
Fall	2B	F_2B_a	293	300	97.6	3.76	0.662
Fall	2B	F_2B_b	391	398	98.3	4.06	0.681
Fall	3A	F_3A_a	1418	1420	99.9	5.97	0.823
Fall	3A	F_3A_b	1147	1150	99.7	5.51	0.782
Fall	3B	F_3B_a	262	267	98.3	3.42	0.614
Fall	3B	F_3B_b	343	346	99.1	3.83	0.656
Fall	4A	F_4A_a	587	593	99.0	2.85	0.448
Fall	4A	F_4A_b	845	851	99.3	3.85	0.572
Fall	4B	F_4B_a	456	460	99.1	3.82	0.624
Fall	4B	F_4B_b	551	554	99.4	4.53	0.718
Fall	5A	F_5A_a	709	716	99.1	3.15	0.480
Fall	5A	F_5A_b	799	803	99.5	4.04	0.605
Fall	5B	F_5B_a	488	492	99.2	3.91	0.632
Fall	5B	F_5B_b	694	697	99.6	4.82	0.736
Fall	6A	F_6A_a	1103	1105	99.8	5.5	0.785
Fall	6A	F_6A_b	1171	1175	99.7	5.79	0.819
Fall	6B	F_6B_a	828	832	99.6	5.34	0.794
Fall	6B	F_6B_b	784	786	99.7	5.4	0.810
Fall	S	F_S_a	209	212	98.7	2.09	0.391
Fall	S	F_S_b	497	500	99.5	2.79	0.450
Fall	Ek	F_Ek	535	537	99.7	4.7	0.748
Fall	Ed	F_Ed	390	393	99.3	3.66	0.613
Fall	K	F_K	341	344	99.2	3.84	0.658
Fall	H	F_H	319	320	99.6	3.03	0.526
Fall	A	F_A	410	412	99.5	4.16	0.692

[a] Sample identifiers "(W-Sp-Su-F)(1-2A-2B-3A-3B-4A-4B-5A-5B-6A-6B-S-Ek-Ed-K-H-A)(a or b)" present information about the sampling time and origin: season (W, winter; Sp, spring; Su, Summer; F, Fall); sampling points within the production line A and B (1, source water; 2A, after rapid sand filtration; 2B, after flotation; 3A, after slow sand filtration; 3B, after double layer filtration; 4A and 4B, after active carbon filter with A for line A and B for line B; 5A and 5B, after UV treatment with A for line A and B for line B; 6A and 6B, after chlorination with A for line A and B for line B; S, storage tank with household tap water from line B; Ek, Ed, K, H and A, household tap water originating from line A); and biological repeat (a or b) (see Fig. 3.1).
[b] Observed richness (S), amount of observed OTUs.
[c] OTU richness estimator based on the number of rare OTUs.
[d] Observed richness/Chao1 estimate * 100.
[e] Shannon-Wiener diversity index (H).
[f] Pielou's evenness (J = H/ln(S)).

3.2.3 DNA extraction, PCR amplification and Illumina MiSeq analysis

For each sample, two subsamples of 0.9 L were filtered over a 0.45 µm filter (mixed sterile cellulose ester filter, Millipore, Billerica, Massachusetts, USA) and used for subsequent DNA extraction using the phenol-chloroform extraction method described by Lievens et al. (2003) using the filters as starting material. Next, obtained DNA was subjected to PCR amplification and Illumina MiSeq sequencing of the bacterial 16S rRNA genes. An amplicon library was made using sample-specific barcode-labeled versions of the primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') generating amplicons covering the hypervariable V4 region of the prokaryotic 16S rRNA gene (Caporaso et al. 2011; dual-index sequencing strategy, Kozich et al.,

2013; Table S3.1, Supporting Information). The primers used represented slightly modified versions (more degenerate bases) of the primers used in Chapter II, widening their spectrum of detection. Amplification was performed in a Bio-Rad T100 thermal cycler in a reaction volume of 20.0 µL containing 1x Titanium Taq PCR buffer, 150 µM of each dNTP, 0.5 µM of each primer, 1x Titanium Taq DNA polymerase (Clontech, Saint-Germain-en-Laye, France) and 1.0 µL 10-times diluted DNA. The reaction was initiated by denaturation at 94 °C for 120 s, followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 59 °C for 45 s and elongation at 72 °C for 45 s, and terminated by a final elongation at 72 °C for 10 min. Amplicons were then purified using Agencourt AMPure XP magnetic beads (Beckman Coulter Genomics GmbH, South Plainfield, UK) according to the manufacturer's instructions. Following quantification of the purified amplicons using a Qubit High Sensitivity Fluorometer kit (Invitrogen, Carlsbad, CA, USA), samples were equimolarly combined in two amplicon libraries, one representing the samples collected in winter and fall and the other containing the samples of spring and summer. Subsequently, libraries were subjected to an ethanol precipitation and loaded on agarose gel. Next, bands of the expected size (~390 bp) were excised and the DNA was purified again, this time using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Finally, both libraries were diluted to 2 nM and sequenced at the Center of Medical Genetics Antwerp (University of Antwerp, Antwerp, Belgium) using an Illumina MiSeq sequencer with v2 500 cycle reagent kit (Illumina, San Diego, CA, USA).

Sequences were received as a de-multiplexed FASTQ file (data deposited in the Sequence Read Archive under BioProject accession PRJNA564131). Paired-end reads were merged using USEARCH (v.8.1) to form consensus sequences (Edgar, 2013) with no more than 10 mismatches allowed in the overlap region. Subsequently, after removal of the barcode and primer sequences, sequences were truncated at the 250th base. Shorter reads or reads with a total expected error threshold above 1.0 were discarded. The "classify.seqs" and "remove.lineage" commands in Mothur (v.1.36.1) and the Silva database (v.1.23) were used to identify and remove potential mitochondrial, chloroplast and eukaryote contaminants. Next, sequences from duplicate DNA extractions were combined, and the number of sequences was rarefied (due to uneven sequencing depth) to that of the sample with the lowest number of reads. Remaining sequences were grouped into operational taxonomic units (OTUs) based on a 3 % sequence dissimilarity cut-off using the UPARSE greedy algorithm in USEARCH, during which chimeric sequences were also removed (Rosselló-Mòra, 2011; Edgar, 2013), as were global singletons (i.e. OTUs with only 1 sequence represented in the entire data set). Global singletons were not taken into account to minimize the risk of retaining sequences from sequencing errors (Waud et al. 2014; Brown et al. 2015). Subsequently, the taxonomic origin of each OTU was determined with the SINTAX algorithm implemented in USEARCH (Edgar, 2016a), based on the RDP 16S rRNA gene trainingset (v.16; Cole et al., 2014). Taxonomic assignments up to genus level were considered reliable when bootstrap confidence values exceeded 0.80.

For each of the 116 investigated samples, a rarefaction curve was constructed using the Vegan package (v.2.4-1) for R (R Development Core Team, 2013; Oksanen et al. 2013). Additionally, OTU richness (defined as the number of bacterial OTUs), the Chao1 richness estimator, the Shannon-Wiener diversity index and Pielou's evenness were calculated using USEARCH (v. 1.36.1) (Schloss et al. 2009) and compared using the 'aov' function in R. Comparable with Ace, the Chao1 estimator is an abundance-based estimator predicting the true number of OTUs in a sample taking into account that there is no equal species distribution (Chao, 1984). The Shannon-Wiener diversity index was transformed (i.e. exp(H)) prior to the statistical analysis. Non-metric multidimensional scaling (NMDS) ordination was applied to visualize the level of similarity in community composition between the different samples based on Bray-Curtis similarities (based on relative abundance data). Additionally, similarities between the bacterial community compositions of the different sampling points studied were quantified using the ANOSIM and ADONIS function of the Vegan package for R (v. 2.4-4). The same analysis was performed to assess differences between the bacterial community compositions from different seasons. Analyses were conducted on different levels, including the level of OTU, genus and phylum. Furthermore, to link specific OTUs to different sampling points, an indicator species analysis was performed using the Indicspecies package (v. 1.7-1) in R (De Cáceres, 2013; R Development Core Team, 2015). Indicator species values are based on how specific and widespread an OTU is within a particular group and are independent of the relative abundance of other bacteria (Dufrêne and Legendre, 1997). Additionally, for each production line, core members of the bacterial community were determined. To this end, first samples were grouped in different categories based on their origin, including (i) source water, (ii) water undergoing treatment in line A or B, and (iii) water at the tap or storage tank for line A and B, respectively. For each production line, core OTUs were defined as OTUs occurring in at least one sample of each category. A Venn diagram was constructed using the VennDiagram package (v. 1.6.19) for R (Chen and Boutros, 2011) to visualize the distribution of the different OTUs over the different sampling categories.

3.2.4 Molecular assessment of Acinetobacter *species*

In order to quantify the *Acinetobacter* populations in the investigated water samples, a quantitative PCR (qPCR) analysis was performed. To this end, first a qPCR assay was developed for detection and quantification of *Acinetobacter* DNA using the primers Aci_gn_F2 (5'-CARCCDGGTGATAARATGGC-3') and Aci_gn_R1 (5'-GTYTCANGAATCTGHCCCAC-3'), which we developed, targeting specific sequences of the RNA polymerase beta-subunit (*rpoB*) gene of *Acinetobacter* species. Specificity of the primers was verified by a basic local alignment search tool (BLAST) analysis against GenBank, and both primers were found to be highly specific. Furthermore, the primers were found to perfectly match *rpoB* sequences of 53 of the 55 *Acinetobacter* species with validly published names at the time of analysis, illustrating their power to detect a broad spectrum of

Acinetobacter species. This was also confirmed by the successful amplification of *rpoB* DNA from pure cultures of diverse *Acinetobacter* species. Next, the assay was optimized to achieve accuracy and reproducibility in qPCR efficiency and copy number quantification (for final qPCR conditions, see below). Once the assay was optimized, the number of *Acinetobacter rpoB* gene copies were determined in all water samples investigated (116 samples) by using a calibration curve generated on the basis of a 10-fold dilution series of purified *rpoB* amplicons of *Acinetobacter* isolate AVA121A2d, which was identified as *Acinetobacter johnsonii*. This strain was selected because most of the *Acinetobacter* isolates obtained in this study were identified as *A. johnsonii* (see further). Calibaration curves generated with other *Acinetobacter* isolates (belonging to *A. calcoaceticus*, *A. guillouiae* and *A. lwoffii*) resulted in identical calibration curves, illustrating the robustness of our assay.

All qPCR amplifications were performed in an ABI StepOnePlus real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). Reaction mixtures were composed of 1.0 µL 10x diluted DNA, 0.5 µL of each forward and reverse primer (20 µM stock), 10.0 µL 2x iTaq universal SYBR Green supermix, and 8.0 µL nuclease-free water. Thermal cycling conditions consisted of an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of a denaturation step at 95 °C for 15 s and an annealing and elongation step at 60 °C for 60 s. Fluorescence (520 nm) was detected at the end of the elongation phase for each cycle. The threshold cycle (C_T), or the PCR cycle where fluorescence was first detected, was determined automatically using the Applied Biosystems software. The baseline was set automatically, while the threshold was manually set at 0.55 (which was above any background). To evaluate amplification specificity, a melting curve analysis was performed at the end of each PCR run as described previously (Bosmans et al. 2016). In each qPCR run a negative control that contained everything but the template DNA (replaced by DNA free water) was included. All reactions were performed in duplicate. A sample was considered positive when the C_T value was below that of the negative control (sterile water) and the melting temperature of the amplicon was as expected within the range of expectation (79.5 – 84.0 °C).

3.2.5 Acinetobacter *enrichment, isolation and identification*

In addition to the molecular analyses described above, water samples were also subjected to a culture-dependent analysis aiming at the isolation and identification of *Acinetobacter* species. To this end, for each of the 116 investigated samples, a subsample of 200 mL was filtered over a 0.45 µm filter (mixed sterile cellulose ester filter, Millipore, Billerica, Massachusetts, USA). The filter was then placed in a mineral medium containing 100 mL of Dijkshoorn's enrichment medium, which consisted of KH_2PO_4 (1.5 g/L), $Na_2HPO_4.2H_2O$ (16.5 g/L), $MgSO_4.7H_2O$ (0.2 g/L), NH_4Cl (2.0 g/L), $CaCl_2$ (0.01 g/L), $FeSO_4.7H_2O$ (0.5 mg/L), and sodium acetate (2.0 g/L) with a pH of 7.5 (Carvalheira et al. 2016). Cultures were subsequently incubated at 22 °C on a shaker at 120 rpm. After 2 or 3 days of incubation (depending on when growth was observed visually), 10 µL of each enrichment culture was plated on

commonly used agar, including R2A and Tryptic Soy Agar (TSA). R2A agar is known to be beneficial for growth support of stressed bacterial cells as, for example, encountered in a drinking water production facility; TSA is a non-selective nutrient-rich agar providing enough nutrients to allow for a wide variety of microorganisms to grow. The fact that in some occasions both agars seem to select for different *Acinetobacter* species also justifies the selection of these two agar media (Van Assche et al. unpublished results). Plates were incubated at 22 °C and checked every day for growth. Colonies from each morphotype recovered were restreaked two times on the same agar as the one from which they were isolated and checked for purity microscopically. Next, pure strains were stored at -80 °C in cryopreservation medium containing nutrient broth N° 2 (25 g/L, Oxoid, Basingstoke, Hampshire, England) and glycerol (150 mL/L, VWR, Fontenay-sous-Bois, France). Obtained isolates were identified by amplifying and sequencing part of the *rpoB* gene, which is more informative than the 16S rRNA gene to distinguish *Acinetobacter* species (Gundi et al. 2013). PCR amplification was performed using the primers Ac696F (5'-TAYCGYAAAGAYTTGAAAGAAG-3') and Ac1598R (5'-CGBGCRTGCATYTTGTCRT-3') (La Scola et al. 2006) and was conducted using a Bio-Rad T100 thermal cycler in a reaction volume of 20.0 µL, consisting of 0.15 mM of each dNTP, 0.5 µM of each primer, 1 unit Titanium Taq DNA polymerase, 1x Titanium Taq PCR buffer and 5 ng genomic DNA. Before amplification, DNA samples were denatured at 94 °C for 2 min, followed by 30 cycles of 45 s at 94 °C, 45 s at 55 °C and 45 s at 72 °C, with a final extension at 72 °C for 10 min. Amplicons were sequenced using the same forward and reverse primer as used for the DNA amplification. Both high-quality forward and reverse sequences were then combined to form a consensus sequence and identified using a BLAST analysis against the nt database in GenBank (excluding uncultured bacteria, unclassified sequences and environmental samples) and positioning of the sequences in a phylogenetic tree containing *rpoB* sequences for all validly named *Acinetobacter* species and previously defined genomic species. Obtained sequences were deposited in GenBank under the accession numbers MN317392-MN317467.

3.2.6 Water chemistry analysis

The household tap water samples were analyzed for a series of chemical and physical parameters, including temperature, pH, electrical conductivity (EC), dissolved oxygen (DO), water hardness, nitrate, orthophosphate, total phosphor, sulfate, (non-purgeable) total organic carbon (TOC), calcium, magnesium and trihalomethanes (i.e. bromodichloromethane, dibromochloromethanes, bromoform and chloroform). All chemical analysis were performed according to the Belgian regulation for drinking water analysis (https://emis.vito.be/nl/wac-2017) within an ISO 17025 certified laboratory (Provincial Institute for Hygiene, Antwerp, Belgium). Free chlorine was spectrophotometrically measured immediately after sampling (Hanna instruments, Temse, Belgium based on ISO 7393-2:2017). Temperature, pH, EC and DO were determined based on standard methods (i.e. DIN 38404/C4:1976,

ISO 10523:2008, ISO 7888:1985, ISO 5814:1990, respectively). Nitrate and orthophosphates were analyzed via a colorimetric method using an automated flow system (Skalar San Plus Analyser, based on EN-ISO 13395:1996 and ISO 15681-2:2003). Total phosphor, calcium and magnesium were determined by inductively couple plasma – optical emission spectroscopy (PerkinElmer Optima 8300DV, based on ISO11885:2007). Water hardness was calculated based on the calcium and magnesium concentration and expressed in French degrees (°fH). Sulfate was analyzed by an ion chromatography method (IC Metrohm 838, based on ISO 10304-1:2007 + ISO 10304-1:2007/Cor1:2010). Total organic carbon was measured via a catalytic oxidation and infrared detector (Shimadzu TOC-Vcph, based on ISO 8245). Finally, trihalomethanes were quantified using a headspace gas chromatograph-mass spectroscopy instrument (Shimadzu GC-MS QP2020, based on EPA 8260B 1996). In order to assess relationships between the abiotic factors and the composition of the bacterial community in the tap water, a redundancy analysis (RDA) between the measured environmental variables and a $\log_{10}$ transformation of the bacterial community composition was performed. The significance of the different chemical parameters was based on the anova.cca function with 1000 iterations.

3.2.7 Statistical analyses

For each statistical analysis the homogeneity of variance and the normal distribution was evaluated by the Levene's test and the Shapiro-Wilk test, respectively. The Levene's test and Shapiro-Wilk test were performed using the R packages 'car' and 'stats'. The results of the Levene's test indicated a homogeneity of variance and the data was normally distributed according to the Shapiro-Wilk test. Hence, significant differences were evaluated using the ANOVA test and a Tukey's test was used to measure the significant difference between groups. Both tests were performed using the R package 'stats'. The same statistical analysis was performed for sampling groups based on sampling point or season within the datasets for relative abundance of phyla and genera, qPCR results for total amount of bacteria and *Acinetobacer rpoB* gene and chemical parameters. Statistical differences were considered significant if the *p* value was < 0.05.

3.3 Results

3.3.1 Bacterial community composition

After rarefying to the minimum number of sequences obtained per sample (~6,000 sequences), a total of 6,764 OTUs were recovered (global singletons excluded), ranging from 129 to 1,522 OTUs per sample (Table 3.1). Based on Chao1, sampling coverage ranged between 84.8 and 99.9 % of the community (in general > 95 %) (Table 3.1), suggesting that the most abundant community members

were covered, as can also be observed from the rarefaction curves that approached saturation (Fig. S3.1, Supporting Information). No significant differences ($p > 0.05$) were found in observed OTU richness, estimated OTU richness (Chao1), Shannon-Wiener index and evenness for the different seasons (Table 3.2). By contrast, when samples were grouped per sampling point, highly significant differences ($p < 0.001$) in diversity measurements were observed for the different sampling points (Table 3.2). OTU richness was higher in line A compared to line B (Fig. 3.2). Furthermore, especially for line B OTU richness increased with every treatment step. For line A, highest OTU richness was observed from sampling point 3A until 6A (Fig. 3.2). Subsequently, following the different treatment steps, both for line A and line B OTU richness drastically decreased in the distribution system reaching an overall average OTU richness of 385 (± 101; SD) and 231 (± 120; SD) OTUs in household tap water A and sampling point S, respectively (Fig. 3.2). Similar observations were made for the Chao1 estimator and the Shannon-Wiener diversity index and evenness.

Table 3.2: Analysis of variance (ANOVA) of the bacterial community diversity indices.

Grouping of samples[a]	**Sobs**[b]		**Chao1**[c]		**Shannon**[d]		**Evenness**[e]	
	F value	*p* value	F value	*p* value	F value	*p* value	F value	*p* value
Sampling point	50.37	< 0.001 ***	50.81	< 0.001 ***	12.65	< 0.001 ***	11.83	< 0.001 ***
Season	0.181	0.909	0.179	0.910	1.109	0.348	0.750	0.524

[a] Sampling point: 1, 2A, 3A, 4A, 5A, 6A, HTW (i.e. A, Ed, Ek, H and K), 2B, 3B, 4B, 5B, 6B and S (see Fig. 3.1); season: winter (W), spring (Sp), summer (Su), and fall (F).
[b] Observed richness (S), amount of observed OTUs.
[c] OTU richness estimator based on the number of rare OTUs.
[d] Transformed Shannon-Wiener diversity index (i.e. exp(H) was used for statistical analysis.
[e] Pielou's evenness.
Asterisks: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

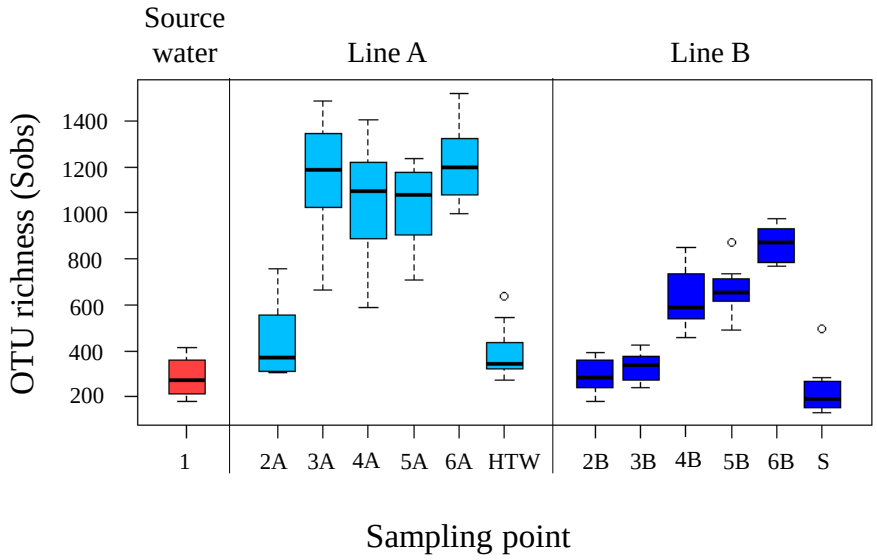


Figure 3.2: Boxplot representation of the OTU richness at different sampling points. The boxplots show the upper and lower quartiles; the whiskers indicate variability outside the upper and lower quartiles which is no more than 1.5 times the interquartile range. Further, the median is plotted as a thick black line. Sampling points: 1, source water (n = 8) (red); 2A, after rapid sand filtration (n = 8); 3A, after slow sand filtration (n = 8); 4A, after activated carbon filtration (n = 8); 5A, after UV treatment (n = 8) ; 6A, after chlorination (n = 8); HTW, combined data from the different household tap waters from line A (i.e. Ek, Ed, K, H, and A) (n = 20) (light blue); 2B, after flotation (n = 8); 3B, after double layer filtration (n = 8); 4B, after activated carbon filtration (n = 8); 5B, after UV treatment (n = 8); 6B, after chlorination (n = 8); and S, storage tank with household tap water from line B (n = 8) (dark blue).

NMDS ordination (Bray-Curtis; stress = 0.141) showed substantial differences in bacterial community composition across many samples (Fig. 3.3). As can be seen from the plot, samples from both production lines were clearly different and are well separated by the first NMDS axis from sampling point 3A on. Nevertheless, bacterial community composition converged again towards the end of the production line (6A vs 6B) (Fig. 3.3). It is also clear that some processes had only little or no influence on the bacterial community composition (i.e. flotation and UV treatment), while other treatment processes such as filtration (slow sand filtration and activated carbon filter treatment) and chlorination had a strong effect (Fig. 3.3). The bacterial community composition in the samples taken in the distribution system (i.e. A, Ed, Ek, H, K and S) was highly similar, but samples were also separated from samples of their respective production line (especially by the second NMDS axis). Differences in bacterial community composition were further tested using ANOSIM and ADONIS. Whereas no significant differences were detected in the diversity measurements between samples from different seasons, both ANOSIM and ADONIS indicated a significant difference ($p < 0.05$) on the OTU and genus level for the bacterial community compositions within seasonal sampling (Table 3.3). On the other hand, no significant difference ($p > 0.05$) was observed on the phylum level. With regard to the different sampling points, the ANOSIM and ADONIS analyses showed significant differences for all three taxonomic levels investigated (i.e. OTU, genus and phylum level) (Table 3.3).

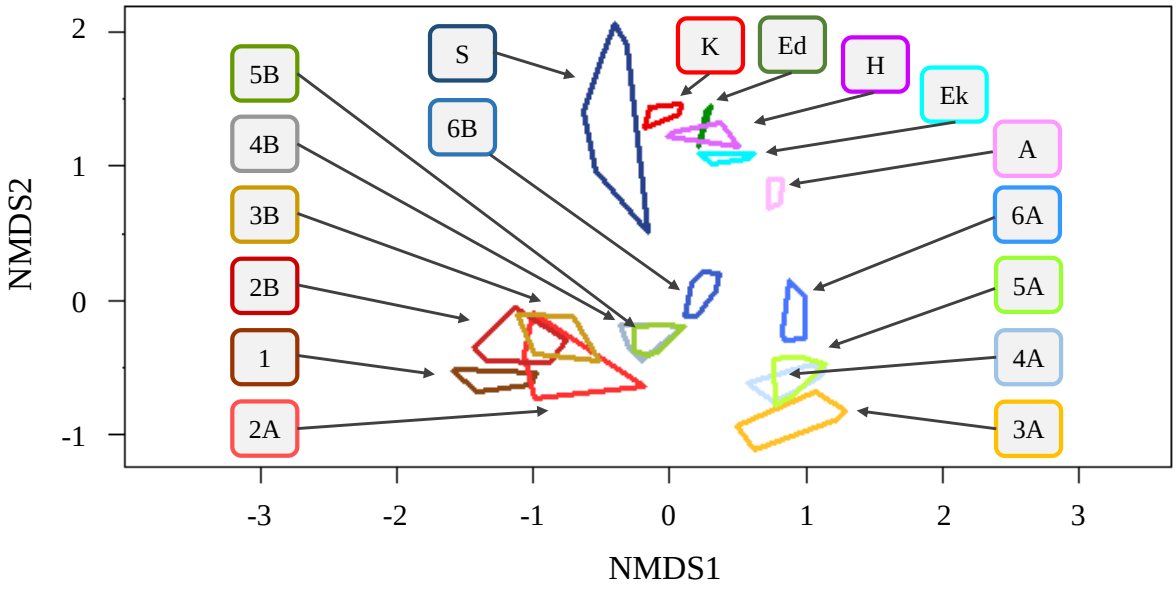


Figure 3.3: Non-metric multidimensional scaling (NMDS) ordination plot based on Bray-Curtis dissimilarities of the bacterial community composition of all water samples studied (stress value 0.141). Samples originate from different sampling points: 1, source water, which is common for line A and B; Line A: 2A, after rapid sand filtration; 3A, after slow sand filtration; 4A, after active carbon filtration; 5A, after UV treatment; 6A, after chlorination; Ek, Ed, K, H, and A, household tap water from line A; Line B: 2B, after flotation; 3B, after double layer filtration; 4B, after active carbon filtration; 5B, after UV treatment; 6B, after chlorination; and S, storage tank with household tap water from line B.

Table 3.3: Values and significance scores of the ANOSIM and ADONIS analysis performed in this study.

Grouping of samples[a]	**ANOSIM**		**ADONIS**	
	R	*p* value	F	*p* value
OTU level				
Sampling point	0.7761	0.001 ***	8.2374	0.001 ***
Season	0.0778	0.001 ***	3.4639	0.001 ***
Genus level				
Sampling point	0.4330	0.001 ***	5.8159	0.001 ***
Season	0.1233	0.001 ***	2.2307	0.001 ***
Phylum level				
Sampling point	0.4937	0.001 ***	8.9071	0.001 ***
Season	0.0155	0.118	1.0574	0.374

[a] Sampling point: 1, 2A, 3A, 4A, 5A, 6A, HTW (i.e. A, Ed, Ek, H and K), 2B, 3B, 4B, 5B, 6B and S (see Fig. 3.1); season: winter (W), spring (Sp), summer (Su), and fall (F).
Asterisks: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Taxonomic assignment of the OTUs using the RDP database revealed the presence of 24 bacterial / archaeal phyla and 167 bacterial / archaeal genera having a confidence bootstrap value > 0.80 (Table S3.2, Supporting Information). *Proteobacteria* was the most abundant phylum in all samples investigated with an average relative abundance of 48.4 %, followed by unclassified sequences (i.e. sequences that could not be identified at a confidence bootstrap value of > 0.80; 20.5 %), *Actinobacteria* (11.5 %) and *Bacteroidetes* (10.8 %). In general, for both production lines relative abundance of *Proteobacteria* increased along the production line, except for the final processed water (i.e. 6A and 6B). Instead, relative abundance of unclassified taxa, *Planctomycetes* and *Parcubacteria* was higher in samples from 6A and 6B compared to samples taken upstream the production line (Fig. 3.4 and Fig. S3.2, Supporting Information). Communities of the samples collected within the distribution system (i.e. household tap water from line A and storage tank water from line B) were dominated again by *Proteobacteria* (> 70 %), together with unclassified sequences covering a relative abundance of 91.2 and 96.1 % in the household tap and storage tank water, respectively (Fig. 3.4; Fig. S3.2, Supporting Information). Further, a clear shift in the bacterial community composition was observed between sampling point 2A and 3A (slow sand filtration), characterized by an increase in relative abundance of unclassified sequences and *Acidobacteria*, and a dramatic decrease in relative abundance of *Actinobacteria* and *Bacteroidetes* (Fig. 3.4; Fig. S3.2, Supporting Information). For line B, not such clear shift at one of the treatment steps was observed. It is also clear from this figure that UV treatment had almost no effect on the bacterial community composition (see 4A vs 5A and 4B vs 5B).

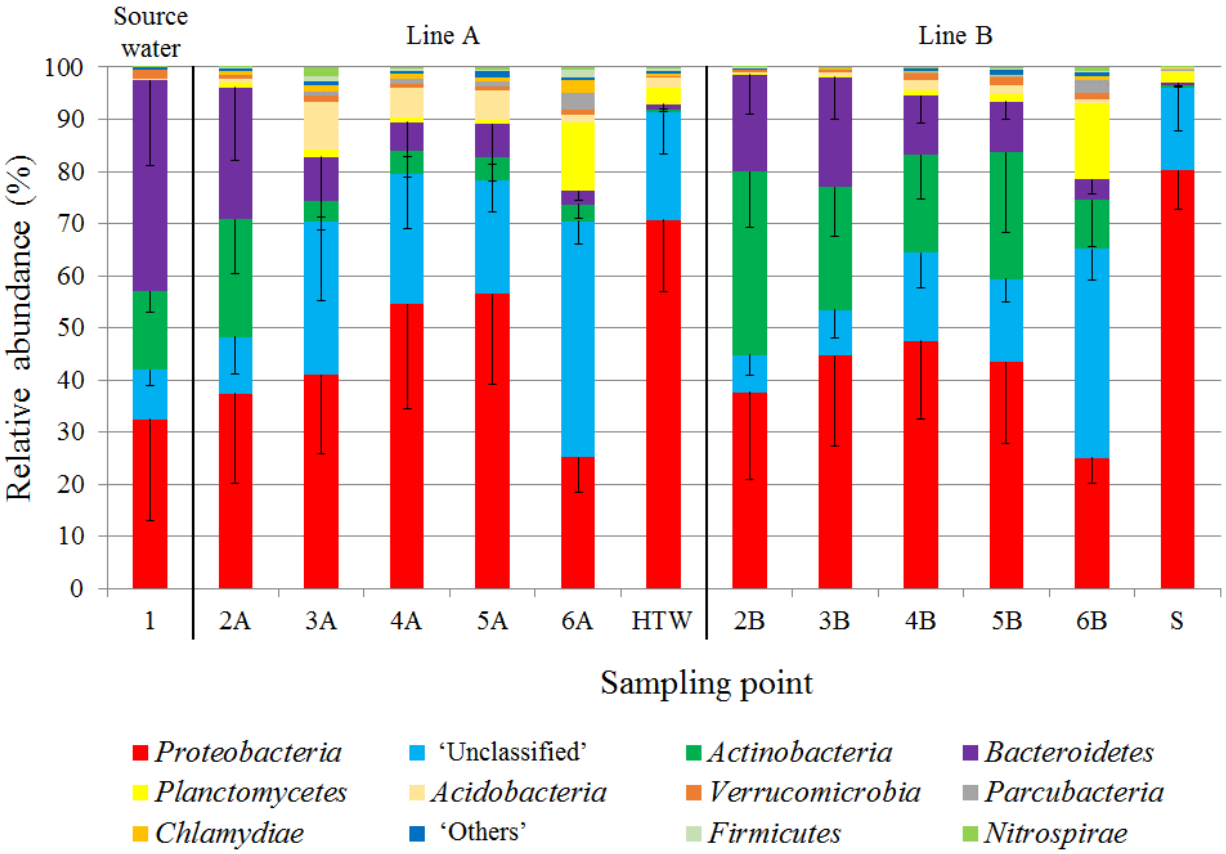


Figure 3.4: Bacterial relative abundance at phylum level in the different samples investigated in this study (samples from different sampling periods are combined per sampling point). Sequences were classified in phyla, when bootstrap confidence values exceeded 0.80. When bootstrap values were lower than 0.80, sequences were grouped in 'Unclassified'. Phyla representing less than 0.2 % of the sequences (in total) are grouped together as 'Others'. Sampling points: 1, source water (n = 8); 2A, after rapid sand filtration (n = 8); 3A, after slow sand filtration (n = 8); 4A, after activated carbon filtration (n = 8); 5A, after UV treatment (n = 8); 6A, after chlorination (n = 8); HTW, combined data for the different household tap waters from line A (i.e. Ek, Ed, K, H, and A) (n = 20); 2B, after flotation (n = 8); 3B, after double layer filtration (n = 8); 4B, after activated carbon filtration (n = 8); 5B, after UV treatment (n = 8); 6B, after chlorination (n = 8); and S, storage tank with household tap water from line B (n = 8). Negative error bars represent the standard deviation and are shown only for the four largest groups.

Among all genera identified with a bootstrap confidence level > 0.80, *Flavobacterium* was the most abundant genus, represented by 1.8 % of all sequences, followed by the genera *Sediminibacterium* (1.7 %), and *Polynucleobacter* (1.6 %) (Table S3.3, Supporting Information). All other genera were present at a relative abundance lower than 1 % of all sequences. In total, 6,417 OTUs, among which 429 could be accurately identified (bootstrap confidence level > 0.80) to the genus level (167 genera in total), occurred throughout the whole production chain from source water until tap water in line A. For line B 3,921 OTUs were found, among which 311 OTUs could be identified to the genus level (138 genera in total). When grouping samples in major categories, i.e. source water, water undergoing process treatment in line A or line B, and water at the tap (for line A) or in the storage tank (for line B), 310 and 226 OTUs were found to occur in each of the categories for line A and B (i.e. occurring in at least one of the samples for each category; Table S3.2, Supporting Information), respectively, and can be considered core taxa for the respective production and distribution circuits. Among these, we found 160

OTUs that were shared by both circuits (from source until tap / storage tank). A considerable amount of OTUs could be exclusively assigned to one of the different categories. More specifically, 60 OTUs were uniquely found in the source water; 2,156 OTUs were exclusively found along line A and 306 OTUs along line B; 369 OTUs were only found in tap water originating from line A and 24 in the storage tank filled with water from line B (Fig. S3.3, Supporting Information). The 160 core OTUs occurring in each of the parallel systems were represented by 48.9 % of all sequences, illustrating their abundant prevalence. These OTUs belong to 9 phyla (i.e. *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Acidobacteria*, *Verrucomicrobia*, *Nitrospirae*, *Planctomycetes*, *Firmicutes*, and *Fusobacteria*) and 34 genera; a number of OTUs could not be accurately identified to genus level (Table S3.2, Supporting Information). When applying a more strict criteria (i.e. OTU must be present in at least 90 % of the samples of the subcategory), 60 OTUs (71.2 % of all source water sequences) can be identified as core members of the source water subcategory. The most important genus of the core community of the source water was *Flavobacterium* (OTU 29, 32, 41 and 154) (Table S3.2, Supporting Information). The core community for line A and line B consisted of 17 (14.4 % of all line A sequences) and 41 (59.0 % of all line B sequences) OTUs, respectively. The low number of OTUs and percent of sequences for the core community of line A reinforces the large diversity of the community present in line A. Furthermore, none of the OTUs within the core community of line A could be identified to the genus level based on the bootstrap value (> 0.80). For line B *Sediminibacterium* (OTU 15), *Algoriphagus* (OTU 25) and *Nitrospira* (OTU 164) were identified at the genus level. Finally, the core community of the household tap water of line A and the storage tank of line B contained 37 (56.6 % of all household tap water sequences of line A) and 23 (88.1 % of all storage tank sequences of line B) OTUs, respectively. The genera *Aquabacterium* (OTU 55), *Bosea* (OTU 57), *Sphingomonas* (OTU 192), *Sphingopyxis* (OTU 213), *Phenylobacterium* (OTU 383) and *Legionella* (OTU 488) were identified as members of the core community for household tap water of line A. The core community of the storage tank of line B included *Sphingomonas* (OTU 67), *Rickettsia* (OTU 580) and *Pseudomonas* (OTU 659) as its members.

In an attempt to link specific genera to certain sampling points, an indicator species analysis was performed (Table S3.4, Supporting Information). As sampling points 2B and 3B, 4A and 5A, and 4B and 5B had a highly similar bacterial community composition (Fig. 3.3 and Fig. 3.4), samples from these sampling points were combined to obtain good indicator genera for the combined group. Additionally, the different household tap waters from line A (i.e. A, Ed, Ek, H, and K) were grouped. Seven genera could be significantly linked (indicator value > 0.500 and $p < 0.050$) to the source water, including from most significant to least *Namhaeicola*, *Haliea*, *Paracoccus*, *Desulfatiglans*, *Cetobacterium*, *Rhodococcus* and *Rudanella*. Further, several genera were found to be good indicators (indicator value > 0.250 and $p < 0.420$) for the different treatments within production line A, while this was less the case for line B (Table S3.4, Supporting Information). Further, ten genera (i.e. *Phenylobacterium*, *Altererythrobacter*, *Parvibacterium*, *Sphingobium*, *Belnapia*, *Bacteriovorax*, *Leptospira*, *Povalibacter*, *Nocardia*, and *Halobacteriovorax*) could be significantly linked to the

household tap water of line A (A, Ed, Ek, H, and K), while none could be significantly linked to water from the storage tank from line B (S). Four genera (i.e. *Bosea*, *Peredibacter*, *Pseudoxanthomonas* and *Flavisolibacter*) were significantly linked with finished water, irrespective of their origin (household tap water from line A or water of the storage tank from line B). The genera *Acidovorax*, *Flavobacterium*, *Sediminibacterium*, *Opitutus*, *Polynucleobacter* and *Diplorickettsia* were good indicators (indicator value > 0.727 and $p < 0.006$) for the combined group of source water and waters that have undergone a treatment process, while excluding the household tap water and water from the storage tank. Vice versa, *Legionella* and *Gemmata* were good indicators for all waters within the different production steps (with the exception of water from sampling point 2B / 3B) combined with the household tap waters and the distribution water, while excluding the source water (Table S3.4, Supporting Information).

3.3.2 Occurrence and absolute abundance of total bacteria and Acinetobacter

qPCR targeting total bacterial 16S rRNA genes revealed that for both line A and line B the bacterial load decreased during each treatment step (Fig. 3.5 A). The lowest amount of bacterial DNA was found after chlorination. However, for line A an increase in total bacteria was again observed after distribution at the house hold taps. This was not the case within the distribution system of line B, where the total amount of bacteria remained constant after chlorination (Fig. 3.5 A). Furtermore, qPCR analysis targeting part of the *Acinetobacter rpoB* gene showed higher amounts of *Acinetobacter* DNA in production line B compared to line A, reaching an average *rpoB* gene log copy number of 1.80 / µL (± 0.54; SD) after the double layer filtration during the summer season (3B) (Fig. 3.5 B). Further, for both lines lowest *Acinetobacter rpoB* gene copy numbers were found after the final process (i.e. at sampling points 6A and 6B). Nevertheless, *Acinetobacter rpoB* gene copy numbers increased again during distribution, especially for the water from line A (Fig. 3.5 B). Overall, *Acinetobacter* DNA concentrations were highest in spring and summer.

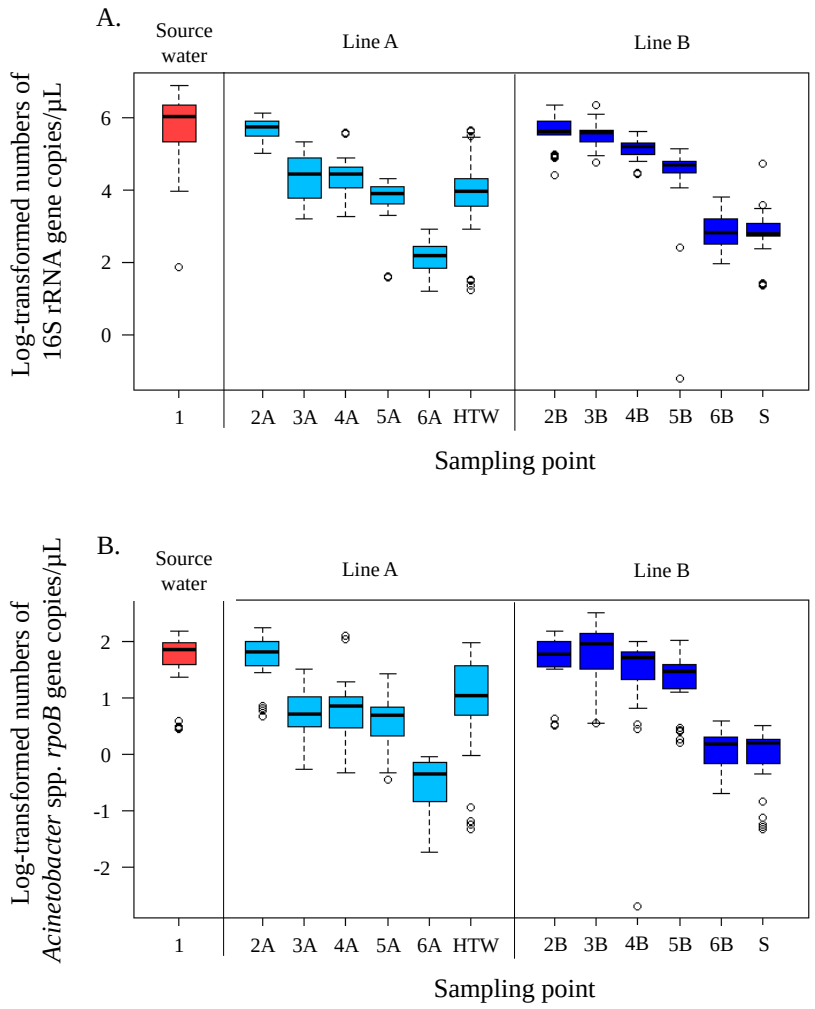


Figure 3.5: Determination of total bacterial 16S rRNA gene copy numbers (**A**) and *Acinetobacter* spp. *rpoB* gene copy numbers (**B**) by qPCR per µL DNA extract. The boxplots show the upper and lower quartiles; the whiskers indicate variability outside the upper and lower quartiles which is no more than 1.5 times the interquartile range. Further, the median is plotted as a thick black line. Sampling points: 1, source water (n = 8) (red); 2A, after rapid sand filtration (n = 8); 3A, after slow sand filtration (n = 8); 4A, after activated carbon filtration (n = 8); 5A, after UV treatment (n = 8); 6A, after chlorination (n = 8); HTW, combined data from the different household tap waters from line A (i.e. Ek, Ed, K, H, and A) (n = 20) (light blue); 2B, after flotation (n = 8); 3B, after double layer filtration (n = 8); 4B, after activated carbon filtration (n = 8); 5B, after UV treatment (n = 8); 6B, after chlorination (n = 8); and S, storage tank with household tap water from line B (n = 8) (dark blue).

Illumina Miseq sequencing and subsequent OTU clustering revealed three OTUs (OTU 328, 471 and 2179) that corresponded to *Acinetobacter* species (Table S3.2, Supporting Information). These OTUs occurred at a relative abundance between 0.0 and 2.8 % per sample. A BLAST search of the different amplicon sequence variants within each of these OTUs (ASVs; unique sequences) against all described *Acinetobacter* type strains and known *Acinetobacter* genomic species revealed that each OTU represented several *Acinetobacter* species (Fig. S3.4, Supporting Information). Among the 173 sequences grouped together in OTU 328, 73 ASVs could be identified that could be assigned to different *Acinetobacter* species. These included (i) *A. lwoffii* (90.2 % of sequences within OTU 328; sequence identity of 98-100 %), (ii) a group of closely related acinetobacters (*A. lactucae*, *A. seifertii*, *A. pittii*, *A.*

nosocomialis and *A. calcoaceticus*) (3.7 %; 99.6-100 %), (iii) *A. harbinensis* (2.6 %; 99.6-100 %), (iv) *A. albensis* (1.6 %; 99.6-100 %), (v) *A. indicus* (1.1 %; 99.6 %), and (vi) *A. radioresistens* / *A. equi* (0.5 %; 100 %) (Fig. S3.4, Supporting Information). OTU 471 contained 315 sequences among which 140 ASVs. BLAST analysis of the ASVs revealed the following species within this OTU: (i) *A. johnsonii* (34.2 % of sequences; sequence identity of 98.4-100 %), (ii) a group of closely related acinetobacters (*A. beijerinckii*, *A. dispersus*, *A. haemolyticus*, *A. parvus*, *A. tandoii*, *A. tjernbergiae*, 'genomic species 15BJ' and 'genomic species 16') (21.8 %; 98.8-100 %), (iii) *A. gandensis* / *A. bouvetii* (20.1 %, 98.8-100 %), (iv) isolate AVA086A3d (16.1 %, 98.8-100 %), (v) a group of closely related acinetobacters (*A. bereziniae*, *A. courvalinii*, *A. colistiniresistens*, *A. gerneri*, *A. guillouiae*, *A. junii*, *A. modestus*, *A. proteolyticus* and *A. vivianii*) (3.7 %, 99.2-100 %), (vi) *A. baumannii* (2.9 %; 99.2-100 %), (vii) *A. bohemicus* (0.6 %; 100 %), (viii) *A. gandensis* / *A. bouvetii* (0.3; 99.6 %) and (ix) *A. schindleri* (0.3 %; 100 %) (Fig. S3.4, Supporting Information). Finally, OTU 2179, which contained 13 sequences, had five ASVs with highest sequence similarity to (i) *A. brisouii* (71.4 %; 98-98.4 %), (ii) *A. ursingii* (21.4 %; 99.6-100 %), and (iii) *A. baumannii* (7.1 %; 99.2 %) (Fig. S3.4, Supporting Information).

After enrichment and isolation, a total of 60 putative *Acinetobacter* isolates were obtained. Sequencing part of the *rpoB* gene revealed that the majority of isolates were closest related to *A. johnsonii* (96.1 – 99.6 % sequence identity; 18 isolates) and *A. bohemicus* (95.8 – 99.2 %; 11 isolates) (Table 3.4). Isolates representing these species were obtained from the source water and from several sampling points within the production process along line A and B. Additionally, *A. johnsonii* was found in the household tap water provided by production line A (Table 3.4). Furthermore, eight isolates showed highest *rpoB* sequence homology with *A. lwoffii* (98.0 – 99.9 %); four with *A. calcoaceticus* (98.1 – 98.7 %); four with *A. guillouiae* (99.1 – 99.9 %); two with *A. albensis* (98.2 – 99.6 %); two with *A. schindleri* (97.3 %); and one with *A. beijerinckii* (98.2 %), *A. dispersus* (98.8 %), *A. harbinensis* (99.2 %), *A. kyonggiensis* (96.2 %), *A. parvus* (98.3 %), *A. pittii* (99.6 %), and *A. tjernbergiae* (96.6 %) (Table 3.4). Four isolates showed highest homology with less than 95 % sequence similarity to an *Acinetobacter* species, and can therefore be considered to represent isolates of not yet validly published *Acinetobacter* species (Table 3.4). Whereas seasonality did not greatly influence the number of isolated acinetobacters, strains identified as *A. bohemicus* were mostly isolated during winter (Table 3.4). Remarkably, isolates identified as *A. lwoffii* were only found in water undergoing a treatment process in line A and the household tap water of line A, while isolates identified as *A. calcoaceticus* were only found in water undergoing a treatment process in line B. No *Acinetobacter* isolates were obtained after UV treatment (sampling points 5A and 5B) as well as in the storage thank (sampling point S). It has to be noted, however, that further analysis is needed to find out whether the different isolates represent different strains, e.g. by sequencing of additional genes or phenotypic analysis (see Chapter IV, Diancourt et al. 2010, Hu et al. 2018).

Table 3.4: List of putative *Acinetobacter* isolates obtained in this study.

Strain identifier	GenBank Accession N°[a]	Water sample[b]	Season	Sampling point[c]	Highest match with GenBank entry[d]	Identity[e] (%)
AVA 013A	MN317403	W_1_a	Winter	Source water	*Acinetobacter johnsonii*, strain DSM 6963^T	99.167
AVA 013B	MN317434	W_1_a	Winter	Source water	*Acinetobacter bohemicus*, strain ANC 3994^T	98.839
AVA 030B2d	MN317439	W_1_b	Winter	Source water	*Acinetobacter bohemicus*, strain ANC 3994^T	98.293
AVA 014A	MN317435	W_2A_a	Winter	Line A	*Acinetobacter bohemicus*, strain ANC 3994^T	98.719
AVA 031A2d	MN317440	W_2A_b	Winter	Line A	*Acinetobacter bohemicus*, strain ANC 3994^T	95.849
AVA 016A	MN317404	W_3A_a	Winter	Line A	*Acinetobacter johnsonii*, strain: DSM 6963^T	98.698
AVA 035A2d	MN317447	W_4A_b	Winter	Line A	*Acinetobacter harbinensis*, strain HITLi 7^T	99.152
AVA 025A2d	MN317405	W_Ek	Winter	HTW, Line A	*Acinetobacter guillouiae*, strain DSM 590^T	99.871
AVA 025B2d	MN317445	W_Ek	Winter	HTW, Line A	*Acinetobacter guillouiae*, strain DSM 590^T	99.127
AVA 015A	MN317436	W_2B_a	Winter	Line B	*Acinetobacter bohemicus*, strain ANC 3994^T	98.955
AVA 032A2d	MN317448	W_2B_b	Winter	Line B	*Acinetobacter albensis*, strain ANC 4874^T	98.235
AVA 017A	MN317437	W_3B_a	Winter	Line B	*Acinetobacter bohemicus*, strain ANC 3994^T	98.549
AVA 034A2d[f]	MN317446	W_3B_b	Winter	Line B	*Acinetobacter guillouiae*, strain DSM 590^T	89.689
AVA 019A	MN317438	W_4B_a	Winter	Line B	*Acinetobacter bohemicus*, strain ANC 3994^T	97.893
AVA 036A2d	MN317441	W_4B_b	Winter	Line B	*Acinetobacter bohemicus*, strain ANC 3994^T	98.710
AVA 042A2d[g]	MN317450	Sp_1_a	Spring	Source water	*Acinetobacter gandensis*, strain ANC 4275^T	89.542
AVA 042B2d	MN317449	Sp_1_a	Spring	Source water	*Acinetobacter albensis*, strain ANC 4874^T	99.639
AVA 059A2d	MN317408	Sp_1_b	Spring	Source water	*Acinetobacter johnsonii*, strain DSM 6963^T	99.305
AVA 060A2d	MN317410	Sp_2A_b	Spring	Line A	*Acinetobacter johnsonii*, strain DSM 6963^T	97.971
AVA 062A2d	MN317442	Sp_3A_b	Spring	Line A	*Acinetobacter bohemicus*, strain ANC 3994^T	99.166
AVA 057A2da	MN317407	Sp_H	Spring	HTW, Line A	*Acinetobacter lwoffii*, strain NCTC 5866^T	98.354
AVA 057A2db	MN317451	Sp_H	Spring	HTW, Line A	*Acinetobacter lwoffii*, strain NCTC 5866^T	98.075
AVA 044A2d	MN317406	Sp_2B_a	Spring	Line B	*Acinetobacter calcoaceticus*, strain CIP 81.8^T	98.084
AVA 061A2d	MN317411	Sp_2B_b	Spring	Line B	*Acinetobacter calcoaceticus*, strain CIP 81.8^T	98.301
AVA 046A2d	MN317454	Sp_3B_a	Spring	Line B	*Acinetobacter kyonggiensis*	96.249
AVA 063A2d	MN317443	Sp_3B_b	Spring	Line B	*Acinetobacter bohemicus*, strain ANC 3994^T	99.187
AVA 065A2d	MN317444	Sp_4B_b	Spring	Line B	*Acinetobacter bohemicus*, strain ANC 3994^T	99.176
AVA 088A2d	MN317464	Su_1_b	Summer	Source water	*Acinetobacter johnsonii*, strain DSM 6963^T	96.662
AVA 072A2d	MN317412	Su_2A_a	Summer	Line A	*Acinetobacter johnsonii*, strain DSM 6963^T	97.139
AVA 089A2d	MN317467	Su_2A_b	Summer	Line A	*Acinetobacter parvus*, strain DSM 16617	98.265
AVA 074A2d	MN317413	Su_3A_a	Summer	Line A	*Acinetobacter johnsonii*, strain DSM 6963^T	96.073
AVA 091A2d	MN317456	Su_3A_b	Summer	Line A	*Acinetobacter pittii*, strain DSM 25618^T	99.637
AVA 076A2d	MN317414	Su_4A_a	Summer	Line A	*Acinetobacter lwoffii*, strain NCTC 5866^T	95.833
AVA 093A2da	MN317457	Su_4A_b	Summer	Line A	*Acinetobacter schindleri*, strain CIP 107287^T	97.291
AVA 093A2db	MN317458	Su_4A_b	Summer	Line A	*Acinetobacter schindleri*, strain CIP 107287^T	97.278
AVA 080A2d	MN317416	Su_6A_a	Summer	Line A	*Acinetobacter lwoffii*, strain NCTC 5866^T	98.032
AVA 080B2d	MN317417	Su_6A_a	Summer	Line A	*Acinetobacter lwoffii*, strain NCTC 5866^T	98.233
AVA 086A3d	MN317418	Su_H	Summer	HTW, Line A	*Acinetobacter johnsonii*, strain DSM 6963^T	99.647
AVA 073A2d	MN317463	Su_2B_a	Summer	Line B	*Acinetobacter calcoaceticus*, strain CIP 81.8^T	98.659

AVA 090A2d	MN317419	Su_2B_b	Summer	Line B	*Acinetobacter johnsonii*, strain DSM 6963^{T}	97.472
AVA 075A2d	MN317455	Su_3B_a	Summer	Line B	*Acinetobacter dispersus*, strain ANC 4105^{T}	98.818
AVA 092A2d	MN317420	Su_3B_b	Summer	Line B	*Acinetobacter johnsonii*, strain DSM 6963^{T}	96.851
AVA 077A2d	MN317415	Su_4B_a	Summer	Line B	*Acinetobacter calcoaceticus*, strain CIP 81.8^{T}	98.193
AVA 094A2d[h]	MN317459	Su_4B_b	Summer	Line B	*Acinetobacter piscicola*	94.971
AVA 098A2d	MN317421	Su_6B_b	Summer	Line B	*Acinetobacter johnsonii*, strain DSM 6963^{T}	98.452
AVA 098B2d	MN317465	Su_6B_b	Summer	Line B	*Acinetobacter johnsonii*, strain DSM 6963^{T}	98.331
AVA 100B2d	MN317422	F_1_a	Fall	Source water	*Acinetobacter johnsonii*, strain DSM 6963^{T}	99.642
AVA 117A2d	MN317425	F_1_b	Fall	Source water	*Acinetobacter johnsonii*, strain DSM 6963^{T}	98.565
AVA 117B2d	MN317426	F_1_b	Fall	Source water	*Acinetobacter guillouiae*, strain DSM 590^{T}	99.164
AVA 101A2d	MN317423	F_2A_a	Fall	Line A	*Acinetobacter johnsonii*, strain DSM 6963^{T}	99.288
AVA 118A2d	MN317427	F_2A_b	Fall	Line A	*Acinetobacter guillouiae*, strain DSM 590^{T}	99.288
AVA 103A2d	MN317452	F_3A_a	Fall	Line A	*Acinetobacter lwoffii*, strain NCTC 5866^{T}	98.333
AVA 113A3d	MN317424	F_Ed	Fall	HTW, Line A	*Acinetobacter lwoffii*, strain NCTC 5866^{T}	99.879
AVA 113B3d	MN317453	F_Ed	Fall	HTW, Line A	*Acinetobacter lwoffii*, strain NCTC 5866^{T}	99.642
AVA 102A2d	MN317466	F_2B_a	Fall	Line B	*Acinetobacter johnsonii*, strain DSM 6963^{T}	99.519
AVA 119A2d	MN317428	F_2B_b	Fall	Line B	*Acinetobacter johnsonii*, strain DSM 6963^{T}	98.582
AVA 104A2d	MN317460	F_3B_a	Fall	Line B	*Acinetobacter beijerinckii*, strain NIPH 838^{T}	98.176
AVA 121A2d	MN317429	F_3B_b	Fall	Line B	*Acinetobacter johnsonii*, strain DSM 6963^{T}	99.149
AVA 123A2d	MN317462	F_4B_b	Fall	Line B	*Acinetobacter tjernbergiae*, strain DSM 14971^{T}	96.610
AVA 110A2d[i]	MN317461	F_6B_a	Fall	Line B	*Acinetobacter pragensis*, strain ANC 4149^{T}	85.818

[a] RNA polymerase beta subunit (*rpoB*) gene.

[b] Sample identifiers "(W-Sp-Su-F)(1-2A-2B-3A-3B-4A-4B-5A-5B-6A-6B-S-Ek-Ed-K-H-A)(a or b)" present information about the sampling time and origin: season (W, winter; Sp, spring; Su, Summer; F, Fall); sampling location within the production line A and B (1, source water; 2A, after rapid sand filtration; 2B, after flotation; 3A, after slow sand filtration; 3B, after double layer filtration; 4A and 4B, after active carbon filter with A for line A and B for line B; 5A and 5B, after UV treatment with A for line A and B for line B; 6A and 6B, after chlorination with A for line A and B for line B; S, storage tank with household tap water from line B; Ek, Ed, K, H and A, household tap water originating from line A); and biological repeat (a or b) (see Fig. 3.1).

[c] HTW, household tap water.

[d] Nearest neighbor based on a BLAST search of partial *rpoB* genes in GenBank against type strains.

[e] Percentage of sequence identity on a total of 805 (± 46) bp. Generally a 95 % sequence similarity is adequate for species identification based on the partial *rpoB* gene sequence (La Scola et al. 2006).

[f] IsolateAVA034A2d has a 90.062 % sequence similarity with *Acinetobacter guillouiae* strain NIPH 769.

[g] Isolate AVA042A2d has a 100 % sequence similarity with *Acinetobacter* sp. strain EP35

[h] No higher match was found for isolate AVA094A2d.

[i] Isolate AVA110A2d has a 99.394 % sequence similarity with *Acinetobacter* sp. strain WCHAc010034.

To further assess whether the obtained isolates were also found by the Illumina amplicon sequencing approach, 16S rRNA gene sequences were determined for the different isolates (Jacquemyn et al. 2013), and compared with all ASVs hold by the three *Acinetobacter* OTUs (OTU 328, 471 and 2179) (Fig. S3.4, Supporting Information). Results revealed that the obtained isolates matched with many of the ASVs obtained using the Illumina amplicon sequencing approach (Fig. S3.4, Supporting Information). Although *rpoB* gene sequencing confirms the species identity for several ASVs, several isolates identified as *A. bohemicus* did not have a > 97 % similarity with an ASVs. This explains why the percentage of *A. bohemicus* ASV (0.6 %) is relatively low compared to the number of *A. bohemicus* strains isolated.

3.3.3 Influence of abiotic factors on the bacterial community composition in household tap waters

In order to assess the influence of the abiotic environment on the bacterial community composition in the household tap waters, a series of physico-chemical parameters were measured. Analysis of variance indicated that most parameters were significantly different based on season rather than sampling location ($p < 0.005$) (Table S3.5, Supporting Information). In general, residual free chlorine levels were lower than the detection limit (< 0.10 mg/L), which in turn is lower than the maximum allowed concentration of 0.25 mg/L. The pH of the drinking water samples was stable during the different seasons and over the different sampling locations, with an overall average of 7.89 (± 0.08, SD). The total organic carbon concentration did not vary significantly between the different seasons and sampling locations, ranging from 1.0 to 3.8 mg C/L. Total phosphor and orthophosphate concentrations were too low to be detected in any of the drinking water samples. Dissolved oxygen (DO) concentrations were the lowest during summer and the highest during winter with an average of 5.34 (±1.67, SD) and 9.50 (±0.73, SD) mg O_2/L, respectively (Fig. S3.5 and Table S3.5, Supporting Information). The average temperature of the drinking water was 21.4 (± 0.5) °C during summer and dropped until 14.5 (± 2.4) °C on average during winter. Nitrate concentrations were highest in winter with an average of 13.4 (± 0.9) mg/L and the lowest in summer with an average of 6.9 (± 1.6) mg/L (Fig. S3.5 and Table S3.5, Supporting Information). Water hardness and EC varied over the different seasons with a significant increase during fall (Fig. S3.5 and Table S3.5, Supporting Information). This was also observed for the calcium, magnesium and sulfate concentration (Table S3.5, Supporting Information). Total trihalomethanes concentration was the only parameter which was significantly different for sampling location ($p = 0.014$) (Fig. S3.6 and Table S3.5, Supporting Information). More specifically, total trihalomethanes concentrations was much lower at sampling location A compared to the other sampling locations. Drinking water from sampling location A contained on average 7 (± 1, SD) µg/L trihalomethanes (irrespective of season), while all other household tap waters showed an average concentration of 31 (± 11, SD) µg/L.

When fitting the environmental variables on a RDA ordination plot of the bacterial community composition of the different household tap waters (Fig. S3.7, Supporting Information), trihalomethanes

concentration, total organic carbon concentration and DO were found to explain a significant proportion of the variation in bacterial community composition. Trihalomethanes was the only environmental variable that was significantly lower in sampling location A (Fig. S3.6 and Table S3.5, Supporting Information). Although, the total organic carbon concentration and DO were not significantly different, they tend to be lower and higher at sampling location A in comparison to the other household tap waters, respectively (Table S3.5, Supporting information). Interestingly, the bacterial community in the drinking water sampled at location A was enriched in *Methylophilus* species (Fig. S3.6, Supporting Information), which are capable of utilizing chlorinated methanes as a substrate (Bader and Leisinger 1994), and may therefore explain the lower trihalomethanes concentration at sampling location A (Fig. S3.6, Supporting Information).

3.4 Discussion

3.4.1 Effect of treatment processes on the drinking water microbiome

In this study, we compared the bacterial communities in two parallel drinking water production circuits using the same source water, but implementing a different set of treatment processes. We hypothesized that different treatment processes and the configuration of these processes result in substantial variation of the microbial communities in the drinking water. Quantification of total bacteria revealed that for both line A and B the amount of bacteria decreased during treatment and the lowest amount of bacteria were present after chlorination. For line A an increase was observed in the household tap water samples. Further, a clear difference in community composition was found between both production lines. In general, OTU richness was higher in line A compared to line B. OTU richness in line A especially increased after slow sand filtration, followed by a stagnation before dropping again in the household tap water. By contrast, OTU richness in line B gradually increased with each process step. Comparable with line A, the number of OTUs dropped again in the finished product. Although the number of OTUs decreased the increase of total amount of bacteria in household tap water of line A suggests that a selection of bacteria are able to regrow during distribution or within the indoor house plumbing. On the other hand, in line B the OTU richness increases each production step while the total amount of bacteria decreases might suggest that the elimination of the most abundant bacteria creates growth opportunity for new species. The difference in overall OTU richness between both circuits is likely to be explained by the fact that line A is substantially older than line B (line B was operational less than one year before the initial sampling, whereas line A was already operational for several decades) holding a more diverse community.

Taxonomy assignment of the OTUs revealed that *Proteobacteria* was the most abundant phylum in our study, which is in line with previous research (Bautista-de los Santos et al. 2016; Vaz-Moreira et al. 2017). The other phyla detected, e.g. *Actinobacteria*, *Bacteroidetes*, *Firmicutes*,

Verrucomicrobia and *Nitrospirae*, are also commonly found in drinking water production and distribution systems (Liu et al. 2013). Likewise, the most abundant genera detected (i.e. *Flavobacterium*, *Sediminibacterium* and *Polynucleobacter*) are known inhabitants of aquatic environments. Our results show that the bacterial community composition gradually changed with the different treatment processes. Especially the slow sand filtration implemented in line A was found to have a strong effect on the community, leading to an increase in relative abundance of unclassified sequences and *Acidobacteria*, and a decrease of *Actinobacteria* and *Bacteroidetes*. The activated carbon filtration as well as chlorination treatment affected the bacterial community composition in both lines. The bacterial community composition shift due to activated carbon filtration was larger for line B than line A and was characterized by an increase of unclassified taxa, *Planctomycetes*, *Acidobacteria*, *Parcubacteria*, *Chlamydiae* and *Latescibacteria*. The chlorination treatment step altered the community composition in both lines, yet resulting in relatively small differences in the bacterial community composition between the delivered products.

Shifts in the bacterial community composition along a drinking water production and distribution system have been reported previously (e.g. Pinto et al. 2012; El-Chakhtoura et al. 2015; Roeselers et al. 2015; Li et al. 2017). The most important reported process steps responsible for these shifts are filtration (i.e. activated carbon, double layer and slow sand filtration) and disinfection (i.e. ozonation and chlorination) (Pinto et al. 2012; Ma et al. 2017; Xu et al. 2017; Hou et al. 2018; Oh et al. 2018, Potgieter et al. 2018). Our results suggest that UV treatment and flotation and double layer filtration had little effect on the bacterial community composition. Similar findings have previously been observed for a flotation treatment step (Poitelon et al. 2010; Xu et al. 2017). In contrast, most studies suggest a significant impact of a double layer filtration step on the bacterial community composition (Pinto et al. 2012; Hou et al. 2018). This discrepancy may be explained by differences in the process configurations of the drinking water treatment plants. In the study of Pinto et al. (2012) the double layer filtration step was preceded by an ozonation treatment, while in our study flotation preceded the double layer filtration. Nevertheless, Hou et al. (2018), used a similar configuration as in our study, but also observed a strong effect of double layer filtration on the bacterial community composition. In this case, however, the study was performed in a subtropical monsoon climate with very mild winters. Differences due to seasonal effects can explain the contradictory observation as the community composition shift were much greater in the wet season in comparison to the dry season. Further, there are other factors that could explain these differences, including age of the treatment plant, history, etc. The absence of a bacterial community composition shift after UV treatment may suggest that the UV treatment is ineffective. Another, more likely, explanation is that the PCR targeted DNA fragments were too short to have undergone enough UV damage to inhibit PCR amplification (Nocker et al. 2018). Typical damage induced by UV irradiation is the formation of thymine dimers, by which amplification is inhibited (Lehle et al., 2013; Beck et al., 2014). The more nucleotides are damaged in the amplified region, the more amplification is inhibited. Therefore, it is likely to assume that when short DNA

fragments have to be amplified as needed for Illumina MiSeq sequencing and qPCR quantification, effects may not be observed, even when the bacteria are inactivated or killed.

In contrast to sampling points, season was found to only have a minor impact on the bacterial community composition. For example, no significant differences were found in OTU richness or total amount of bacteria between the different seasons. Further, no significant differences in community composition were found among the different seasons at phylum level. However, at the level of OTUs or genus, significant differences were found. It has to be noted, however, that temporal dynamics were only monitored over one year in this study. Therefore, further research conducted over multiple years is needed before drawing strong conclusions. Indeed, in other studies, differences based on seasonality have been reported (Pinto et al. 2014; Ling et al. 2015), yet often the differences in community composition are larger for sampling point than season (Ma et al. 2017). Perhaps, these opposing observations can be explained by differences in the source water. In Chapter II, for example, we have shown that the impact of season on the bacterial community composition is bigger when surface water is used as source water in comparison to groundwater (Van Assche et al. 2018). Additionally, temporal dynamics in the bacterial community composition are known to vary less the further the drinking water is sampled from its treatment facility (Potgieter et al. 2018).

3.4.2 Potential health risks and presence of Acinetobacter

Dispersion of opportunistic pathogens via drinking water systems is a major public concern. Within the amplicon sequences of the household tap water (line A) and the water of the storage tank (line B), several sequences were related to bacterial genera containing opportunistic pathogens. These genera included *Legionella*, *Mycobacterium*, *Pseudomonas* and *Acinetobacter*, which are known to harbor opportunistic premise plumbing pathogens (Falkinham III et al. 2015). Relative abundance of these genera, however, was low, i.e. on average 0.25, 0.24, 0.14 and 0.07 % for *Pseudomonas*, *Legionella*, *Mycobacterium*, and *Acinetobacter*, respectively. So far, no information is available about their absolute abundance, viability or pathogenicity, making it difficult to truly decipher their risk for public health. Anyway, it is estimated that the hospital costs of infections caused by opportunistic premise plumbing pathogens are about 0.6 billion USD per year in the USA, mainly for elderly and immunocompromised patients (Naumova et al. 2016). Several studies confirm the presence of *Legionella* species in drinking water (Rodríguez-Martínez et al. 2015; De Fillipis et al. 2018). Additionally, *Mycobacterium* species are increasingly isolated in tap waters (Donohue et al. 2015) and have shown to be correlated with isolates from clinical samples (Dovriki et al. 2016). Vaz-Moreira et al. (2017) studied resistance of bacterial isolates from drinking water against antibiotics and metals, and found that predominantly *Acinetobacter* and *Pseudomonas* presented the highest minimum inhibitory concentrations (MIC), illustrating the resilience of these genera to stresses. Both genera are known to have multidrug efflux pumps which might be beneficial for survival during chlorination (Shi et al. 2013, Karumathil et al. 2014). Moreover, both

genera are known to contain multidrug resistant bacteria, reinforcing their importance for public health (Potron et al. 2015).

In contrast to Chapter II, *Acinetobacter* was found at low relative abundance in the water samples investigated in this study, reaching a maximum relative abundance of 2.8 % in sample Su_6B_b. Further research is needed to find out the factors underlying these huge differences in relative abundance. Highest relative abundance of *Acinetobacter* was found after the chlorination step, which is in agreement with previous research showing an increase in *Acinetobacter* relative abundance after chlorine disinfection as well as its survival during chlorine exposure (Mathieu et al. 2009; Karumathil et al. 2014; Ma et al. 2017). Nevertheless, although relative abundance increased after chlorination, the results from the qPCR analysis suggest a decrease in absolute *Acinetobacter* abundance after chlorination, followed by an increase again during distribution. Compared to amplicon sequencing approaches using universal primers, qPCR methods using specific primer sets enable more accurate determinations of the relative and absolute abundance of community members (Zhang et al. 2017).

Culturing and isolation resulted in a broad collection of putative acinetobacters. Sequencing of the *rpoB* gene suggested the presence of 14 known *Acinetobacter* species (> 95 % *rpoB* sequence identity with type strains, La Scola et al. 2006) and a number of unclassified strains. These unclassified isolates could represent novel species, yet further genotypic and biochemical characterization is needed for proper identification (Diancourt et al. 2010, Hu et al. 2018). Several species found in this study have been found in aquatic environments, including drinking water. For example, *A. albensis*, *A. bohemicus* and *A. harbinensis* and have been regularly encountered in environmental water and soils (Krizova et al. 2014; Li et al. 2014; Krizova et al. 2015). Further, species such as *A. calcoaceticus, A. dispersus*, *A. guillouiae* and *A. pittii* have been isolated from soil, water and human samples (Bouvet and Grimont. 1986; Bouvet and Jeanjean. 1989 Nemec et al. 2010; Nemec et al. 2011; Nemec et al. 2016). Three *Acinetobacter* species were isolated in the final drinking water, including *A. guillouiae*, *A. johnsonii* and *A. lwoffi*i. *Acinetobacter lwoffii* and *A. johnsonii* have been previously found in drinking water (Narciso-da-Rocha et al. 2013). Furthermore, *A. lwoffii* has been isolated from immunocompromised patients with bacteremia (Ku et al. 2000; Tega et al. 2007). Similar observations have been made for *A. johnsonii*, which is also associated with vascular catheter-related bloodstream infections (Seifert et al. 1993a). Both species were also found in an 8-years survey of *Acinetobacter* infections in a Dutch university hospital (van den Broek et al. 2009). Furthermore, *A. johnsonii* has been isolated from human feces samples (Dijkshoorn et al. 2005). More generally, it has been shown that *Acinetobacter* increased in feces of mice that drank tap water (Dias et al. 2018), showing its strong capability to invade and colonize animal guts. *Acinetobacter baumannii* is the most common species to cause *Acinetobacter* infection, (Visca et al. 2011), but this species was not isolated from our water samples. Nevertheless, the amplicon sequencing approach revealed a number of sequences that showed high similarity with *A. baumannii*. Generally *A. baumannii* is not believed to be an inhabitant of aquatic environments, however, recently it has been recovered from water of a wastewater treatment plant and river water (Higgins et al. 2018;

Tsai et al. 2018). In addition to its opportunistic pathogenic character, *Acinetobacter* is known for its multi-drug resistance (Perez et al. 2007). Importantly, in a previous study it was shown that microorganisms with antibiotic resistance genes may persist during drinking water treatment systems and are carried by bacteria which are able to grow in the distribution system (Xi et al. 2009; Su et al. 2018). It has also been observed that multidrug resistant bacteria better withstand chlorination treatment (Armstrong et al. 1980, 1981; Murray et al. 1984). Further investigation on the diversity of *Acinetobacter* species, their persistence in drinking water systems, their potential pathogenicity and their antibiotic resistance profiles should clarify the importance of these species in drinking water.

3.4.3 Impact of physicochemical parameters on the bacterial community composition (or vice versa)

Total organic carbon (TOC), dissolved oxygen (DO) and trihalomethanes concentration were found to explain the variation in the household tap water bacterial community composition. During summer lower concentrations of DO and trihalomethanes were observed compared to winter. Indeed, TOC concentration is known to vary between different seasons and is correlated with differences in water temperature, and may therefore alter the bacterial community composition or influence microbial activity (Revetta et al. 2016). This is especially the case when surface water is used as source water which might result in an increase of dissolved organic matter, micropollutants and pathogens (Delpla et al. 2009). Trihalomethanes concentrations were lower in tap water from sampling location A in comparison to household tap waters from other locations (Ed, Ek, H, K). Interestingly, the bacterial community in the drinking water sampled at location A showed a higher relative abundance of *Methylophilus* species (Fig. S3.6, Supporting Information). Members of *Methylophilus* are known to be able to use methane as a carbon source and have been shown to dehalogenate chlorinated methanes (Bader and Leisinger 1994), and may therefore explain the lower trihalomethanes concentration at sampling location A. Higher abundance of *Methylophilus* in tap water of sampling location A may also be explained by the absence of free chlorine in the delivered drinking water. It has been shown that under chloramination disinfection regimes members of the family *Methylophilaceae* are more dominant than under chlorination disinfection regimes, probably due to lower levels of free chlorine after chloramination than after chlorination (Hwang et al. 2012).

Altogether, from this study it can be concluded that production line A had a more diverse bacterial community composition than production line B. However, the final drinking waters had a similar bacterial community composition. For line A, a clear shift in bacterial community composition was observed after the slow sand filter, which drastically altered the community composition (increase in unclassified sequences, *Acidobacteria*, *Chlamydiae*, *Firmicutes*, *Latescibacteria*, *Nitrospirae*, *Parcubacteria* and *Verrucomicrobia*; decrease in *Actinobacteria* and *Bacteroidetes*); for line B not such clear shifts were observed, except for the final chlorination step. In both production lines, chlorination was found to strongly affect the bacterial community composition, leading to a decrease in relative

abundance of *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, and *Latescibacteria* and an increase in unclassified OTUs, *Planctomycetes*, *Parcubacteria*, *Chlamydiae*, and *Firmicutes*. In both line the total amount of bacteria decreased after each treatment step and the lowest amount of total bacteria were found after chlorination. An increase was noticed within the household tap water of line A but not in the storage tank of line B. Further, qPCR analysis revealed a higher presence of *Acinetobacter* DNA in line B in comparison to line A. Additionally, more *Acinetobacter* isolates were obtained from line B than line A. More research is needed to find out the underlying mechanisms confirming and explaining these observations. In the finished waters, three *Acinetobacter* species were isolated, including *A. johnsonii*, *A. lwoffii* and *A. guillouiae*. In general, most environmental variables measured in the household tap water showed seasonal variation rather than geographical variation with the exception of the trihalomethanes concentration. The household tap water in location A showed a reduced concentration of trihalomethanes which could be explained by an increase of the relative abundance of *Methylophilus* species.

Chapter IV: Genotypic and phenotypic diversity of *Acinetobacter* isolates from clinical and environmental sources, with special emphasis on (drinking) water

4.1 Introduction

The *Acinetobacter* genus comprises a large group of species that are known to flourish in diverse natural ecosystems as well many as man-made environments (Doughari et al. 2011; Touchon et al. 2014). *Acinetobacter* spp. frequently occur in soil, aquatic environments including drinking water, insect guts and plant related environments such as nectar and tree bark (e.g. Anandham et al. 2010; Vaz-Moreira et al. 2011; Álvarez-Pérez et al. 2013; Choi et al. 2013; Narciso-da-Rocha et al. 2013; Kim et al. 2014; Krizova et al. 2014; Li et al. 2015; Adewoyin and Okoh, 2018). Also in this PhD study, *Acinetobacter* was commonly found in drinking water and drinking water systems (Van Assche et al. 2018; Chapter II and Chapter III). Especially their versatile metabolism, biofilm formation ability and resistance to disinfectants make them ideally suited to thrive and persist in the drinking water environment (Rodríguez-Baño et al. 2008; Bhargava et al. 2010; Simões et al. 2010; Peleg et al. 2012). Furthermore, particular *Acinetobacter* species are well adapted to different human body sites, often causing opportunistic infections in certain patient populations (e.g. immunocompromised and critically ill patients, and those undergoing long-term care following severe burns or traumatic injury) (Dijkshoorn et al. 2007). Pneumonia, bacteremia, urinary tract infection, and skin, soft-tissue and wound infections are the most common manifestations of *Acinetobacter* (Dijkshoorn et al. 2007; Wong et al. 2017). Also, globally it has been noticed that multi-drug resistant *Acinetobacter* strains have increasingly emerged, thereby representing an additional threat to public health (Van Looveren et al. 2004; Zhang et al. 2009; Roca et al. 2012, Harding et al. 2018). This is partially due to the ability of *Acinetobacter* spp. to acquire genes that encode diverse resistance mechanisms through horizontal transfer of plasmids and integrons. A wide variety of drug-inactivating enzymes such as β-lactamases and aminoglycoside-modifying enzymes may be transferred through horizontal acquisition. Within the genus, *A. baumannii* is most often the causative agent of infections (Towner, 2009). Moreover, whereas previously *Acinetobacter* spp. have been associated with opportunistic infections that were rare and of modest severity, the last decades have seen an increase in both the incidence and severity of *A. baumannii* infections, with the main targets being patients in intensive-care units (Gootz and Marra, 2008). Yet, several other species such as members of the *A. calcoaceticus-A. baumannii* (Acb) complex as well as species like *A. haemolyticus*, *A. lwoffii*, *A. junii*, *A. guillouiae* and *A. johnsonii* can cause nosocomial infections (Seifert et al. 1993a; Ku et al. 2000; Tega et al. 2007; van den Broek et al. 2009; Visca et al. 2011).

Molecular (sub)typing methods such as ribotyping, RAPD-PCR, repetitive element sequence-based (REP)-PCR, AFLP, PFGE and MLST and more recently whole genome sequencing (WGS) have been developed for *Acinetobacter* spp. and have been used to study relationships between clinical isolates in epidemiological studies (Gerner-Smidt, 1992; Gerner-Smidt and Tjernberg, 1993; Seifert and Gerner-Swidt, 1995; Wisplinghoff et al. 2000; van den Broek et al. 2009; Fitzpatrick et al. 2016). Despite the acknowledged importance of many *Acinetobacter* species as nosocomial pathogens, still relatively little is known about their epidemiology. It is, however, reasonable to assume that

Acinetobacter strains find their way into clinical environments through different environmental sources, including contaminated water supplies. To test this hypothesis and to determine the level of relatedness between isolates from different environments a comprehensive study of the relatedness of 58 isolates belonging to four *Acinetobacter* species that are associated with human infections was carried out using phenotypic and genotypic methods. Isolates were phenotyped using Biolog's GENIII identification microplate, which analyzes the performance of a microorganism in 94 phenotypic tests, including 71 carbon source utilization assays and 23 chemical sensitivity assays. The same technology has also been used to investigate the presence of a phylogenetic signal in phenotypic traits in *Acinetobacter* spp (Van Assche et al. 2017; Chapter V). Spearman rank correlation analysis was performed to determine whether the ability to use different carbon sources co-vary between the different isolates. Additionally, antibiotic susceptibility testings were generated using 15 antibiotic compounds. Further, isolates were genotyped by partial sequencing of the RNA polymerase beta subunit gene (*rpoB*), and a Mantel test was performed to assess correlations between phenotypic and *rpoB* gene sequence similarity.

4.2 Materials and Methods

4.2.1 Bacterial isolates

A collection of 58 *Acinetobacter* isolates was used in this study (Table 4.1), representing four species that have been associated with human opportunistic infections, including *A. calcoaceticus* (14 isolates), *A. guillouiae* (11 isolates), *A. johnsonii* (24 isolates) and *A. lwoffii* (9 isolates) (Table 4.1). Among these, 22 isolates were isolated from human or veterinary clinical samples, 32 from water samples (surface water, water from a drinking water production facility, and tap water), and four from other environmental samples (Table 4.1). A number of isolates were obtained previously in this doctoral research (isolates with designation "AVA"; Chapter III) or were isolated in this study from the lab's tap water (Sint Katelijne Waver, Belgium). Isolation was performed with the Dijkshoorn's medium and plating on R2A or TSA agar as previously described (Chapter III). The rest of the isolates were kindly provided by colleague researchers or obtained from international culture collections. Identifications were performed or confirmed by AFLP fingerprinting, sequencing part of the 16S ribosomal RNA (rRNA) gene and/or sequencing part of the *rpoB* gene. Isolates were stored at -80 °C in nutrient broth No. 2 (Oxoid, Basingstoke, UK, 25 g/L), containing 15 % (v/v) glycerol (VWR, Fontenay-sous-Bois, France).

Table 4.1: Overview of Acinetobacter isolates used in this study.

Strain identifier[a]	Highest match *rpoB* gene[b]	Source of isolation[c]	Origin[d]	Geographical origin	Year of isolation	GenBank accession N° *rpoB* gene[e]	Non-WT traits[f]
AVA 009B	*A. calcoaceticus*, strain CIP 81.8^T (98.4 %)	Aq	HTW	Belgium	2015	MN317400	S
AVA 012A	*A. calcoaceticus*, strain CIP 81.8^T (98.1 %)	Aq	HTW	Belgium	2015	MN317402	AML
AVA 044A2d	*A. calcoaceticus*, strain CIP 81.8^T (98.2 %)	Aq	DWTP	Belgium	2016	MN317406	
AVA 061A2d	*A. calcoaceticus*, strain CIP 81.8^T (98.4 %)	Aq	DWTP	Belgium	2016	MN317411	
AVA 077A2d	*A. calcoaceticus*, strain CIP 81.8^T (98.2 %)	Aq	DWTP	Belgium	2016	MN317415	S
DSM 30006^T	*A. calcoaceticus*, strain CIP 81.8^T (100 %)	Env	Soil	The Netherlands	Before 1911	EF611388	
LUH 2005	*A. calcoaceticus*, strain CIP 81.8^T (98.6 %)	Cli	Amputation stump	The Netherlands	1994	HQ123420	
LUH 9144	*A. calcoaceticus*, strain CIP 81.8^T (99.7 %)	Cli	Catheter	The Netherlands	2004	MN317430	S
LUH 12679	*A. calcoaceticus*, strain CIP 81.8^T (98.6 %)	Cli	Sputum	Czech Republic	2005	HQ123423	
LUH 14369	*A. calcoaceticus*, strain CIP 81.8^T (98.9 %)	Aq	Water	Czech Republic	2008	KU961609	
M 23	*A. calcoaceticus*, strain CIP 81.8^T (97.9 %)	Env	Nectar	Belgium	2013	MN317431	
RUH 944	*A. calcoaceticus*, strain CIP 81.8^T (99.7 %)	Cli	Drain	The Netherlands	1984	HQ123417	
RUH 2202	*A. calcoaceticus*, strain CIP 81.8^T (99.7 %)	Cli	Wound	Sweden	Unknown	HQ123418	AML
RUH 2203	*A. calcoaceticus*, strain CIP 81.8^T (98.5 %)	Cli	Wound	Sweden	Unknown	HQ123419	
AVA 011A	*A. guillouiae*, strain DSM 590^T (99.5 %)	Aq	HTW	Belgium	2015	MN317401	K, AML
AVA 025A2d	*A. guillouiae*, strain DSM 590^T (99.9 %)	Aq	HTW	Belgium	2016	MN317405	
AVA 059B2d	*A. guillouiae*, strain DSM 590^T (99.5 %)	Aq	Surface water	Belgium	2016	MN317409	AML
AVA 117B2d	*A. guillouiae*, strain DSM 590^T (99.2 %)	Aq	Surface water	Belgium	2016	MN317426	K, AML
AVA 118A2d	*A. guillouiae*, strain DSM 590^T (99.3 %)	Aq	DWTP	Belgium	2016	MN317427	AML
LUH 5606	*A. guillouiae*, strain DSM 590^T (98.3 %)	Aq	Fresh water and sediment	Denmark	1997	FJ754451	CIP, TE, SXT
LUH 5653	*A. guillouiae*, strain DSM 590^T (99.2 %)	Cli	Blood	The Netherlands	1999	FJ754453	AML
LUH 7830	*A. guillouiae*, strain DSM 590^T (99.3 %)	Vet	Eye cat	The Netherlands	2001	FJ754457	AML
M 24	*A. guillouiae*, strain DSM 590^T (98.6 %)	Env	Nectar	Belgium	2013	MN317432	
RUH 2234	*A. guillouiae*, strain DSM 590^T (99.4 %)	Cli	Contact lens	Sweden	Unknown	MN317433	AML
RUH 2861^T	*A. guillouiae*, strain DSM 590^T (100 %)	Env	Sewage	Unknown	Before 1951	EU477117	K
AVA 013A	*A. johnsonii*, strain DSM 6963^T (99.2 %)	Aq	Surface water	Belgium	2016	MN317403	NET, TE
AVA 016A	*A. johnsonii*, strain DSM 6963^T (98.7 %)	Aq	DWTP	Belgium	2016	MN317404	
AVA 059A2d	*A. johnsonii*, strain DSM 6963^T (99.3 %)	Aq	Surface water	Belgium	2016	MN317408	

AVA 060A2d	*A. johnsonii*, strain DSM 6963^T (99.4 %)	Aq	DWTP	Belgium	2016	MN317410	
AVA 072A2d	*A. johnsonii*, strain DSM 6963^T (97.9 %)	Aq	DWTP	Belgium	2016	MN317412	S
AVA 074A2d	*A. johnsonii*, strain DSM 6963^T (98.4 %)	Aq	DWTP	Belgium	2016	MN317413	
AVA 086A3d	*A. johnsonii*, strain DSM 6963^T (99.8 %)	Aq	HTW	Belgium	2016	MN317418	SXT
AVA 090A2d	*A. johnsonii*, strain DSM 6963^T (99.2 %)	Aq	DWTP	Belgium	2016	MN317419	SXT, S
AVA 092A2d	*A. johnsonii*, strain DSM 6963^T (98.0 %)	Aq	DWTP	Belgium	2016	MN317420	
AVA 098A2d	*A. johnsonii*, strain DSM 6963^T (98.5 %)	Aq	DW	Belgium	2016	MN317421	SXT
AVA 100B2d	*A. johnsonii*, strain DSM 6963^T (99.6 %)	Aq	Surface water	Belgium	2016	MN317422	
AVA 101A2d	*A. johnsonii*, strain DSM 6963^T (99.3 %)	Aq	DWTP	Belgium	2016	MN317423	DOR, IMI, MRP, AML
AVA 117A2d	*A. johnsonii*, strain DSM 6963^T (98.6 %)	Aq	Surface water	Belgium	2016	MN317425	
AVA 119A2d	*A. johnsonii*, strain DSM 6963^T (98.6 %)	Aq	DWTP	Belgium	2016	MN317428	
AVA 121A2d	*A. johnsonii*, strain DSM 6963^T (99.1 %)	Aq	DWTP	Belgium	2016	MN317429	
CCUG 58904	*A. johnsonii*, strain DSM 6963^T (98.9 %)	Cli	Blood	Sweden	2009	MN317392	
CCUG 60467	*A. johnsonii*, strain DSM 6963^T (99.4 %)	Cli	Larynx	Sweden	2010	MN317393	
CCUG 60882	*A. johnsonii*, strain DSM 6963^T (98.8 %)	Cli	Blood	Sweden	2011	MN317394	
CCUG 61200	*A. johnsonii*, strain DSM 6963^T (98.3 %)	Cli	Blood	Sweden	2011	MN317395	
LMG 1018	*A. johnsonii*, strain DSM 6963^T (99.6 %)	Cli	Chronic conjunctival suppuration	UK	Before 1967	MN317396	
LMG 1302	*A. johnsonii*, strain DSM 6963^T (98.3 %)	Cli	Clinical material	Hungary	Unknown	MN317397	S
RUH 2231^T	*A. johnsonii*, strain DSM 6963^T (100 %)	Cli	Duodenum	Unknown	Before 1963	EU477113	S
RUH 2857	*A. johnsonii*, strain DSM 6963^T (99.0 %)	Cli	Urine	Sweden	1980-81	KU961616	SXT
RUH 2859	*A. johnsonii*, strain DSM 6963^T (99.5 %)	Cli	Urine	Sweden	1980-81	KU961617	
AVA 057A2da	*A. lwoffii*, strain NCTC 5866^T (98.6 %)	Aq	HTW	Belgium	2016	MN317407	SXT, S
AVA 076A2d	*A. lwoffii*, strain NCTC 5866^T (95.8 %)	Aq	DWTP	Belgium	2016	MN317414	
AVA 080A2d	*A. lwoffii*, strain NCTC 5866^T (98.6 %)	Aq	DW	Belgium	2016	MN317416	
AVA 080B2d	*A. lwoffii*, strain NCTC 5866^T (98.6 %)	Aq	DW	Belgium	2016	MN317417	
AVA 113A3d	*A. lwoffii*, strain NCTC 5866^T (100 %)	Aq	HTW	Belgium	2016	MN317424	
LMG 1136	*A. lwoffii*, strain NCTC 5866^T (99.1 %)	Cli	Urine	U.S.S.R.	Before 1967	MN317398	
LMG 1301	*A. lwoffii*, strain NCTC 5866^T (98.3 %)	Cli	Clinical material	Hungary	Unknown	MN317399	SXT, S
LUH 1710	*A. lwoffii*, strain NCTC 5866^T (98.6 %)	Cli	Gangrenous lesion	Italy	Before 1945	KU961624	
RUH 2219^T	*A. lwoffii*, strain NCTC 5866^T (100 %)	Cli	Unknown	France	Before 1940	EU477111	SXT

[a] AVA, isolated in this PhD study; CCUG, Culture Collection University of Gothenburg, Sweden; DSM, Leibinz Institute DSMZ-German collection of Microorganisms and Cell Culture, Germany; LMG, Belgium Co-ordinated Collections of Microorganisms – Laboratory of Microbiology Ghent, Belgium; M, Collection of the Laboratory for Process Microbial Ecology and Bioinspirational Management, KU Leuven, Belgium; LUH, Leiden University Hospital, The Netherlands; RUH, Rotterdam University Hospital, The Netherlands.
[b] Nearest neighbor based on a BLAST search of partial *rpoB* genes in GenBank against type strains. The percentage of sequence identity on a total of 840 (± 32, SD) bp is given between brackets. Generally a 95 % sequence similarity is adequate for species identification based on *rpoB* gene sequences (La Scola et al. 2006).
[c] aq, aquatic; env, environmental; cli, clinical; vet, veterinary.
[d] HTW, household tap water; DWTP, non-finished drinking water from a drinking water production facility; DW, finished drinking water, sampled within a drinking water production facility. See Table 3.4 for additional information on the sampling location.
[e] RNA polymerase beta subunit (*rpoB*) gene.
[f] Antibiotics are shown for which the isolates had a non-wild type trait. DOR, Doripenem; IMI, Imipenem; MRP, Meropenem; CIP, Ciprofloxacin; K, Kanamycin; NET, Netilmicin; TE, Tetracyclin; AML, Amoxicillin; SXT, Sulfamethoxazole/tri-methoprim; CS, Colistin sulfate; S, Streptomycin. See Table S4.2 (Supporting Information) for an overview of the average inhibition zones.

4.2.2 Genotypic analysis

Isolates for which no *rpoB* gene sequence was available in GenBank were subjected to partial *rpoB* gene sequencing as described in Chapter III. Briefly, following DNA extraction, PCR amplification was performed using the primers Ac696F (5'-TAYCGYAAAGAYTTGAAAGAAG-3') and Ac1598R (5'-CGBGCRTGCATYTTGTCRT-3') (La Scola et al. 2006) in a reaction volume of 20.0 µL, consisting of 0.15 mM of each dNTP, 0.5 µM of each primer, 1 unit Titanium Taq DNA polymerase, 1x Titanium Taq PCR buffer and 5 ng genomic DNA. Before amplification, DNA samples were denatured at 94 °C for 2 min, followed by 30 cycles of 45 s at 94 °C, 45 s at 55 °C and 45 s at 72 °C, with a final extension at 72 °C for 10 min. Amplicons were sequenced using the same forward and reverse primer as used for the DNA amplification. Subsequently, forward and reverse sequences were combined to form a consensus sequence, which was then subjected to a BLAST search against type materials in GenBank. For all other isolates, *rpoB* gene sequences were downloaded from GenBank and included in the study (Table 4.1). All *rpoB* gene sequences of the isolates investigated in this study were used to construct a phylogenetic tree, together with *rpoB* gene sequences for all validly named *Acinetobacter* species and previously defined genomic species. Together, this not only allowed (presumptive) identification of the isolates, but also enabled to visualize the (phylo)genetic relationships between the different isolates. Several studies have demonstrated the usefulness of *rpoB* gene sequences for the identification and taxonomic classification of various bacterial species, including acinetobacters (La Scola et al. 2006).

4.2.3 Phenotypic analysis

Biolog's GENIII microplate test (Biolog, Hayward, CA, USA) was carried out for all studied isolates, which provides a standardized method using 94 biochemical tests to profile and characterize a broad range of bacteria (Bochner, 1989). More particularly, the test panel contains 71 carbon sources and 23 chemical sensitivity assays (Table 4.2). The analysis was performed as described in Van Assche et al. (2017), according to the manufacturer's instructions. Briefly, strains were grown from a -80 °C stock culture on BUG agar for 24 hours at 33 °C. Subsequently, the strains were restreaked on BUG agar and incubated for another 24 hours at 33 °C. Next, inocula were prepared by swabbing cells from the agar surface and suspending them in Inoculation Fluid A (IF-A, Biolog, Hayward, CA, USA) until an optical density of 95 % was reached, which was determined using Biolog's turbidimeter. Each well of the GENIII microplates was then inoculated with 100 µL of the cell suspension. Subsequently, the GENIII microplates were incubated at 33 °C for 36 hours and read every 15 minutes using the OmniLog incubator/reader. Raw kinetic data were retrieved using the OmniLog – OL_PM_FM/Kin 1.30-: File Management/ Kinetic Plot Version software of Biolog. Next, area under the curve (AUC) was calculated by the OPM package (version 1.1.0, 2014-04-22) in R v.3.1.3 (R Core Team, 2015) using the 'splines' method and 'p.splines' option (Schumaker 2015). For each isolate two independent experiments were

performed which resulted in very similar kinetic curves and kinetic parameters. Therefore, data obtained for both replicates were averaged.

Table 4.2: Overview of the carbon source assimilation assays (columns 1 to 9) and chemical sensitivity assays (columns 10, 11, and 12) on Biolog's GENIII microplate (Colour legend: yellow = carbon sources; orange = osmotic stressors; blue = acidic stressors; red = toxins; green = chaotropes; grey = inhibitors with unknown mechanism of inhibition; and blank = positive control, negative control or the tetrazolium dyes used to evaluate the cellular responses (reduced to purple formazan for a positive response)).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Negative control	Dextrin	D-Maltose	D-Trehalose	D-Cellobiose	Gentiobiose	Sucrose	D-Turanose	Stachyose	Positive control	pH 6	pH 5
B	D-Raffinose	α-D-Lactose	D-Melibiose	β-Methyl-D-Glucoside	D-Salicin	N-Acetyl-D-Glucosamine	N-Acetyl-D-Mannosamine	N-Acetyl-D-Lactososamine	N-Acetyl Neuraminic Acid	1 % NaCl	4 % NaCl	8 % NaCl
C	α-D-Glucose	D-Mannose	D-Fructose	D-Galactose	3-Methyl Glucose	D-Fucose	L-Fucose	L-Rhamnose	Inosine	1 % Sodium Lactate	Fusidic Acid	D-Serine
D	D-Sorbitol	D-Mannitol	D-Arabitol	Myo-Inositol	Glycerol	D-Glucose-6-PO4	D-Fructose-6-PO4	D-Aspartic Acid	D-Serine	Troleandomycin	Rifamycin SV	Minocycline
E	Gelatin	Glycyl-L-Proline	L-Alanine	L-Arginine	L-Aspartic Acid	L-Glutamic Acid	L-Histidine	L-Pyroglutamic Acid	L-Serine	Lincomycin	Guanidine HCl	Niaproof 4
F	Pectin	D-Galacturonic acid	L-Galactonic Acid Lactone	D-Gluconic Acid	D-Glucuronic Acid	Glucuronamide	Mucic Acid	Quinic Acid	D-Saccharic Acid	Vancomycin[a]	Tetrazolium Violet	Tetrazolium Blue
G	P-Hydroxy-Phenylacetic Acid	Methyl Pyruvate	D-Lactic Acid Methyl Ester	L-Lactic Acid	Citric Acid	α-Keto-Glutaric Acid	D-Malic Acid	L-Malic Acid	Bromo-Succinic Acid	Nalidixic Acid[a]	Lithium Chloride	Potassium Tellurite
H	Tween 40	γ-Amino-Butyric Acid	α-Hydroxy-Butyric Acid	β-Hydroxy-D,L-Butyric Acid	α-Keto-Butyric Acid	Acetoacetic Acid	Propionic Acid	Acetic Acid	Formic Acid	Aztreonam	Sodium Butyrate	Sodium Bromate

[a] Vancomycin and nalidixic acid are particularly used to differentiate Gram positive from Gram negative bacteria.

In addition, isolates were subjected to antibiotic susceptibility testing. To this end, the disk diffusion method described by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (also known as the Kirby-Bauer method) was used (Hudzicki, 2009; EUCAST, 2019). Antibiotics were tested at commonly used concentrations and/or concentrations prescribed by the Clinical and Laboratory Standards Institute (CLSI)/EUCAST guidelines, and included doripenem (10 µg), imipenem (10 µg), meropenem (10 µg), ciprofloxacin (5 µg), amakacin (30 µg), gentamicin (10 µg), kanamycin (30 µg), netilmicin (10 µg), streptomycin (10 µg), tobramycin (10 µg), tetracyclin (30 µg), amoxicillin (25 µg), cephalothin (30 µg), colistin sulfate (50 µg), and a combination of sulfamethoxazole (23.75 µg) and trimethoprim (1.25 µg) (Liofilchem, Italy). In this way, in total, eight different groups of antibiotics were tested, including carbapenemases (doripenem, imipenem and meropenem), fluoroquinolones (ciprofloxacin), aminoglycosides (amakacin, gentamicin, kanamycin, netilmicin, streptomycin and tobracmycin), tetracyclines (tetracyclin), penicillin (amoxicillin), cephalosporins (cephalothin), polypeptide antibiotics (colistin sulfate), and sulfonamide antibiotic/dihydrofolate reductase inhibitors (combination of sulfamethoxazole and trimethoprim). *Pseudomonas aeruginosa* CCUG 17619 was used as a quality control strain and yielded the expected results, indicating that all experimental conditions were met to obtain robust data. For each isolate, two technical replicates were included. The diameter of the inhibition zone was measured and averaged across replicates. In order to evaluate whether the isolates tested had acquired tolerance against one or more antibiotics, for each antibiotic, strains were categorized as wild type (WT) or non-wild type (non-WT), according to the epidemiological cut-off (ECOFF, microbiological breakpoint) values from the EUCAST website (http://mic.eucast.org/Eucast2/), or when not present in the database, based on cut-off values previously determined by Hombach et al. (2012) and Narciso-da-Rocha et al. (2013). When inhibition zones were obtained smaller than the cut-off value, isolates were considered non-WT; when inhibition zones were larger than the cut-off value, isolates were considered WT (http://mic.eucast.org/Eucast2/). For the antibiotics doripenem, kanamycin, netilmicin and cephalotin no cut-off values were found for *Acinetobacter* spp. in previous studies. In this case, outliers were defined by a box plot representation and isolates which had an inhibition zone smaller than the first quartile subtracted with 1.5x the inter quartile range (IQR) were classified as non-WT (Narciso-da-Rocha et al. 2013). In general, this criterion was in good agreement with the values found by EUCAST when applied on the other antibiotics tested with a known ECOFF value. Isolates with an inhibition zone within the box plot and 1.5x the IQR value were considered WT.

4.2.3 Data analysis

GENIII trait differences were evaluated using a binary trait score (0/1) and the AUC values. A trait was considered positive (i.e. 1) if the AUC value exceeded 1.5x the value of the negative control (well A01). When this criterion was not met, the trait was considered negative (0). First, phenotypic differences were

evaluated based on substrate richness (i.e. the number of positive traits) and average well color development (AWCD) (i.e. the average AUC for traits) for both the metabolic phenotypes (nutrient score, NS) (column 1 to 9 excluding the negative control well, Table 4.2) and the chemical sensitivity traits (tolerance score, TS) (column 10 to 12, excluding the positive control well, Table 4.2). Statistical significance was evaluated for three different groupings: the first group was based on the taxonomic grouping of the isolates at species level, the second group was based on the isolation source of the strains (i.e. environmental/aquatic versus human clinical/veterinary), and the third group was based on species classification and the origin of the isolate. For each statistical analysis the homogeneity of variance and the normal distribution was evaluated by the Levene's test and the Shapiro-Wilk test, respectively. The Levene's test and Shapiro-Wilk test were performed using the R packages 'car' and 'stats'. Although most of the results of the Levene's test indicated a homogeneity of variance, usually one of the groups was not normally distributed according to the Shapiro-Wilk test. Hence, significant differences were evaluated using the Kruskal-Wallis test and a pairwise Wilcoxon rank sum test with Bonferroni *p* adjustment was used to measure the significant difference between groups. Both tests were performed using the R package 'stats'. The same statistical analysis was performed for each phenotype individually. Statistical differences were considered significant if the *p* value was < 0.05. Further, GENIII phenotype similarities/dissimilarities between isolates were explored by heatmap clustering. The distance between isolates was calculated with the Euclidean method and the clustering was performed with the ward.D2 method. The heatmap was generated using the 'ggplot2' package of R. In order to assess whether certain phenotypes were correlated, the Spearman rank correlation was measured using the 'Hmisc' R package. The results were visualized via the 'corrplot' R package and clustering of the ρ value was done by the ward.D2 method. Finally, to assess correlations between the variability of *rpoB* gene sequences and trait differences a Mantel test was performed. After sequence alignment, sequence similarities were evaluated using the 'bio3d' package of R and the distances were calculated based on the Euclidean method. The Mantel test was performed using the 'ape' package for R with 1000 permutations.

4.3 Results

4.3.1 Phylogenetic analysis

The phylogenetic tree created using the neighbor-joining (NJ) method and based on partial *rpoB* gene sequences (631 bp) revealed four distinct clades within our collection of isolates (Fig. 4.1), showing most homology with type strains of *A. calcoaceticus* (97.9 – 99.7 %), *A. guillouiae* (98.3 – 99.9 %), *A. johnsonii* (97.9 – 99.8 %) and *A. lwoffii* (95.8 – 99.1 %) (Table 4.1). Figure 4.1 further indicates that isolate RUH 2857 was somewhat different from the other isolates in the *A. johnsonii* clade. This isolate was obtained from the collection of the Leiden University Medical Center and has been classified as *A. johnsonii* by AFLP fingerprinting (pers. com. Lenie Dijkshoorn). Within the *A. lwoffii* clade, isolate

AVA 076A2d (obtained from a drinking water production facility) clustered slightly away from the other isolates, having a sequence identity percentage of 95.8 % with the *A. lwoffii* type strain. All isolates presumptively identified as *A. calcoaceticus* clustered together in the NJ tree with the *A. calcoaceticus* type strain. The same is also true for the isolates identified as *A. guillouiae*, which all fell in a single clade (Fig. 4.1).

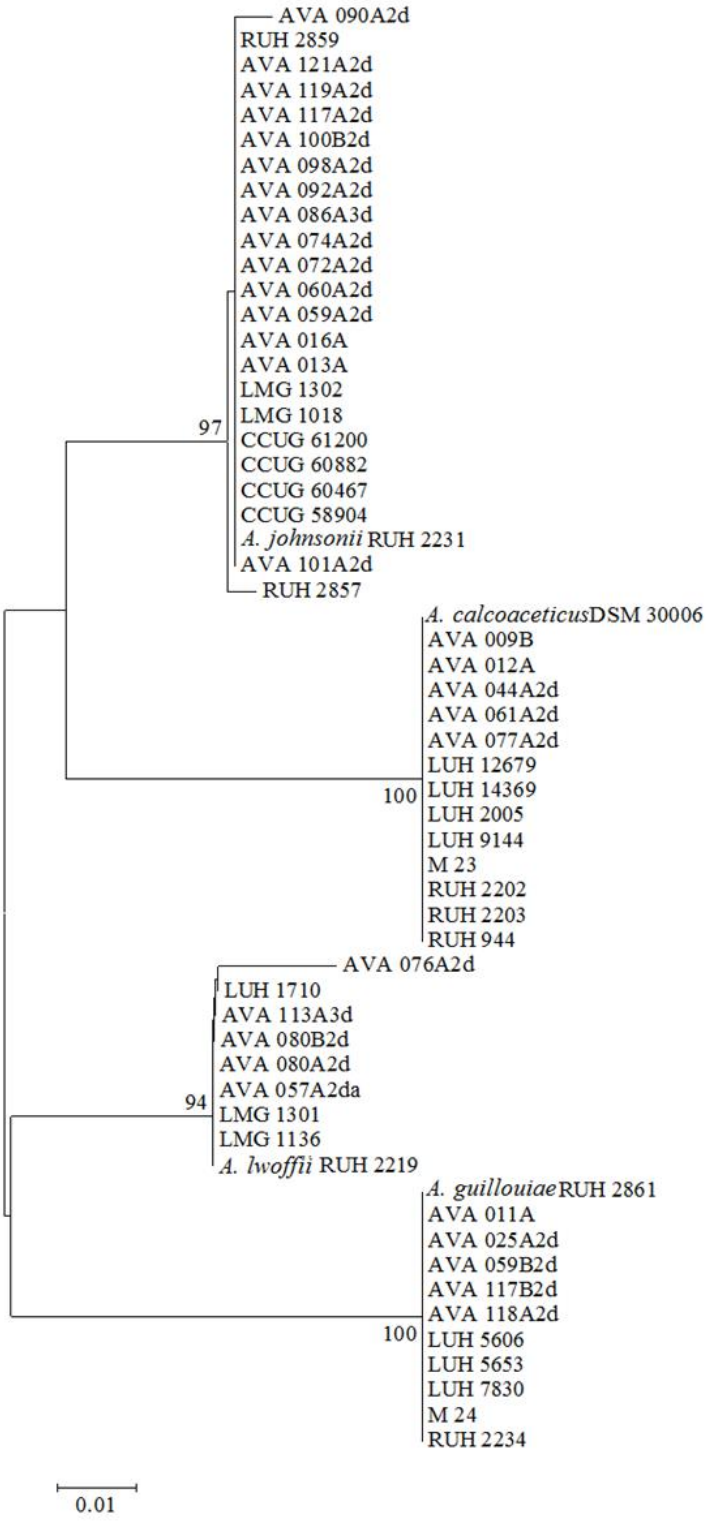
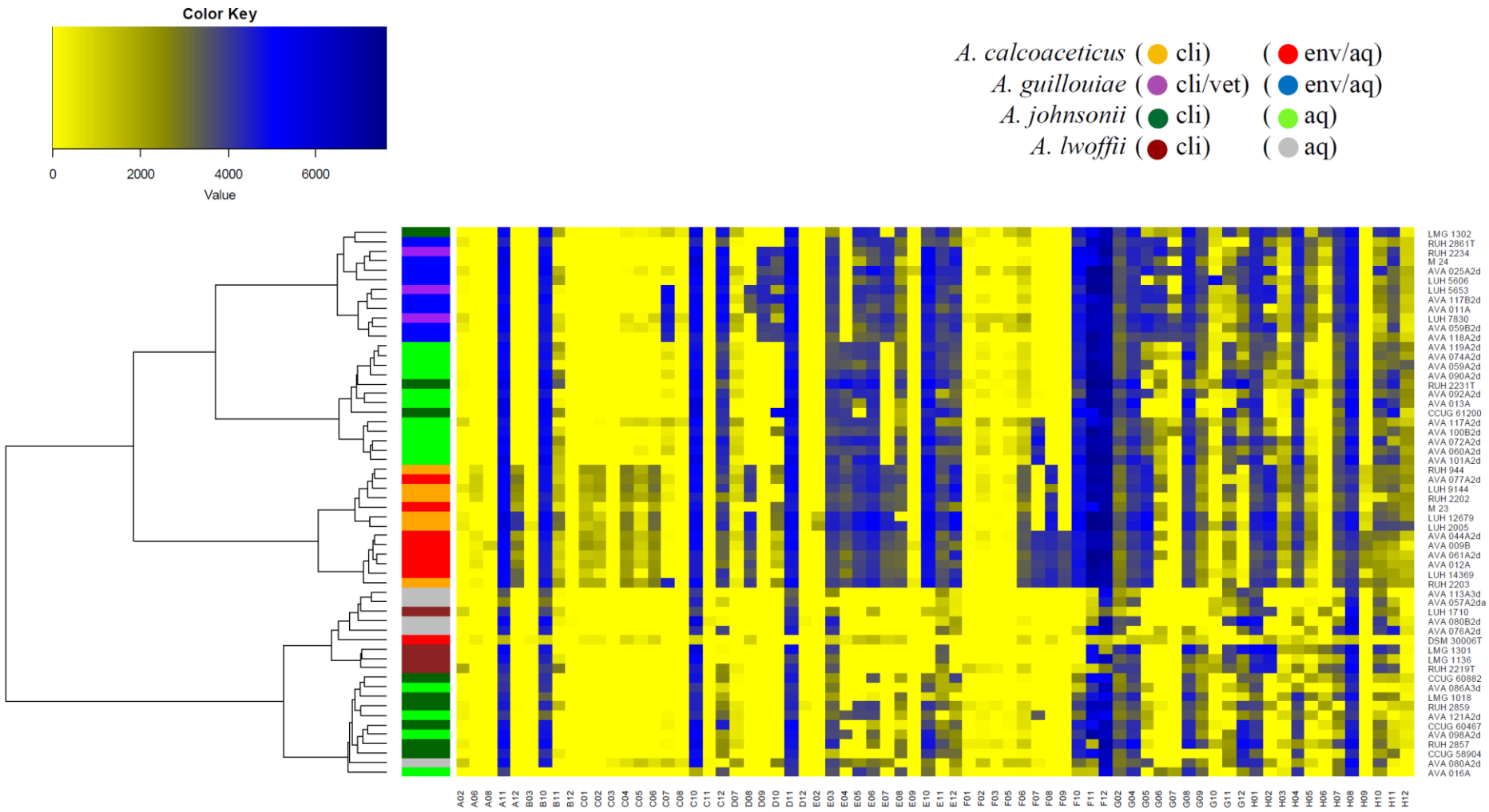


Figure 4.1: Neighbour-joining tree, based on partial *rpoB* gene sequences, showing the relationships of the different *Acinetobacter* isolates. Evolutionary distances were computed using the maximum composite likelihood method and are in units of the number of base substitutions per site. There were a total of 631 positions in the final dataset. Node support values (bootstrap percentages, based on 1000 simulations) ≥ 90 % are shown next to the branches.

4.3.2. Phenotypic profiling (Biolog GENIII microplates)

Phenotypic profiling using Biolog GENIII revealed four major clusters among the isolates tested (Fig. 4.2). While most isolates grouped based on species classification according to their *rpoB* gene sequence similarity, *A. johnsonii* isolates were divided over two separate groups, i.e. one linked to *A. guillouiae*, and the other one linked to *A. lwoffii*. Additionally, within each species isolates from a similar origin (aquatic/environmental vs clinical/veterinary) mostly yielded a similar metabolic profile (Fig. 4.2). The separation of the *A. johnsonii* isolates in two separate groups is mainly due to the fact that part of the isolates produced less strong signal in comparison to the other *A. johnsonii* isolates, thereby clustering more closely to the *A. lwoffii* isolates (Fig. 4.2). Yet, several phenotypes indicate that these isolates still had a number of traits in common with the other *A. johnsonii* isolates. For example, most *A. johnsonii* isolates were able to use L-glutamic acid (E06) and L-pyroglutamic acid (E08), while only some *A. lwoffii* isolates were able to use L-glutamic acid (E06), and none could use L-pyroglutamic acid (E08) (Table S4.1, Supporting Information). Furthermore, *A. lwoffii* isolates were sensitive to the chemical stressors D-serine (C12), niaproof 4 (E12), vancomycin (F10) and sodium bromate (H12), while *A. johnsonii* isolates were able to resist them. Additionally, all *A. johnsonii* isolates had a clear phenotypic difference with *A. guillouiae* and *A. calcoaceticus*. In comparison to *A. guillouiae*, isolates of *A. johnsonii* were not able to use D-serine (D09), and to use C6 sugars and formic acid (H09) in comparison to *A. calcoaceticus* (Table S4.1, Supporting Information). Strikingly, three isolates did not group together with strains from the same species. In particular, type strain *Acinetobacter calcoaceticus* DSM 30006^T did not cluster with the other *A. calcoaceticus* isolates, but instead clustered among the *A. lwoffii* isolates. This strain was isolated before 1911 and in its early years it was stored on slants and subcultured for many years. Therefore, this may have led to an altered phenotype, yielding a strong background signal in the GENIII microplate test. Further, isolate AVA 080A2d which was identified as *A. lwoffii* (Table 4.1; Fig. 4.1) grouped together with *A. johnsonii* isolates. Isolate AVA 080A2d was able to use some carbon sources such as L-histidine (E07) and citric acid (G05), while all other *A. lwoffii* isolates did not. Finally, based on GENIII phenotyping *A. johnsonii* LMG 1302 grouped together with the *A. guillouiae* isolates (Fig. 4.2).

Figure 4.2: Heatmap



representation showing differences in phenotypic traits determined by GENIII plates for the different *Acinetobacter* isolates investigated in this study (columns represent different phenotypes; for a list of the different test substrates and corresponding positions in GENIII plates, see Table 4.2). The color key (yellow-blue) is based on area under the curve (AUC) values. A Bray-Curtis distance matrix was used and the samples were clustered by a Ward.D2 agglomeration method. The origin and species classification of the isolates is indicated by different colors. Metabolic profiling using Biolog GENIII revealed four major clusters among the isolates tested. While most isolates grouped based on species classification, *A. johnsonii* isolates were divided over two separate groups, i.e. one linked to *A. guillouiae*, and the other one linked to *A. lwoffii*. Additionally, the clustering seems to suggest that within each species isolates from a similar origin (aquatic/environmental vs clinical/veterinary) yielded a similar metabolic profile.

From all carbon sources tested, 46 carbon sources were used (i.e. generating an average AUC value greater than 1.5 times that of the blank) by at least one *Acinetobacter* isolate (Table S4.1, Supporting Information). Carbon sources that did not yield a positive signal for any of the isolates tested included all sugar alcohols and polysaccharides (four compounds each). Acetic acid, tween 40 and methyl pyruvate were the only carbon sources resulting in a positive signal for all tested isolates. Grouping of isolates per species revealed significant differences in substrate richness based on the Kruskal-Wallis test ($p < 0.001$) (Fig. 4.3). *Acinetobacter calcoaceticus* had the highest substrate richness with an average of 31.8 (± 2.5, SD) carbon sources used. By contrast, *A. lwoffii* showed the lowest substrate richness using an average of 12.8 (± 7.5) carbon sources. *Acinetobacter guillouiae* and *A. johnsonii* showed an average substrate richness of 24.7 (± 4.1) and 20.0 (± 5.6), respectively. Results were significantly different ($p < 0.05$) between *A. calcoaceticus* and *A. lwoffii*, and between *A. calcoaceticus*/*A. lwoffii* and the other two species tested. Yet, based on AWCD all species were significantly different from each other ($p < 0.001$). The highest average AWCD nutrient score was observed for *A. calcoaceticus* (1,233 (± 284, SD), followed by *A. guillouiae*, *A. johnsonii* and *A. lwoffii* (1,101 (± 140), 748 (± 198) and 462 (± 219), respectively). When isolates were grouped based on species and origin, the substrate richness and AWCD scores were also significantly different according to the Kruskal-Wallis test ($p < 0.001$). Yet, the pairwise Wilcoxon test revealed this was between groups of different species and not between two groups of the same species but different origin. When isolates were grouped based on origin (environmental/aquatic versus clinical/veterinary) no significant differences were observed ($p > 0.05$).

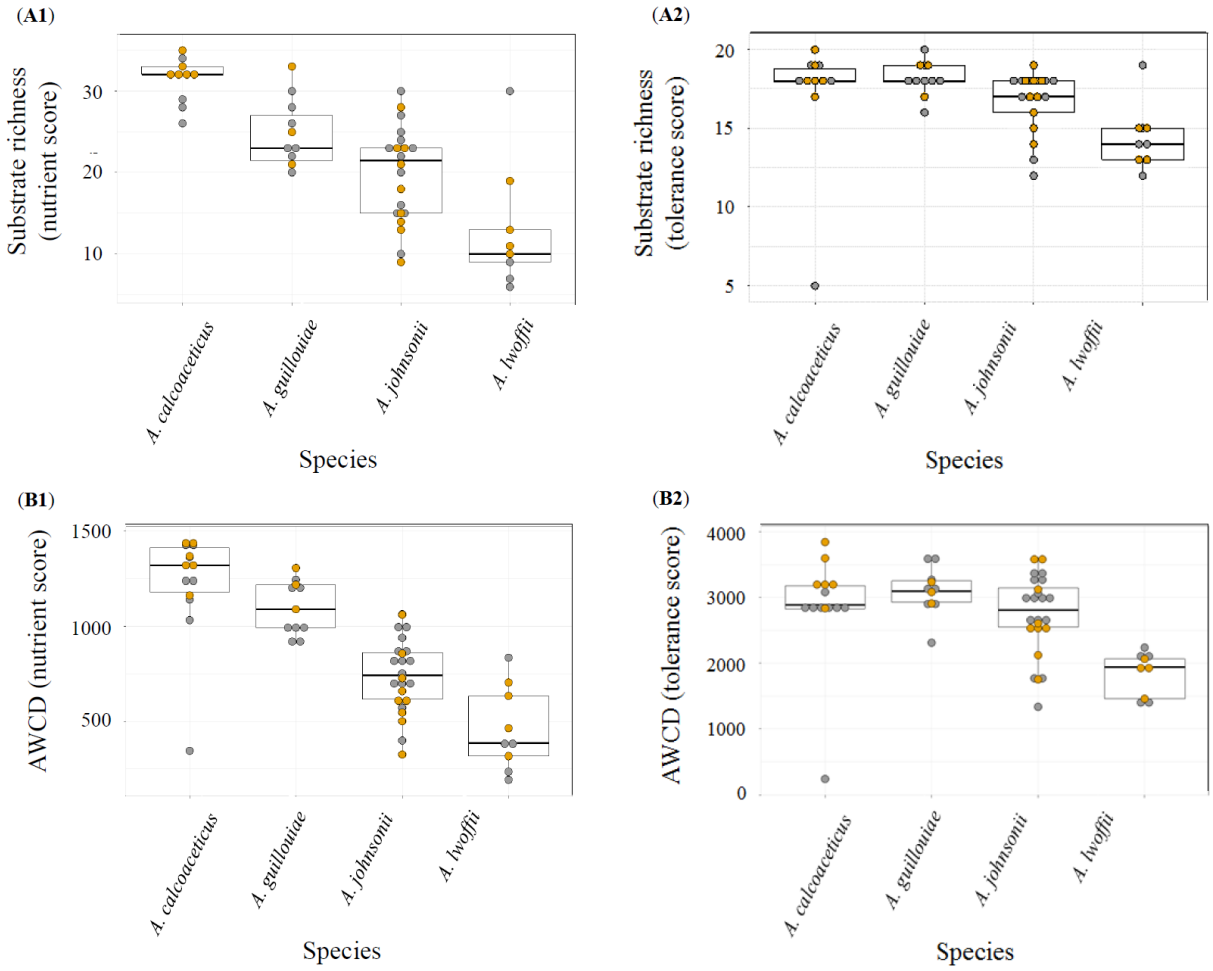


Figure 4.3: Boxplot representation of substrate richness (**A**) and average well color development (AWCD) (**B**) based on nutrient score (**1**) and tolerance score (**2**) when isolates were subjected to GENIII phenotyping. *Acinetobacter* isolates (n = 58) were grouped per species: *A. calcoaceticus* (n = 14), *A. guillouiae* (n = 11), *A. johnsonii* (n = 24) and *A. lwoffii* (n = 9) . The origin of isolates is indicated by color: orange, clinical/veterinary; grey, aquatic/environmental. The boxplots show the upper and lower quartiles. Values outside the upper and lower quartile but within 1.5 times the interquartile range are connected with a full line. Further, the median is plotted as a thick black line.

All chemical sensitivity assays (23 assays) gave a signal for at least one isolate. All isolates generated a positive signal for growth at pH 6 (A11), 1 % sodium lactate (C10) and the redox dyes tetrazolium violet (F11) and tetrazolium blue (F12) (Table S4.1, Supporting Information). Significant differences in richness (tolerance score) were found when isolates were grouped per species (p = 0.001). Tolerance to inhibitory chemicals for *A. lwoffii* was significantly lower than for *A. calcoaceticus* and *A. guillouiae* (p = 0.033 and 0.011, respectively), but not in comparison with *A. johnsonii* (p = 0.051) (Fig. 4.3). No differences were found between *A. calcoaceticus*, *A. guillouiae* and *A. johnsonii*. On average, *A. lwoffii* isolates tolerated 14.4 (± 2.0 SD) stressors, *A. johnsonii* 16.8 (± 1.8), *A. calcoaceticus* 17.4 (± 3.6) and *A. guillouiae* 18.2 (± 1.1) (Fig. 4.3). Based on AWCD values, *A. lwoffii* was also significantly different compared to the three other species (p < 0.005). The other *Acinetobacter* species were not significantly different from each other. The average AWCD value for *A. lwoffii* was 1,848 (± 335, SD) in comparison to 2,731 (± 614), 2,871 (± 820) and 3,095 (± 355) for *A. johnsonii*, *A. calcoaceticus* and

A. guillouiae, respectively (Fig. 4.3). A similar observation as for the nutrient scores was observed when isolates were grouped based on species and origin ($p \leq 0.01$). The pairwise Wilcoxon test revealed that the AWCD value (tolerance score) of the environmental/aquatic isolates of *A. guillouiae* was significantly different from the aquatic isolates of *A. lwoffii* ($p = 0.044$). No significant differences in richness and AWCD values of the tolerance score were observed when isolates were grouped by origin.

Evaluating each phenotype individually, eight carbon sources and seven chemical sensitivity assays did not generate significant differences according to the Kruskal-Wallis test when isolates were grouped based on species classification, origin (i.e. environmental/aquatic versus clinical/veterinary), or species classified by origin (combination of species classification and origin of isolation) (Fig. 4.4). Results for three carbon sources (i.e. D-melibiose (B03), L-fucose (C07) and Tween 40 (H01)) and one chemical stressor (aztreonam (H10) were significantly different based on only species classification. The carbon sources glycyl-L-proline (E02) and mucic acid (F07) were significantly different based on both species classification and origin. Mucic acid (F07) was utilized by several environmental/aquatic isolates belonging to the species *A. calcoaceticus* and *A. johnsonii*, while only one clinical isolate (belonging to *A. calcoaceticus*) was found to do so (Fig. S4.1, Supporting Information). Additionally, five other carbon sources (i.e. pectin (F01), L-galactonic acid lactone (F03), methyl pyruvate (G02), L-lactic acid (G04) and acetoacetic acid (H06)) resulted in significant differences when isolates were grouped per origin (Fig. 4.4). For pectin, L-galactonic acid lactone and acetoacetic acid, metabolic activity was related to isolates from clinical settings. Further, clinical isolates tended to yield a stronger signal for methyl pyruvate and L-lactic acid (Fig. S4.1, Supporting Information). Other phenotypes were significantly different based on species classification with or without subdivision according to their origin of isolation (Fig. 4.4). A pairwise Wilcoxon test indicated that this was rather due to species classification than to their origin of isolation. For example, some amino acids were found as key elements to distinguish the different *Acinetobacter* species studied. L-Arginine (E04) was used by most *A. calcoaceticus* and aquatic *A. johnsonii* isolates (Fig. S4.2, Supporting Information), whereas L-histidine (E07) was utilized by most *A. calcoaceticus* and *A. guillouiae* isolates but not by *A. johnsonii* and *A. lwoffii* isolates (Fig. S4.2, Supporting Information). Additionally, most *A. lwoffii* isolates did not consume L-aspartic acid (E05), L-glutamic acid (E06) and L-pyroglutamic acid (E08) while other species generally did (Fig. S4.2, Supporting Information).

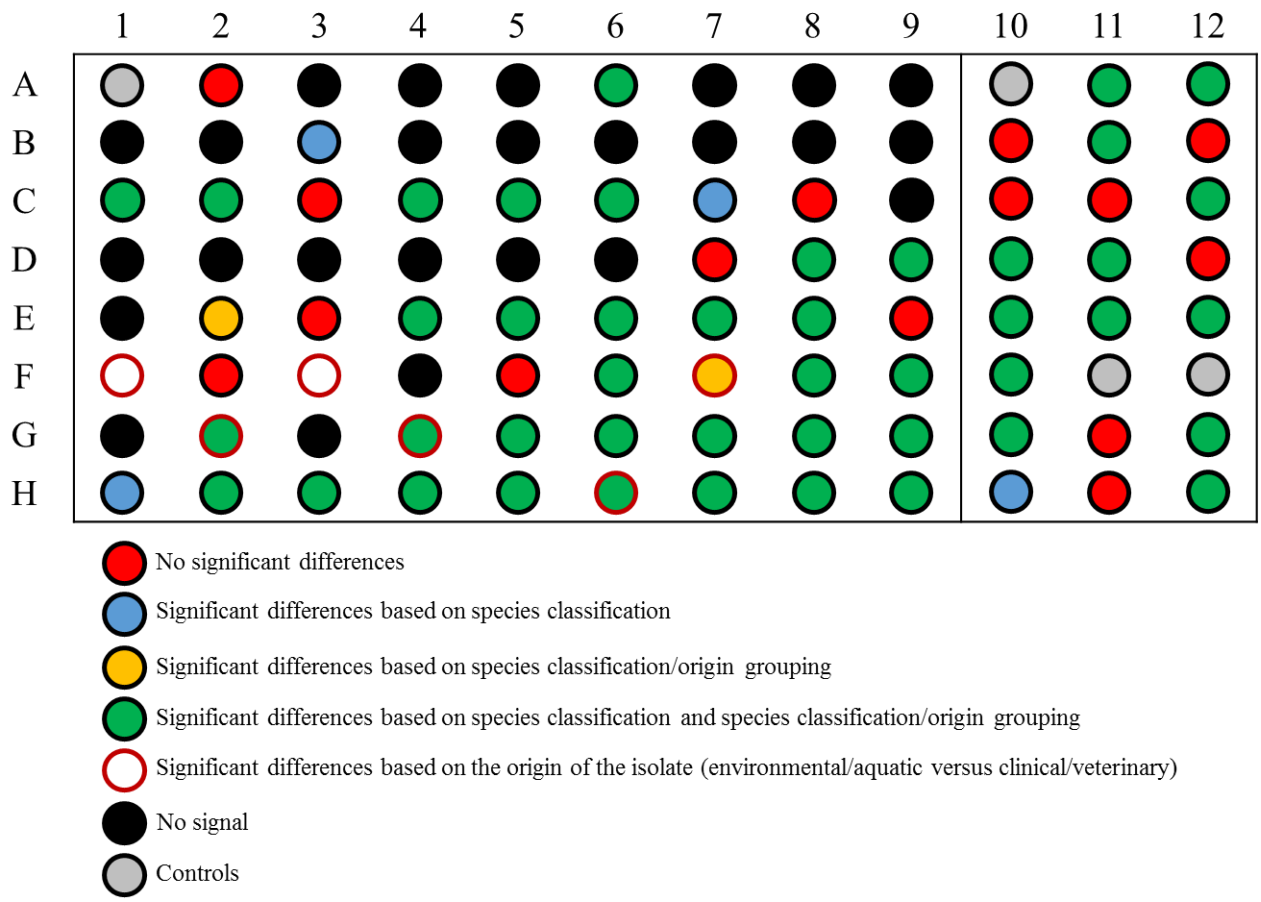


Figure 4.4: Overview of phenotypic traits determined using GENIII plates with significant difference ($p < 0.05$) as determined using the Kruskal-Wallis analysis. Grouping was done based on species classification (i.e. *A. calcoaceticus* (n = 14), *A. guillouiae* (n = 11); *A. johnsonii* (n = 24) and *A. lwoffii* (n = 9)), origin (i.e. environmental/aquatic (n = 36) and clinical/veterinary (n = 22) and species classification/origin (i.e. environmental/aquatic *A. calcoaceticus* isolates (n = 8); clinical *A. calcoaceticus* isolates (n = 6); environmental/aquatic *A. guillouiae* isolates (n = 8); clinical/veterinary *A. guillouiae* isolates (n = 3); aquatic *A. johnsonii* isolates (n = 15); clinical *A. johnsonii* isolates (n = 9); aquatic *A. lwoffii* isolates (n = 5) and clinical *A. lwoffii* isolates (n = 4)). Phenotypes that did not generate any response for any strain are presented by black dots. Wells A01, A10, F11 and F12 are the negative control well, the positive control well and the tetrazolium violet and tetrazolium blue control wells, respectively. See Table 4.2 for the different test substrates and corresponding positions in GENIII plates.

Comparison of the different phenotypes based on binary traits confirms the importance of certain carbon sources for species differentiation. Pectin (F01), L-galactonic acid lactone (F03), mucic acid (F07) and acetoacetic acid (H08) allow differentiation of isolates based on grouping by origin. Nevertheless, no single GENIII assay allowed to clearly differentiate between clinical/veterinary isolates and aquatic/environmental isolates (Table 4.3). On the other hand, the carbon sources gentiobiose (A06), α-D-glucose (C01), D-mannose (C02), quinic acid (F08) and formic acid (H09) represented carbon sources that were mostly used by isolates identified as *A. calcoaceticus*. D-Serine (D09) was used by almost all *A. guillouiae* isolates (10 out of 11 isolates) while it was not used by any other species tested. *Acinetobacter lwoffii* isolates were not able to use L-pyroglutamic acid (E08), while most isolates of other species did. Additionally, D-malic acid (F07) was used by most *A. guillouiae* isolates and some *A. johnsonii* isolates, but not by the other two species. The most versatile phenotype was observed for *A. johnsonii* isolates and based on the binary traits no carbon source was exclusively used or not used by *A. johnsonii* (Table 4.3).

Table 4.3: Percentage of isolates positive for GENIII phenotypic assays[a] based on binary data[b].

	Grouping[c]	1	2	3	4	5	6	7	8	9	10	11	12
A	cli	45.5	0	0	0	0	18.2	0	0	0	100	100	31.8
	non-cli	22.2	0	0	0	0	19.4	0	2.8	0	100	100	25.0
	A. calcoaceticus	21.4	0	0	0	0	71.4	0	7.1	0	100	100	92.9
	A. guillouiae	54.5	0	0	0	0	0	0	0	0	100	100	9.1
	A. johnsonii	25.0	0	0	0	0	0	0	0	0	100	100	4.2
	A. lwoffii	33.3	0	0	0	0	11.1	0	0	0	100	100	11.1
B	cli	0	0	9.1	0	0	0	0	0	0	100	72.7	0
	non-cli	0	0	2.8	0	0	0	0	0	0	97.2	63.9	2.8
	A. calcoaceticus	0	0	21.4	0	0	0	0	0	0	100	92.9	0
	A. guillouiae	0	0	0	0	0	0	0	0	0	100	45.5	0
	A. johnsonii	0	0	0	0	0	0	0	0	0	95.8	66.7	0
	A. lwoffii	0	0	0	0	0	0	0	0	0	100	55.6	11.1
C	cli	27.3	27.3	0	36.4	36.4	36.4	40.9	9.1	0	100	0	90.9
	non-cli	22.2	25.0	2.8	33.3	33.3	30.6	50	11.1	0	100	11.1	91.7
	A. calcoaceticus	100	100	0	100	85.7	100	42.9	0	0	100	0	92.9
	A. guillouiae	0	0	0	27.3	27.3	18.2	63.6	18.2	0	100	0	100
	A. johnsonii	0	4.2	0	8.3	16.7	8.3	54.2	12.5	0	100	8.3	100
	A. lwoffii	0	0	11.1	11.1	11.1	11.1	11.1	11.1	0	100	22.2	55.6
D	cli	0	0	0	0	0	0	68.2	31.8	13.6	36.4	100	4.5
	non-cli	0	0	0	0	0	0	72.2	25.0	19.4	41.7	97.2	2.8
	A. calcoaceticus	0	0	0	0	0	0	78.6	92.9	0	71.4	92.9	0
	A. guillouiae	0	0	0	0	0	0	81.8	27.3	90.9	90.9	100	0
	A. johnsonii	0	0	0	0	0	0	79.2	0	0	8.3	100	4.2
	A. lwoffii	0	0	0	0	0	0	22.2	0	0	11.1	100	11.1
E	cli	0	9.1	100	36.4	54.5	81.8	40.9	77.3	9.1	86.4	100	81.8
	non-cli	0	0	97.2	61.1	83.3	86.1	47.2	83.3	5.6	86.1	88.9	97.2

	A. calcoaceticus	0	14.3	100	100	100	100	100	92.9	21.4	92.9	92.9	92.9
	A. guillouiae	0	0	100	0	100	100	100	100	9.1	100	100	100
	A. johnsonii	0	0	100	62.5	66.7	91.7	0	95.8	0	100	91.7	95.8
	A. lwoffii	0	0	88.9	11.1	11.1	22.2	11.1	0	0	22.2	88.9	66.7
F	cli	27.3	40.9	27.3	0	27.3	72.7	4.5	27.3	4.5	86.4	100	100
	non-cli	2.8	50	0	0	36.1	86.1	36.1	22.2	13.9	88.9	100	100
	A. calcoaceticus	7.1	42.9	7.1	0	28.6	100	42.9	100	42.9	92.9	100	100
	A. guillouiae	18.2	36.4	9.1	0	36.4	81.8	0	0	0	100	100	100
	A. johnsonii	12.5	62.5	12.5	0	41.7	87.5	29.2	0	0	100	100	100
	A. lwoffii	11.1	22.2	11.1	0	11.1	33.3	11.1	0	0	33.3	100	100
G	cli	0	100	0	100	59.1	40.9	13.6	90.9	90.9	45.5	90.9	86.4
	non-cli	0	100	0	97.2	77.8	69.4	27.8	91.7	88.9	41.7	86.1	83.3
	A. calcoaceticus	0	100	0	100	100	64.3	0	100	100	14.3	92.9	35.7
	A. guillouiae	0	100	0	100	90.9	100	63.6	100	100	81.8	90.9	100
	A. johnsonii	0	100	0	100	66.7	54.2	25.0	95.8	91.7	41.7	83.3	100
	A. lwoffii	0	100	0	88.9	11.1	11.1	0	55.6	55.6	44.4	88.9	100
H	cli	100	72.7	77.3	95.5	72.7	63.6	90.9	100	27.3	100	100	81.8
	non-cli	100	69.4	77.8	91.7	77.8	11.1	100	100	16.7	94.4	88.9	86.1
	A. calcoaceticus	100	100	100	100	100	7.1	100	100	85.7	92.9	92.9	92.9
	A. guillouiae	100	90.9	81.8	100	100	45.5	100	100	0	100	100	100
	A. johnsonii	100	50.0	75.0	95.8	54.2	29.2	95.8	100	0	95.8	91.7	91.7
	A. lwoffii	100	55.6	44.4	66.7	66.7	55.6	88.9	100	0	100	88.9	33.3

[a] Data are presented according to the position of the test substrates in the GENIII plates. See Table 4.2 for the different test substrates and corresponding positions in GENIII plates.
[b] GENIII phenotypes were considered positive when the AUC value exceeded 1.5 times the AUC value of the negative control well.
[c] The grouping was based on origin (cli = clinical and veterinary isolates (n = 22) versus non-cli = aquatic and environmental isolates (n = 36)) and species classification (*A. calcoaceticus* (n = 14), *A. guillouiae* (n = 11), *A. johnsonii* (n = 24) and *A. lwoffii* (n = 9), see Table 4.1).

4.3.3 Correlation between carbon source utilization

A Spearman rank correlation test revealed strong correlations in carbon source utilization for a number of carbon sources (Fig. 4.5). Clustering of the data revealed two clear groups of carbon sources, whose consumption was strongly correlated ($p < 0.05$) (Fig. 4.5). The first group (Group I) consisted of 15 carbon sources, mainly representing monosaccharides like α-D-glucose (C01), D-mannose (C02), D-galactose (C04), 3-methyl-glucose (C05) and D-fucose (C06) (Fig. 4.5). Notably, these carbon sources were predominantly consumed by *A. calcoaceticus* (Table S4.1, Supporting Information). Additionally, strong correlations were found within a group (Group II) of five monosaccharides with an altered configuration on their sixth carbon (i.e. L-fucose (C07), L-rhamnose (C08), D-fructose-6-phosphate (D07), D-galacturonic acid (F02) and D-glucuronic acid (F05)) (Group II) (Fig. 4.5). Further, most carboxylic acids/esters and amino acids such as L-alanine (E03), L-histidine (E07), methyl pyruvate (G02), L-lactic acid (G04) and acetic acid (H08) were grouped together, but not all sources were significantly correlated with another carbon source ($p > 0.05$).

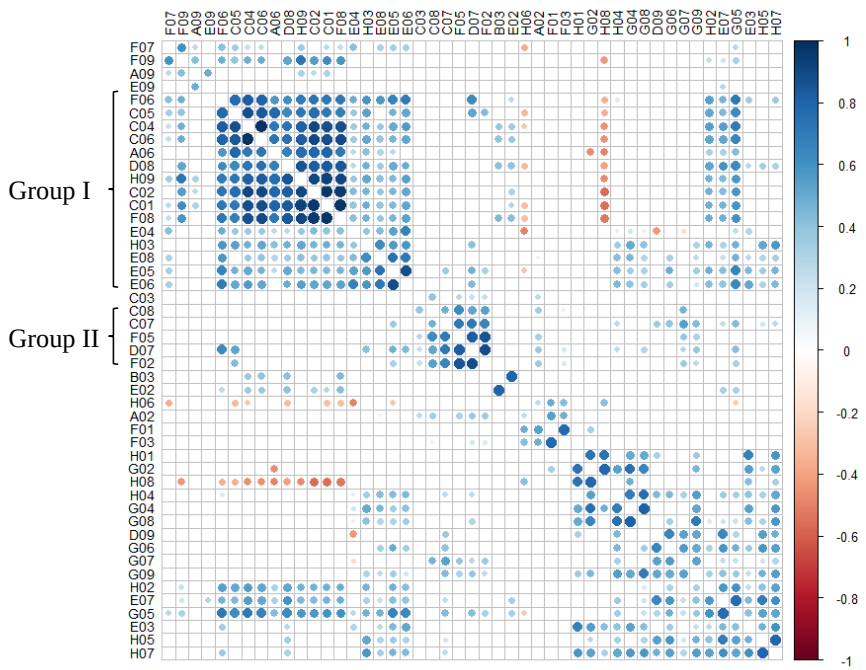


Figure 4.5: Spearman rank correlation analysis of the utilization (area under the curve (AUC) values) of the different carbon sources tested on GENIII plates (indicated by their location on the test plate). Data are combined for all *Acinetobacter* isolates tested ($n = 58$). The size of the dots is representative for the ρ value of the test and are not shown for results with a confidence level < 0.05. The results were clustered by a Ward.D2 agglomeration method. See Table 4.2 for the different test substrates and corresponding positions in GENIII plates. Group I contains several monosaccharides and are mostly used by *A. calcoaceticus* isolates. Group II contains five monosaccharides with an altered configuration on their sixth carbon.

4.3.4 Antibiotic susceptibility testing

An overview of the number of WT and non-WT isolates per tested antibiotic is given in Table 4.4, and the average diameter of the inhibition zone is given in Table S4.2 (Supporting Information). In total, 26 out of the 58 isolates were classified as non-WT isolates for at least one of the compounds tested, among which eight isolates demonstrated multidrug resistance (i.e. non-WT phenotype for more than one antibiotic). These isolates belonged to *A. guillouiae* (AVA 011A, non-WT for kanamycin and amoxicillin; AVA 117B2d, non-WT for kanamycin and amoxicillin; and LUH 5606, non-WT for ciprofloxacin, tetracyclin and sulfamethoxazole/tri-methoprim), *A. johnsonii* (AVA 013A, non-WT for netilmicin and tetracyclin; AVA 098A2d, non-WT for sulfamethoxazole/tri-methoprim and streptomycin; and AVA 101, non-WT for amoxicillin, doripenem, imipenem and meropenem), *A. lwoffii* (AVA 057A2da, non-WT for sulfamethoxazole/tri-methoprim and streptomycin; and LMG 1301, non-WT for sulfamethoxazole/tri-methoprim and streptomycin) (Table 4.1). Most non-WT phenotypes were observed against amoxicillin (10 isolates, among which seven belonging to *A. guillouiae*), streptomycin (nine isolates) and sulfamethoxazole/tri-methoprim (eight isolates) (Table 4.4). Most non-WTs were found in *A. johnsonii*, which had non-WT traits against even eight antibiotics in at least one isolate (amoxicillin, doripenem, imipenem, meropenem, netilmicin, sulfamethoxazole/tri-methoprim and streptomycin), followed by *A. guillouiae* which had non-WT behaviors against five antibiotics in at least one isolate (amoxicillin, ciprofloxacin, kanamycin, tetracyclin and sulfamethoxazole/tri-methoprim). *Acinetobacter calcoaceticus* (amoxicillin and streptomycin) and *A. lwoffii* (sulfamethoxazole/tri-methoprim and streptomycin) had non-WT traits against two antibiotics in at least one isolate (Table 4.1). No non-WTs were observed for amakacin, cephalothin, colistin sulfate, gentamicin and tobramycin (Table 4.4).

Table 4.4: Distribution of wild type (WT) and non-wild type (non-WT) *Acinetobacter* phenotypes when subjected to different antibiotics.

Antibiotic[a]	DOR	IMI	MRP	CIP	K	AK	CN	NET	TOB	TE	AML	SXT	KF	CS	S
Concentration (µg)	10	10	10	5	30	30	10	10	10	30	25	23.75/1.25	30	50	10
ECOFF (mm)	< 18[b]	< 23[c]	< 20[d]	< 20[d]	< 19[b]	< 18[e]	< 15[e]	< 19[e]	< 17[d]	< 18[d]	< 12[d]	< 16[d]	< 0[b]	< 13[d]	< 15[d]
# WT	57	57	57	57	55	58	58	57	58	56	48	50	58	58	49
% WT	98.3	98.3	98.3	98.3	94.8	100	100	98.3	100	96.6	82.8	86.2	100	100	84.5
# non-WT	1	1	1	1	3	0	0	1	0	2	10	8	0	0	9
% non-WT	1.7	1.7	1.7	1.7	5.2	0	0	1.7	0	3.4	17.2	13.8	0	0	15.5

[a] DOR, Doripenem; IMI, Imipenem; MRP, Meropenem; CIP, Ciprofloxacin; K, Kanamycin; AK, Amakacin; SN, Gentamicin; NET, Netilmicin; TOB, Tobramycin; TE, Tetracyclin; AML, Amoxicillin; SXT, Sulfamethoxazole/tri-methoprim; KF, Cephalothin; CS, Colistin sulfate; S, Streptomycin.

[b] Value determined in this study. Outliers were defined by a box plot representation and isolates which had an inhibition zone smaller than the first quartile subtracted with 1.5x the inter quartile range were classified as non-wild types (non-WT). In general, this criterion was in good agreement with the values found by EUCAST when applied to the other antibiotics tested. Isolates with an inhibition zone within the box plot and 1.5x the IQR value were considered wild type (WT) (Narciso-da-Rocha et al. 2013).

[c] Value determined by Hombach et al (2012).

[d] Value determined by Narciso-da-Rocha et al (2013).

[e] EUCAST value.

Ten out of the 22 isolates from clinical or veterinary origin displayed a non-WT phenotype for at least one antibiotic. For isolates from environmental or aquatic origin, 16 out of the 36 isolates were non-WT for at least one antibiotic, among which six out of ten isolates were from household tap water or finished drinking water within the drinking water production plant (Table 4.1). For example, isolate *A. johnsonii* AVA 101A2d, isolated from the effluent of a rapid sand filter of a drinking water treatment plant, displayed a non-WT phenotype to amoxicillin, doripenem, imipenem and meropenem (Table 4.1).

4.3.5 Correlation between rpoB *gene sequences and phenotypes*

In order to determine whether similarity in certain phenotypes was mirrored in the *rpoB* gene sequence tree, a Mantel test was performed between pairwise phylogenetic distances and phenotype differentiation. A p value < 0.05 indicates that phylogenetic distance has some predictive value of phenotype/trait differentiation. When all GENIII phenotypes were taken into consideration a p value of 0.157 was obtained. When only considering results for the different carbon sources or chemical stressors, p values were 0.046 and 0.315, respectively. For the antibiotic testing, an overall p value of 0.516 was obtained. Table 4.5 gives an overview of the different p values for each phenotype individually. For four carbon sources and one chemical stressor a p value less than 0.05 was obtained, i.e. for the carbon sources L-arginine (E04), L-histidine (E07), citric acid (G05), and γ-amino-butyric acid (H02), and the chemical stressor sodium bromate (H12). Further, one antibiotic (imipenem) had a p value close to 0.05 (0.051). L-Arginine was mainly used by isolates belonging to *A. calcoaceticus* (14 out of 14 isolates) and *A. johnsonii* (14/24 isolates). For the latter, this was mostly the case for isolates from an aquatic origin, although not significantly different (p > 0.1) (Table S4.1 and Fig. S4.3, Supporting Information). L-Histidine was utilized by all *A. calcoaceticus* and *A. guillouiae* isolates and not by isolates classified as *A. johnsonii* or *A. lwoffii* (with one exception for the latter) (Table S4.1 and Fig. S4.3, Supporting Information). Additionally, the majority of *A. lwoffii* isolates were not able to use citric acid (1/8 isolates), while the isolates of the other species tested could (14/14 for *A. calcoaceticus*, 10/11 for *A. guillouiae*, 16/24 for *A. johnsonii*). For *A. johnsonii*, results seem to suggest that citric acid could be slightly better used by aquatic isolates (used by 12/15 aquatic isolates vs. 4/9 clinical isolates), yet not significantly different (p = 0.230) (Table S4.1 and Fig. S4.3, Supporting Information). Furthermore, *A. lwoffii* isolates originating from clinical samples (4) were able to use γ-amino-butyric acid as a sole carbon source, while only one aquatic isolate (out of a total of 5) did. *Acinetobacter calcoaceticus* and *A. guillouiae* isolates generated strong signals for this carbon source. For *A. johnsonii,* results seem to vary again within the group of isolates tested (Table S4.1 and Fig. S4.3, Supporting Information). Most of the *Acinetobacter* isolates tested in this study seem to tolerate sodium bromate as a stressor. However, all *Acinetobacter lwoffii* isolates from a clinical setting were sensitive to it (but, not significantly different from the isolates originating from aquatic samples (p > 0.1)) (Table S4.1 and Fig. S4.3, Supporting Information). For the antibiotic imipenem only one isolate was designated as a non-

WT strain (*Acinetobacter johnsonii* AVA 101A2d). Clinical isolates of *A. lwoffii* were more sensitive to imipenem than aquatic isolates, yet again this was not significantly different (p = 0.440) (Table S4.1 and Fig. S4.3, Supporting Information).

Table 4.5: Overview of the *p* values of the Mantel test between the pairwise similarity distance matrix of the *rpoB* gene sequences and the phenotypes obtained for all *Acinetobacter* isolates studied (n = 58). Data are presented for phenotypic traits determined using GENIII plates[a] and antibiotic resistance testing[b].

Phenotypes determined by GENIII microplates:

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	Nd	0.448	nd	nd	nd	0.148	nd	nd	nd	nd	0.480	0.174
B	Nd	nd	0.061	nd	nd	nd	nd	nd	nd	0.862	0.369	0.565
C	0.138	0.175	0.605	0.159	0.188	0.148	0.217	0.860	nd	0.766	0.602	0.391
D	Nd	nd	nd	nd	nd	nd	0.927	0.222	0.162	0.252	0.517	0.776
E	Nd	0.057	0.551	0.020*	0.373	0.343	0.001*	0.404	0.170	0.249	0.605	0.318
F	0.650	0.789	0.624	nd	0.852	0.196	0.252	0.197	0.096	0.307	0.201	0.417
G	Nd	0.496	nd	0.551	0.006*	0.159	0.214	0.409	0.450	0.582	0.790	0.085
H	0.167	0.032*	0.565	0.416	0.110	0.686	0.549	0.308	0.143	0.779	0.918	0.011*

Antibiotic susceptibility testing:

DOR	IMI	MRP	CIP	K	AK	CN	NET	TOB	TE	AML	SXT	KF	CS	S
0.278	0.051	0.313	0.951	0.526	0.738	0.822	0.887	0.885	0.730	0.626	0.540	0.441	0.907	0.820

[a] Data are presented according to the position of the test substrates in the GENIII plates. See Table 4.2 for the different test substrates and corresponding positions in GENIII plates. nd, not determined (no signal for these phenotypes).
[b] DOR, Doripenem; IMI, Imipenem; MRP, Meropenem; CIP, Ciprofloxacin; K, Kanamycin; AK, Amakacin; SN, Gentamicin; NET, Netilmicin; TOB, Tobramycin; TE, Tetracyclin; AML, Amoxicillin; SXT, Sulfamethoxazole/tri-methoprim; KF, Cephalothin; CS, Colistin sulfate; S, Streptomycin.
* p value < 0.05.

4.4 Discussion

The omnipresence of *Acinetobacter* species within natural environments such as soil, water and plant- and insect-related environments has become well documented in recent years (Anandham et al. 2010; Vaz-Moreira et al. 2011; Álvarez-Pérez et al. 2013; Choi et al. 2013; Kim et al. 2014; Krizova et al. 2014; Li et al. 2015; Adewoyin and Okoh, 2018). Furthermore, several *Acinetobacter* members are known to be the causative agent of opportunistic human and animal infections (Seifert et al. 1993a; Dijkshoorn et al. 2007; Ku et al. 2000; Tega et al. 2007; Towner, 2009; van den Broek et al. 2009; Visca et al. 2011). Therefore, and also because antibiotic resistance is increasingly reported in *Acinetobacter*, the genus has gained increasing attention on how strains may be dispersed from the environment to potential hosts. It is generally considered that personal contact and ventilator-associated spread is a major cause of transmission (Evans Patterson et al. 1991; Mulin et al. 1997; van den Broek et al. 2006). However, alternative routes of *Acinetobacter* transmission have been observed via drinking water

distribution systems and water usage for personal hygiene (Umezawa et al. 2015). Indeed, several studies have shown that *Acinetobacter* is a common inhabitant of drinking water systems (Villarreal et al. 2010; Vaz-Moreira et al. 2013, 2017; Narciso-de-Rocha et al. 2013; Van Assche et al. 2018; Chapters II and III). Furthermore, *Acinetobacter* shows several characteristics aiding survival in these systems (e.g. biofilm formation capability, resistance against disinfectants, siderophore production) (Chaves Simões et al. 2008; Karumathil et al. 2014; Lee et al. 2017; Biswas and Mettlach, 2019). Narciso-de-Rocha and colleagues (2013) found up to 11 different *Acinetobacter* species with validly published names in potable water, reaching densities of 10^4 CFU/ml. Likewise, our data obtained in Chapter III suggest that several *Acinetobacter* species occur in drinking water production systems in Belgium and their distribution networks, among which a number of species that are documented as opportunistic pathogens, including *A. calcoaceticus*, *A. guillouiae*, *A. johnsonii* and *A. lwoffii*. Here, we aimed to obtain more insights into the ecology and epidemiology of opportunistic pathogenic acinetobacters, and investigated the relatedness between isolates from aquatic/environmental habitats and clinical origin using an integrated genotypic-phenotypic approach. We hypothesized that a number of phenotypic traits could be identified that were indicative for species classification or the origin of the isolates.

GENIII phenotyping revealed that *Acinetobacter* uses a wide diversity of carbon sources and generated signals for the majority of the chemical sensitivity assays. These findings are in agreement with previous studies, showing that *Acinetobacter* strains have a high versatility to use different carbon sources (Bernards et al. 1995). Furthermore, our results are in line with previous observations from taxonomic studies of *Acinetobacter* species (Krizova et al. 2015a). Nevertheless, slightly deviating results were observed for some parameters. For example, while our results suggest that *A. calcoaceticus* is able to use α-D-glucose, taxonomic classification studies using classical growth based assays report that *A. calcoaceticus* is not able to completely assimilate α-D-glucose, and the majority of *A. calcoaceticus* strains are reported to acidify the carbon source (Krizova et al. 2015a). It is known that within the *Acinetobacter* genus some species are able to oxidize certain carbon sources without growing on them (Müller and Babel, 1986). In this way NADH is produced without growth observation which may cause the reduction of the tetrazolium dye used in the Biolog analysis, thereby generating a positive signal (Bernards et al. 1995, Van Assche et al. 2017). Our results also suggest that *A. calcoaceticus* is able to use more carbon sources then the other species tested, as also observed previously based on the taxonomic classification scheme of Nemec and coworkers (Krizova et al. 2015a). The taxonomic classification scheme consists of temperature growth assays, acidification of D-glucose, hemolysis of sheep blood, liquefaction of gelatin and the assimilation of 36 carbon sources in a minimal medium. Assimilation is evaluated based on visual growth after six and ten days. Based on this scheme, a higher metabolic diversity should be observed for *A. guillouiae* in comparison to *A. lwoffii*, which is also in agreement with the higher average substrate richness and AWCD of the nutrient scores obtained in this study for this species. Nevertheless, while *A. guillouiae* isolates had a higher average nutrient score based on AWCD data than *A. johnsonii* isolates, they did not have a significant higher average richness

value for the nutrient score. This may indicate that kinetic measurements are also important to evaluate metabolic differences between species, rather than endpoint measurements alone (Vaas et al. 2012). Furthermore, carbon sources like formic acid, gentiobiose, α-D-glucose, D-mannose and quinic acid were almost exclusively used by *A. calcoaceticus* isolates, whereas almost all *A. guillouiae* isolates were the only isolates that used D-serine. The majority of *A. guillouiae* isolates were also able to use D-malic acid, which was not the case for the other isolates tested (with the exception of some *A. johnsonii* isolates), corroborating earlier findings (Krizova et al. 2015a). On the other hand, isolates of *A. lwoffii* were not able to oxidize L-pyroglutamic acid while only a few isolates belonging to the other species could. In line with the classification scheme of Nemec (Nemec et al. 2010), it is also clear from our results that *A. johnsonii* is a highly versatile species.

It is known that expression of genes in an organism and microbial metabolism can be influenced by the environment, resulting in niche-specific phenotypes. For example, niche-specific regulation results in the activation of the glyoxylate cycle and gluconeogenesis within the fungal pathogen *Candida albicans*, when the yeast is phagocytosed by macrophages and neutrophils and as a reaction of entering an environment relatively free of glucose (Barelle et al. 2006). Strain-specific niche adaptation has also been observed for many other microbes, among which aquatic and terrestrial microorganisms (Kolton et al. 2013; Monk et al. 2013; Di Cenzo et al. 2016; Lazar et al. 2016). When our results obtained for the different carbon sources were evaluated based on the origin of the isolates irrespective of species classification, six carbon sources were found to be significantly different. Acetoacetic acid generated a positive signal for a large group of clinical isolates except for the clinical isolates of *A. calcoaceticus*. Only three non-clinical isolates generated positive signals for acetoacetic acid, including two isolates from nectar (*A. calcoaceticus* and *A. guillouiae*) and one isolate from sewage (*A. guillouiae*). It has to be noted, however, that signals obtained for these isolates were weaker compared to those obtained for the clinical strains. These observations thus suggest that the ability to utilize acetoacetic acid as a sole carbon source is not only a species characteristic, but is also related to the origin of the isolates. Acetoacetic acid is a by-product of the partial degradation of fatty acids in the liver (Bora et al. 2019) and therefore is linked to warm-blooded animals rather than aquatic habitats. Similar observations were made for pectin and L-galactonic acid lactone, which were predominantly used by clinical isolates. Both carbon sources are plant-related compounds. L-galactonic acid lactone is an intermediate compound in the production of L-ascorbic acid and pectin is a plant cell wall polysaccharide that allows primary cell wall extension and plant growth (Harholt et al. 2010; Wheeler et al. 2015). By contrast, mucic acid (also known as galactaric acid) resulted in positive signals for strains mostly isolated from aquatic environments. Mucic acid is a hexaric acid resulting from the oxidative ring cleavage of galactose. It is not clear so far why this compound could be particularly used by strains from aquatic environments, and not by clinical strains. Similar observations were found for *Acinetobacter* strains associated with mosquitoes when compared to their free-living counterparts. Biolog analysis revealed that mosquito strains tended to utilize fewer substrates than free living isolates of the same species (28.5 % vs 36.3 %

for *A. calcoaceticus*, 18.8 % vs 20.7 % for *A. johnsonii*, 15.1 % vs 22.2 % for *A. lwoffii* and 29.1 % vs 28.5 % for *A. baumannii*, respectively), but some substrates known as blood or plant components were specifically utilized by mosquito isolates (Minard et al. 2013).

Methyl pyruvate and L-lactic acid generated positive signals for almost all isolates tested (*A. lwoffii* AVA 076A2d was the only isolate that did not use L-lactic acid). Nevertheless, on average isolates from clinical samples generated stronger signals for these two carbon sources. These differences in signal intensity may be caused by subtle differences in optimal growth temperature. The standard protocol for Biolog's GENIII microplates is set at 33 °C which might favor the metabolic activity of clinical strains of human origin which are adapted to higher temperature (37 °C) versus environmental strains which typically perform better at lower temperature (5 – 25 °C). Both compounds were also correlated according to the Spearman rank test. Additionally, the Spearman rank test resulted in a clustering of several monosaccharides mostly utilized by *A. calcoaceticus* isolates. It is known that several *Acinetobacter* species can acidify these carbon sources, yet they often do not grow on them (Bernards et al. 1995; Van Assche et al. 2017). Finally, a group of C6 altered monosaccharides (L-rhamnose, L-fucose, D-glucuronic acid, D-fructose-6-phosphate and D-galacturonic acid) which are often found in mammalian or plant related environments were found to correlate based on the Spearman rank test. Remarkably, only L-fucose was significantly different based on species classification and was mostly used by *A. guillouiae* isolates. Other carbon sources were not significantly different for any of the groupings, suggesting that these traits are random, rather than related to species classification or origin of isolation.

Antibiotic resistance among bacteria is gaining worldwide attention, especially because multidrug resistance is increasingly reported (Gaynes and Edwards 2005; McGowan 2006; Peleg et al. 2008). It is reasonable to assume that antibiotic resistance is more common in clinical isolates over isolates originating from other habitats. This was also observed in this study, where 45.5 % of the clinical isolates had a non-WT phenotype versus 38.5 % of the environmental isolates (excluding isolates obtained after disinfection or from the household tap water). Recent studies have shown that drinking water treatment selects for the presence of antibiotic resistant bacteria (Xi et al. 2009; Shi et al. 2013; Vaz-Moreira et al. 2017). These findings are confirmed by our results where 60 % of the isolates that were obtained after disinfection within a water treatment plant or from household tap water were non-WT. Non-WT phenotypes were particularly found for the antibiotics amoxicillin, streptomycin and sulfamethoxazole/tri-methoprim, which is in agreement with previous studies (Narciso-da-Rocha et al. 2013). However, in contrast to Narciso-da-Rocha et al. (2013) non-WT phenotypes were not found for the antibiotic colistin. Non-WT phenotypes were mostly found in *A. guillouiae* and occurred the least among *A. lwoffii* isolates. It has been noticed that *A. lwoffii* strains are more susceptible to antibiotics (Seifert et al. 1993b).

A Mantel test was used to assess significant relationships between the phylogenetic distance and trait differentiation among isolates. A significant relationship was found for the carbon sources L-

arginine, L-histidine, citric acid and γ-amino-butyric acid as well as the chemical stressor sodium bromate. The most significant result was found for L-histidine which was used by *A. calcoaceticus* and *A. guillouiae* isolates, but not by the other species with the exception of one *A. lwoffii* isolate. This observation is in line with the taxonomic classification scheme of prof. Alexandr Nemec (Charles University, Prague) (Nemec et al. 2010). Additionally, L-arginine gave a positive Mantel test result for all *A. calcoaceticus* isolates, most of the *A. johnsonii* isolates and one *A. lwoffii* isolate which is also in agreement with the taxonomic classification scheme of Nemec (Krizova et al. 2015a). Similarly, the majority of the *A. calcoaceticus*, *A. guillouiae* and *A. johnsonii* isolates gave a positive Mantel test result for citric acid, while most *A. lwoffii* isolates did not. In contrast, γ-amino-butyric acid, which is assumed to be assimilated by most strains of the four species investigated here (Krizova et al. 2015a), generated a signal for approximately 50 % of the isolates classified as *A. johnsonii* and *A. lwoffii* and resulted in a significant correlation with the phylogenetic position of the isolates. Our findings are more correlated with the initial reporting for species description of *A. johnsonii* and *A. guillouiae* which observed less strains able to use this compound (Bouvet and Grimont, 1986; Nemec et al. 2010).

In summary, in this Chapter we have shown that certain phenotypes can be linked to specific groups of *Acinetobacter* species. We primarily observed phenotypic differences between species rather than when isolates were grouped based on origin of isolation. However, some phenotypes, mostly carbon source related traits, were found to differentiate strains isolated from different environments. Differences were either determined by a number of carbon sources that were predominantly used by clinical strains, or based on a higher metabolic activity rate (resulting in stronger signals) for isolates originating from a clinical or veterinary setting. However, in order to draw stronger conclusions, further investigation is required on a larger set of isolates to confirm these results. Further, future studies using genomics and transcriptomics may help us to better understanding the metabolic differences between strains.. Further, our results suggest that phenotypic traits are more species- than habitat-dependent. Further research is needed to evaluate if similar trait differentiation is common between several other *Acinetobacter* species and whether a higher resolution would be obtained by using whole genome sequence information rather than only partial *rpoB* gene sequences. Additionally, other relevant curve parameters such as lag phase and slope could be useful to evaluate the different phenotypic traits.

Chapter V: Phylogenetic signal in phenotypic traits related to carbon source assimilation and chemical sensitivity in *Acinetobacter* species[2]

[2] This chapter is based on the following publication:
Van Assche, A., Álvarez-Pérez, S., de Breij, A., De Brabanter, J., Willems, K.A., Dijkshoorn, L. and B. Lievens. 2017. Phylogenetic signal in phenotypic traits related to carbon assimilation and chemical sensitivity in *Acinetobacter* species. Applied Microbiology and Biotechnology. 101:367-379.

5.1 Introduction

Recent studies indicate that the phylogeny of Bacteria and Archaea may reflect molecular functions and phenotypic characteristics, pointing towards phylogenetic conservatism of phenotypic traits. The term 'phylogenetic signal' is commonly used to describe a pattern where evolutionary-related taxa have more similar traits than expected by chance (Blomberg et al. 2003; Langille et al. 2013; Martiny et al. 2013). This likely arises from the fact that microbial evolution mainly proceeds by vertical gene inheritance. However, evolutionary events such as gene loss, horizontal gene transfer (HGT) and convergent evolution may result in the distribution of functional traits across multiple phylogenetic groups (Snel et al. 2002), leading to random associations between phylogenetic and functional relatedness and, consequently, to less phylogenetic signal (Boucher et al. 2003).

The genus *Acinetobacter* is an ancient and heterogeneous group of bacteria that occupy different natural ecosystems and play an increasing causative role in opportunistic human infections (Dijkshoorn et al. 2007; Doughari et al. 2011; Touchon et al. 2014). Up to now, 58 *Acinetobacter* species are on the List of Prokaryotic Names with Standing in Nomenclature (LPSN) (http://www.bacterio.net/acinetobacter.html, last accessed July, 2019). Meanwhile, several additional species have been described and are waiting to be added to the LPSN list (http://apps.szu.cz/anemec/Classification.pdf), which brings the total number of species with validly published names at 63. Apart from these, there are also a number of provisionally termed genomic species based on DNA–DNA hybridization (DDH) profiles, and a number of putative new species based on (phylo)genetic data (Bouvet and Grimont 1986; Bouvet and Jeanjean 1989; Kang et al. 2011; Tjernberg and Ursing 1989; Yamahira et al. 2008). Additionally, novel strains are frequently isolated from an as-yet unknown taxonomic status, for which in depth genetic and/or phenotypic data is lacking. Apart from the well-known human and animal-pathogenic *Acinetobacter* species, several novel species have recently been described based on isolates from diverse habitats such as soils, wastewater, wetlands, insect guts, and plant related environments including tree bark and floral nectar (e.g. Álvarez-Pérez et al. 2013; Anandham et al. 2010; Choi et al. 2013; Kim et al. 2014; Krizova et al. 2014; 2015; Li et al. 2013; 2014; 2015; Vaz-Moreira et al. 2011). *Acinetobacter* phylogenies have been generally reconstructed based on phylogenetic marker genes such as the 16S ribosomal RNA (rRNA) gene and genes encoding DNA gyrase subunit B (*gyrB*), DNA recombinase A (*recA*) and RNA polymerase subunit B (*rpoB*) (Krawczyk et al. 2002; Krizova et al. 2015; La Scola et al. 2006; Nemec et al. 2011, 2016). More recently, phylogenies have been reconstructed based on genome-wide analyses (Chan et al. 2012; Touchon et al. 2014). Altogether, these studies have greatly contributed to our understanding of the population structure and evolutionary relationships within the genus *Acinetobacter*. Nevertheless, despite these studies and an increasing number of *Acinetobacter* strains that have been subjected to in-depth phenotypic characterization using high-throughput phenotypic screening technologies (Álvarez-Pérez et al. 2013; Bernards et al. 1995; Farrugia et al. 2013; Feng et al. 2014; Fondi et al. 2016; Mara et

al. 2012; Peleg et al. 2012) such as Biolog's Phenotype MicroArray (PM) technology (Bochner et al. 2001), little is still known about whether phenotypic traits are phylogenetically conserved within the genus *Acinetobacter*. So far, a comprehensive phenotypic scheme does not allow a clearcut delimitation of most of the currently described taxa (Bouvet and Grimont 1986; Nemec et al. 2011).

Here, we assessed whether phylogenetic distance of *Acinetobacter* species is related to variation in carbon source assimilation and chemical sensitivity as determined by Biolog GENIII phenotyping (Bochner 2009). Using Biolog GENIII phenotyping, microbial strains are simultaneously subjected to 94 phenotypic assays, covering several categories of biochemical compounds (including, amongst some others, carbon substrates, osmotic stressors, acidic stressors, toxins and chaotropes) (Cray et al. 2015; Hallsworth et al. 2003). This phenotyping method provides an easy-to-use, highly reproducible and robust system to characterize a wide range of bacteria, including *Acinetobacter*. To perform the study, a comprehensive collection of isolates from *Acinetobacter* species with validly published names, genomic species and of an as-yet unknown taxonomic status was used. In a first phase, we estimated the strength and significance of the phylogenetic signal of each trait across phylogenetic reconstructions based on partial *rpoB* sequences and the core genome of diverse *Acinetobacter* species. Secondly, we tested whether phylogenetic distance was a good predictor of trait differentiation for this bacterial group by Mantel test analysis. And finally, evolutionary model fitting was used to determine if the data for each phenotypic character was consistent with a phylogenetic or an essentially random model of trait distribution. Although we particularly choose *Acinetobacter* as our model organism due to its ecological and clinical importance as well as its relevance in drinking water (See previous chapters), it has to be noted that the approach used in this study is applicable on diverse microbial systems.

5.2 Material and methods

5.2.1 Bacterial strains

A collection of 133 *Acinetobacter* strains was used in this study (Table S5.1), representing 33 species with validly published names (119 strains), two different provisionally termed genomic species ('genomic species 6' (3 strains) and 'genomic species between 1 and 3' which will be referred to as '*A. calcoaceticus*-like' (3 strains)), as well as the not validly named '*A. oleivorans*' species (1 strain), which is closely related to one of the *A. calcoaceticus*-like strains (Touchon et al. 2014). Additionally, the collection consisted of seven yet unclassified strains which were selected because of their non-clinical origin or their close relatedness to species with validly published names based on the *rpoB* clustering. Isolates with designations LUH and RUH originate from the Leiden University Medical Centre culture collection; most of these have been isolated or received and investigated in previous studies. If possible, for each species strains were selected that have been isolated from different habitats (i.e. human, animal, soil and aquatic isolates), so as to account for possible habitat-related differences in phenotypic traits.

For each species with a validly published name the type strain was included. The other strains were identified to the species level by amplification and sequencing of the variable zones 1 and 2 of the *rpoB* gene (representing a fragment of 861 bp), and positioning of the sequences in a phylogenetic tree based on the *rpoB* sequences of the type strains of all known *Acinetobacter* species (La Scola et al. 2006; Nemec et al. 2009, 2011). Identification was confirmed by AFLP fingerprinting, performed as described previously, and isolates that clustered at a level of ≥50% AFLP similarity were considered to belong to the same species (Dijkshoorn et al. 2007). AFLP analysis for delineation of *Acinetobacter* species was originally developed and validated by Janssen et al. (1997). In later studies, the protocol was simplified by doing digestion with *Eco*RI and *Mse*I and adapter ligation in one step and by using non-radioactive labelling (Nemec et al. 2001). Clustering according to the AFLP banding patterns has been shown to correlate well with species delineation within *Acinetobacter* when using a cut-off level of ≥ 50% AFLP similarity (Dijkshoorn et al. 2007). Some species such as *A. nectaris* and *A. boissieri* were not selected because of their inability to grow on Biolog's Universal Growth (BUG) agar without modification (i.e. addition of 10 % (w/v) of sucrose), which would hinder direct comparison of results with those obtained for the other species. Isolates were stored at -80 °C in nutrient broth No. 2 (Oxoid, Basingstoke, UK, 25 g/L), containing 15 % (v/v) glycerol (VWR, Fontenay-sous-Bois, France).

5.2.2 Phenotypic analysis using Biolog's GENIII microplates

Phenotyping using Biolog's GENIII microplates (Biolog, Hayward, CA, USA), providing a panel of 94 biochemical tests, including 71 carbon source and 23 chemical sensitivity assays (including sensitivity to osmotic stressors, acidic stressors, toxins and chaotropes (Cray et al. 2015; Hallsworth et al. 2003)) (Table 5.1), was performed according to the GENIII microplate protocol A provided by the manufacturer. Briefly, strains were grown from a -80 °C stock culture on BUG agar for 24 hours at 33 °C. Subsequently, the strains were restreaked on BUG agar and incubated for another 24 hours at 33 °C. Due to slow growth rate and small colony size, *A. parvus* strains were incubated two times 48 hours instead of 24 hours. Next, inocula were prepared by swabbing cells from the agar surface and suspending them in Inoculation Fluid A (IF-A, Biolog, Hayward, CA, USA) until an optical density of 95 % was reached, which was determined using Biolog's turbidimeter. Each well of the GENIII microplates was then inoculated with 100 µL of cell suspension. Subsequently, the GENIII microplates were incubated at 33 °C for 36 hours and read every 15 minutes using the OmniLog incubator/reader. Raw kinetic data were retrieved using the OmniLog – OL_PM_FM/Kin 1.30-: File Management/ Kinetic Plot Version software of Biolog. Different kinetic parameters, including area under the curve (AUC), lag time and slope (μ_{max}) were calculated by the OPM package (version 1.1.0, 2014-04-22) in R v.3.1.3 (R Core Team, 2015) using the 'splines' method and 'p.splines' option (Schumaker, 2015). Two independent experiments were performed for each strain. For further analysis, kinetic parameters for both replicates were averaged (for details see Table S5.2).

Table 5.1: Overview of the carbon source assimilation assays (columns 1 to 9) and chemical sensitivity assays (columns 10, 11, and 12) on Biolog's GENIII microplate (Colour legend: yellow = carbon sources; orange = osmotic stressors; blue = acidic stressors; red = toxins; green = chaotropes; grey = inhibitors with unknown mechanism of inhibition; and blank = positive control, negative control or the tetrazolium dyes used to evaluate the cellular responses (reduced to purple formazan for a positive response)).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Negative Control	Dextrin	D-Maltose	D-Trehalose	D-Cellobiose	Gentiobiose	Sucrose	D-Turanose	Stachyose	Positive control	pH 6	pH 5
B	D-Raffinose	α-D-Lactose	D-Melibiose	β-Methyl-D-Glucoside	D-Salicin	N-Acetyl-D-Glucosamine	N-Acetyl-D-Mannosamine	N-Acetyl-D-Lactososamine	N-Acetyl Neuraminic Acid	1 % NaCl	4 % NaCl	8 % NaCl
C	α-D-Glucose	D-Mannose	D-Fructose	D-Galactose	3-Methyl Glucose	D-Fucose	L-Fucose	L-Rhamnose	Inosine	1 % Sodium Lactate	Fusidic Acid	D-Serine
D	D-Sorbitol	D-Mannitol	D-Arabitol	Myo-Inositol	Glycerol	D-Glucose-6-PO4	D-Fructose-6-PO4	D-Aspartic Acid	D-Serine	Troleandomycin	Rifamycin SV	Minocycline
E	Gelatin	Glycyl-L-Proline	L-Alanine	L-Arginine	L-Aspartic Acid	L-Glutamic Acid	L-Histidine	L-Pyroglutamic Acid	L-Serine	Lincomycin	Guanidine HCl	Niaproof 4
F	Pectin	D-Galacturonic acid	L-Galactonic Acid Lactone	D-Gluconic Acid	D-Glucuronic Acid	Glucuronamide	Mucic Acid	Quinic Acid	D-Saccharic Acid	Vancomycin[a]	Tetrazolium Violet	Tetrazolium Blue
G	P-Hydroxy-Phenylacetic Acid	Methyl Pyruvate	D-Lactic Acid Methyl Ester	L-Lactic Acid	Citric Acid	α-Keto-Glutaric Acid	D-Malic Acid	L-Malic Acid	Bromo-Succinic Acid	Nalidixic Acid[a]	Lithium Chloride	Potassium Tellurite
H	Tween 40	γ-Amino-Butyric Acid	α-Hydroxy-Butyric Acid	β-Hydroxy-D,L-Butyric Acid	α-Keto-Butyric Acid	Acetoacetic Acid	Propionic Acid	Acetic Acid	Formic Acid	Aztreonam	Sodium Butyrate	Sodium Bromate

[a] Vancomycin and nalidixic acid are particularly used to differentiate Gram positive from Gram negative bacteria.

5.2.3 Testing for phylogenetic signal in binary traits

Presence of phylogenetic signal of the 94 GENIII phenotypic traits was determined by calculating the D metric (Fritz and Purvis, 2010) from AUC data with the 'phylo.d' function of the 'caper' package (Orme et al. 2013) in R. Each trait was coded as binary (1/0) data where a trait was considered positive (1) when the average value exceeded 1.5 times that for the blank. Vice versa, the trait was considered negative (0) if this was not the case. The robustness of our scoring system was verified with data available from classic tests (e.g. the carbon assimilation tests for species descriptions) for a subset of carbon sources (e.g. from Bouvet and Grimont, 1986, 1987; Krizova et al. 2015; Nemec et al. 2011). However, it has to be noted that our system is based on cellular respiration and substrate oxidation while the other phenotypic assays used are commonly based on evaluation of microbial growth. Interpretation of estimated D values was as follows: D = 1, the distribution of the binary trait is random with respect to the phylogeny; D = 0, the trait follows a distribution pattern expected under a Brownian motion evolutionary model (i.e. in a random walk with constant trait variance over time; Felsenstein, 1985; Fritz and Purvis, 2010); D > 1, the trait is more over-dispersed than expected at random; and D < 0, the trait is highly phylogenetically clustered (Fritz and Purvis 2010). Two different phylogenies were used in these calculations: i) a recently published tree based on the alignment of protein encoding genes of the core genome of *Acinetobacter* species (Fig. 5.1) (Touchon et al. 2014); and ii) a set of 100 maximum likelihood (ML) trees built from an alignment of partial *rpoB* sequences (Table S5.1). In all cases, estimated D values were compared with simulated distributions (1000 permutations) of D under randomly reshuffled trait values across the tips of the tree, and trait evolution under Brownian motion. As extreme levels of trait prevalence can affect the statistical power of D estimates (Fritz and Purvis, 2010), only traits with each count of state (i.e. 0 or 1) accounting for > 10 % of tested strains were considered. p values < 0.01 were considered significant.

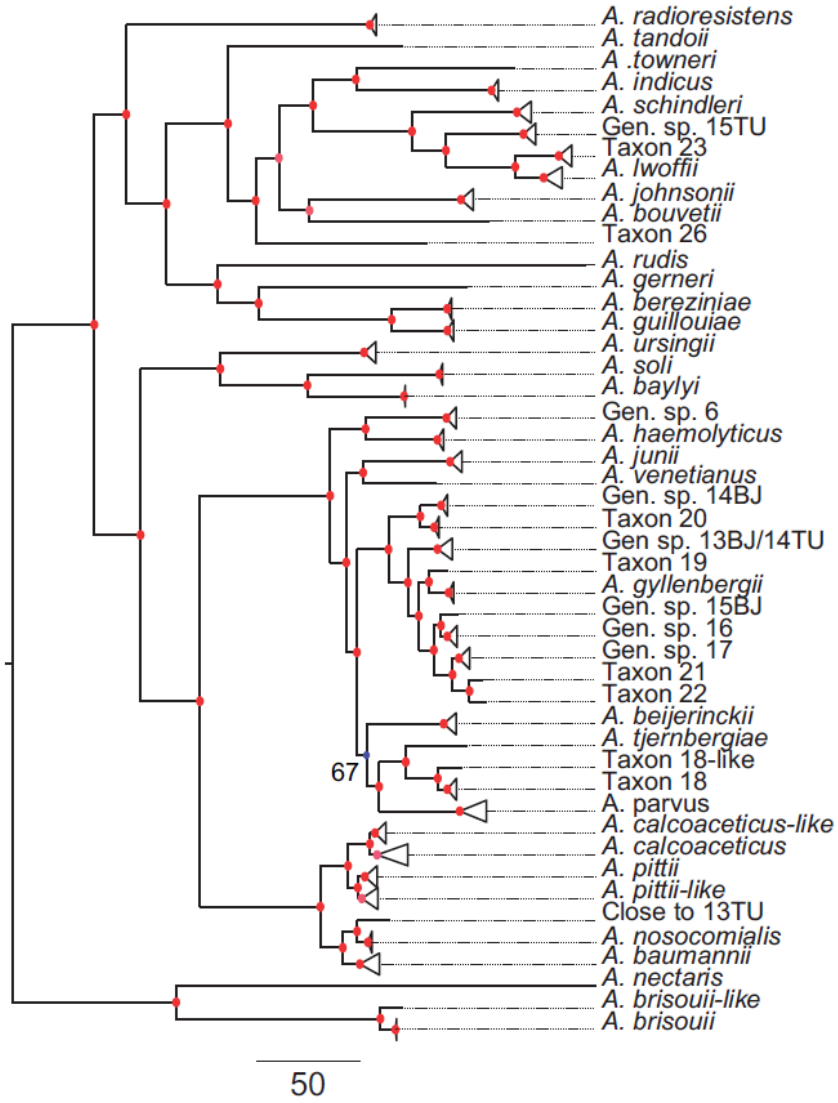


Figure 5.1: Phylogeny of the *Acinetobacter* genus based on the alignment of the protein families of the core-genome. Triangles mark groups of taxa that are from the same species or have more than 95 % ANI values and therefore might be regarded as coming from the same species. The nodes in red have bootstrap supports higher than 95 %. (As published in Touchon et al. 2014)

5.2.4 Phylogenetic signal of kinetic parameters

In order to get further insight into the phylogenetic dependence of the traits determined using GENIII plates, we evaluated the presence and strength of phylogenetic signal in continuous kinetic parameters associated with the different strains (AUC, μ_{max} and lag time) by calculating four commonly used indices: Blomberg's K (K), Moran's I (I), Abouheif's C_{mean} (C_{mean}), and Pagel's λ (λ) (Abouheif 1999; Blomberg et al. 2003; Moran 1950; Münkemüller et al. 2012; Pagel, 1999). In general, these indices differ in their performance, robustness to phylogeny size, degree of resolution of tree structure and sensitivity to true underlying patterns of phylogenetic signal, but are robust to missing branch length information (Kamilar and Cooper, 2013; Münkemüller et al. 2012). Briefly, Blomberg's K and Pagel's λ assume a Brownian motion model of trait evolution. For both metrics, the closest the value to zero the

more phylogenetically independent is a trait; a value of 1 corresponds to a Brownian motion expectation, and values > 1 (which for Pagel's λ are restricted in practice due to calculation reasons) mean that close relatives are more similar than expected under Brownian motion (Kamilar and Cooper, 2013; Münkemüller et al. 2012). In contrast, Moran's I and Abouheif's C_{mean} are autocorrelation indices that are not based on any evolutionary model and are unable to provide information on the strength of the phylogenetic signal (Alonso et al. 2015; Münkemüller et al. 2012). Computations were performed with functions in the packages 'adephylo' (Jombart et al. 2010), 'picante' (Kembel et al. 2010) and 'phytools' (Revell, 2012) in R, using the same phylogenetic trees as for binary traits. Statistical significance was tested in all cases by randomization with 1000 repetitions, and only *p* values < 0.01 were considered significant.

5.2.5 Mantel test

Significant relationships between pairwise phylogenetic distances and trait differentiation among species were assessed using Mantel tests, as implemented in the 'ape' package (Paradis et al. 2004) in R. Trait distances were calculated as Euclidean distances using the 'dist' function, while the complement of Abouheif proximity was selected as measure of phylogenetic distances. This alternative phylogenetic distance metric provides improved testing power in relation to patristic distances (i.e. sum of branch lengths linking species; Hardy and Pavoine, 2012). In this case, calculations were only performed using the phylogenetic tree of Touchon et al. (2014), based on protein encoding genes of the core genome of multiple *Acinetobacter* strains, so as to better reflect the true evolutionary relationships among studied species (Nater et al. 2015; Nichols, 2001). Computations were performed as described in Hardy and Pavoine (2012), and statistical significance was set at *p* values < 0.01.

5.2.6 Model fitting

In order to determine whether the Brownian motion model of evolution was a good fit to the GENIII data or alternative models provided a better explanation of trait variation, we tested for each kinetic parameter of measured traits the following nine models of continuous trait evolution using the 'geiger' package in R: Brownian motion, Ornstein-Uhlenbeck, Early-burst, trend, lambda, kappa, δ tree-transformation, drift, and white noise model (Harmon et al. 2008). Again, only the core-genome tree of Touchon et al. (2014) was used in the analyses. The relative likelihood (Charles, 2014) of the tested models was assessed by calculating their Akaike's Information Criterion (AIC) and Akaike weights (Akaike, 1974). When no evolutionary model achieved an AIC weight ≥ 0.5, it was concluded that none of them performed substantially better than the others (Narwani et al. 2015).

5.3 Results

5.3.1 Phenotypic properties of the studied isolates

Out of a total of 71 carbon sources tested, 49 carbon sources were used (i.e. generating an average area under the curve (AUC) exceeding 1.5 times that of the blank) by at least one *Acinetobacter* strain, whereas 22 carbon sources were not used by any strain at all (including all tested sugar alcohols) (Tables S5.2 and S5.3). Tween 40 was the only carbon source that was used by all strains; acetic acid was used by all strains with the exception of *A. towneri* LUH 9347^{T}. Although, it is generally presumed that almost all acinetobacters can use acetic acid as sole carbon source (Krizova et al. 2015), similar observations have been made by Carr and colleagues (2003) who also found that not all *Acinetobacter* strains tested in their study were able to oxidize acetic acid using Biolog's technology. Among all *Acinetobacter* species, *A. soli* ranked first for the number of carbon sources used (42 ± 2, SD, n = 5), whereas *A. parvus* ranked last (4 ± 1, n = 3) (Fig. S5.1). With regard to the chemical sensitivity assays, all strains gave a positive signal (AUC exceeding 1.5 times that of the blank) for growth at pH 6 while sensitivity to other stressors varied among strains (Tables S5.2 and S5.4). In general, GENIII profiling resulted in the presence of 70 variable biochemical characteristics (mainly belonging to amino acids, carboxylic acids and sugars) that differentiated the studied *Acinetobacter* species based on AUC data (Tables S5.2, S5.3 and S5.4).

5.3.2 Phylogenetic signal of phenotypic traits

Estimated D values for the studied binary traits varied widely depending on the trait and phylogenetic tree used in the analysis (Tables 5.2 and S5.5). Similarly, variation was also found in the number of traits for which D values were significantly < 0 (1.1 % and 13.8 % of traits for the *rpoB* and genomic tree, respectively; see Table 5.2). In general, the traits displaying lower D values (i.e. with the highest phylogenetic signal) referred to the utilization of different carbon sources such as organic acids (citric acid), amino acids (L-arginine, L-histidine and L-pyroglutamic acid) and sugars (α-D-glucose and D-melibiose), while D values for chemical sensitivity assays were in most cases higher than zero (Table S5.6 and Fig. 5.2). An overview of the three traits scoring best for the corresponding phylogenetic signal index is presented in Table S5.6.

Table 5.2: Overview of the results of phylogenetic signal tests for presence/absence of phenotypic traits determined using GENIII plates.

Index values[a]	**Phylogenetic tree (*n*)**[b]	
	***rpoB* (133)**	**Genomic (40)**
Range	-0.044 – 0.990	-0.749 – 1.159
Mean	0.425	0.285
D < 0	1 (1.1 %)	13 (13.8 %)

[a] The range of D values and the mean D value obtained for the whole set of GENIII traits (i.e. 94) is indicated. Additionally, the number (and percentage) of traits for which D values were < 0 (i.e. highly phylogenetically clustered) is presented.
[b] Phylogenetic tree used for computation of phylogenetic signal. *n*, number of *Acinetobacter* strains included in the analyses.

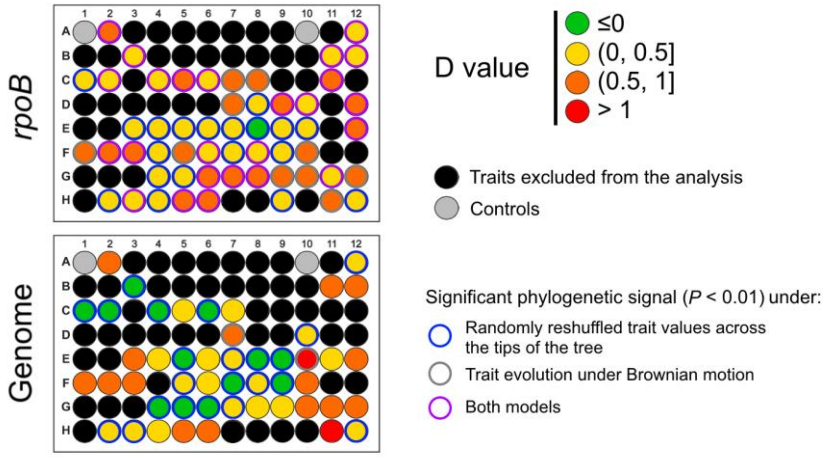


Figure 5.2: Overview of binary (0/1) traits derived from the area under the curve (AUC) data set displaying significant phylogenetic signal, as determined by calculating Fritz and Purvis' D metric (Fritz and Purvis, 2010) in analyses performed using trees based on partial *rpoB* and core genome sequences of *Acinetobacter* species (see Materials and Methods). D values ≤ 0, > 0 but ≤ 0.5, > 0.5 but ≤ 1, and > 1 are indicated by green, yellow, orange and red filling of wells, respectively. Black-filled wells represent those traits excluded from the analysis (< 10 % of tested strains showed one of the two possible states), while A1 and A10 (shown in grey) are the negative and positive growth controls, respectively. Significant signals ($p < 0.01$) under randomly reshuffled trait values across the tips of trees, trait evolution under Brownian motion or both are indicated by blue-, grey- and violet-colored outer rings, respectively. See Table 5.1 for the different test substrates and corresponding positions in GENIII plates.

Substantial variation was also observed in the four phylogenetic signal metrics for continuous traits considered in this study (Tables 5.3 and S5.7). When the *rpoB* / genomic trees were used for computations (n = 282; i.e. three kinetic parameters × 94 traits determined by PM using GENIII plates), statistically significant results were obtained in the following percent of tests: Blomberg's K, 16.6% / 7.8 %; Moran's I, 48.6 % / 24.5 %; Abouheif's C_{mean}, 47.5 % / 22.3 %; and Pagel's λ, 19.5 % / 14.9 % (Fig. 5.3). For most analysis combinations (i.e. phylogenetic tree and kinetic parameter considered), there was not much difference in the values of phylogenetic signal metrics between carbon source assimilation and chemical sensitivity assays, but the percentage of significant traits was in most cases higher for the former than for the latter (Table S5.7). In any case, Blomberg's K values were in most cases very low (especially in the analyses based on the set of *rpoB* ML trees; see Tables 5.3 and S5.7), indicating that the strength of the phylogenetic signal was weaker than would be expected under a Brownian motion model of evolution. Moran's I and Abouheif's C_{mean} yielded similar results in most cases (Tables 5.3 and S5.7). Table S5.8 provides an overview of the top three traits for the four phylogenetic signal indices.

Table 5.3: Overview of the results of phylogenetic signal tests for different kinetic parameters of phenotypic traits determined using GENIII plates.

Phylogenetic tree (*n*)[a]	**Kinetic parameter**	**Index values[b]**	**Phylogenetic signal index**			
			Blomberg's K	**Moran's I**	**Abouheif's C_{mean}**	**Pagel's λ**
rpoB (133)	AUC	Range	$3.5 \cdot 10^{-7}$ – $1.9 \cdot 10^{-5}$	-0.096 – 0.775	-0.091– 0.777	0.073 – 0.983
		Mean	$3.2 \cdot 10^{-6}$	0.451	0.455	0.758
		Significant traits	38 (40.4 %)	90 (95.7 %)	90 (95.7 %)	36 (38.3 %)
	μ_{max}	Range	$9.1 \cdot 10^{-8}$ – $2.5 \cdot 10^{-4}$	-0.058 – 0.393	-0.051 – 0.396	0.023 – 0.809
		Mean	$1.1 \cdot 10^{-5}$	0.025	0.031	0.126
		Significant traits	0 (0 %)	4 (4.3 %)	2 (2.1 %)	0 (0 %)
	lag time	Range	$2.2 \cdot 10^{-7}$ – $3.2 \cdot 10^{-5}$	-0.097 – 0.597	-0.081 – 0.601	0.057 – 0.871
		Mean	$1.7 \cdot 10^{-6}$	0.179	0.186	0.381
		Significant traits	9 (9.6 %)	43 (45.7 %)	42 (44.7 %)	19 (20.2 %)
	Combined[c]	Significant traits for all kinetic parameters	47 (16.6 %)	137 (48.6 %)	134 (47.5 %)	55 (19.5 %)
Genomic (40)	AUC	Range	0.056 – 0.848	-0.209 – 0.670	-0.204 – 0.677	0 – 1
		Mean	0.226	0.273	0.285	0.410
		Significant traits	18 (19.1 %)	54 (57.4 %)	49 (52.1 %)	29 (30.9 %)
	μ_{max}	Range	0.020 – 0.571	-0.167 – 0.500	-0.137 – 0.507	0 – 1
		Mean	0.152	0.029	0.048	0.160
		Significant traits	1 (1.1 %)	8 (8.5 %)	7 (7.4 %)	9 (9.6 %)
	lag time	Range	0.019 – 0.551	-0.227 – 0.434	-0.187 – 0.459	0 – 0.947
		Mean	0.115	0.059	0.076	0.127
		Significant traits	3 (3.2 %)	7 (7.4 %)	7 (7.4 %)	4 (4.3 %)
	Combined[c]	Significant traits for all kinetic parameters	22 (7.8 %)	69 (24.5 %)	63 (22.3 %)	42 (14.9 %)

[a] Phylogenetic tree used for computation of phylogenetic signal indices. *n*, number of *Acinetobacter* strains included in the analyses.
[b] For each phylogenetic signal metric, the range of values and the mean value obtained for the whole set of GENIII traits is indicated. Additionally, the number (and percentage) of traits for which the corresponding phylogenetic signal index is statistically significant ($p < 0.01$) is presented.

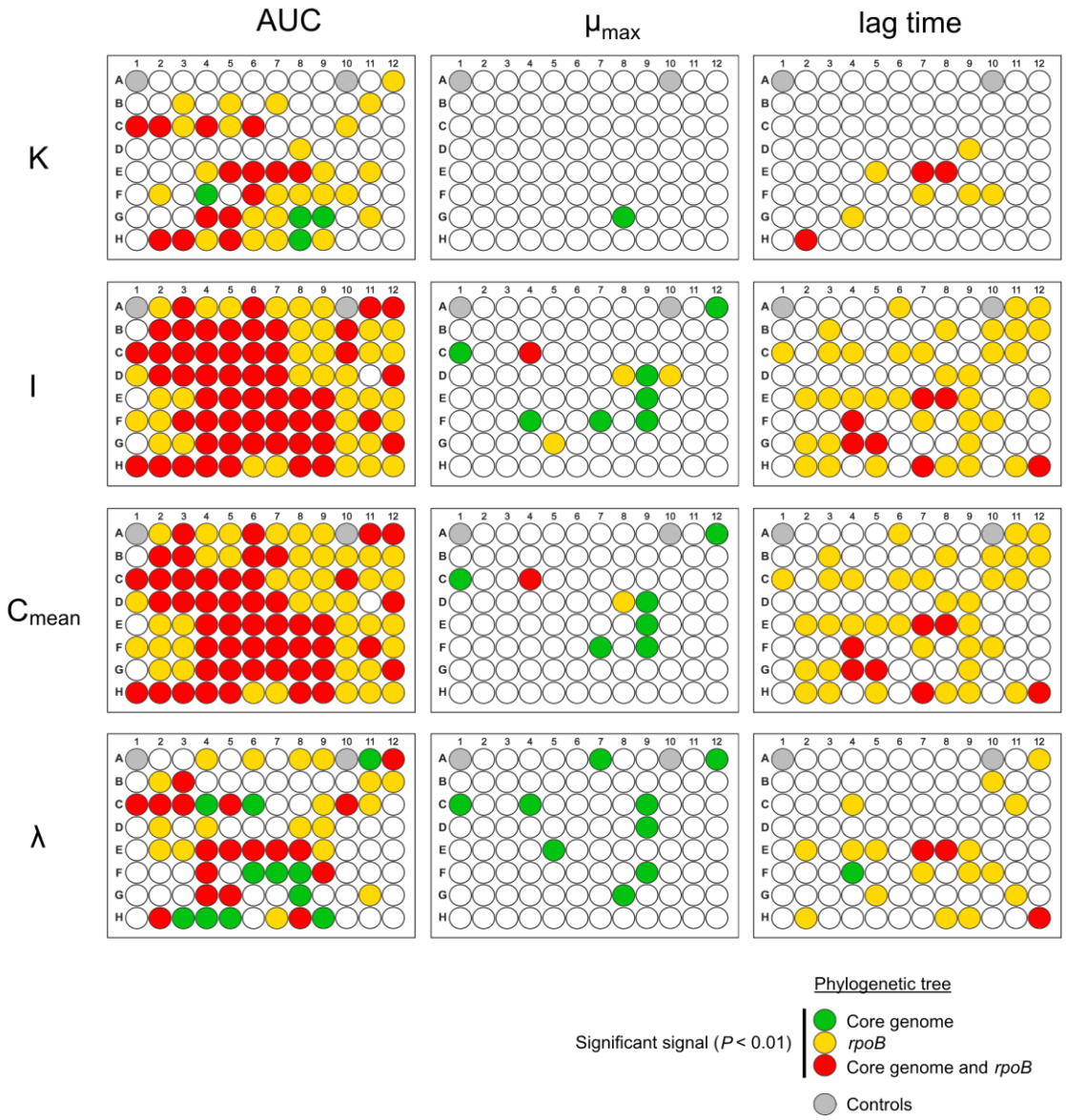


Figure 5.3: Overview of continuous traits, including area under the curve (AUC), slope (μ_{max}) and lag time, displaying significant phylogenetic signal, as determined by four different metrics: Blomberg's K, Moran's I, Abouheif's C_{mean} and Pagel's λ (see Materials and Methods). Significant signal ($p < 0.01$) for each GENIII trait and kinetic parameter found in tests using trees based on the core genome of *Acinetobacter* species, partial *rpoB* sequences or both are indicated by green, yellow and red filling of wells, respectively. See Table 5.1 for the different test substrates and corresponding positions in GENIII plates.

The Mantel test results confirmed that phylogenetic relatedness had some predictive value for variation of 52.1 %, 9.6 % and 11.7 % of GENIII traits when the AUC, μ_{max} and lag time parameters were considered (Fig. 5.4 and Tables S5.9 and S5.10). Notably, utilization of α-D-glucose (well C1), citric acid (well G5) and L-serine (well E9), and growth at pH values of 5 (well A12) yielded significant results for the three kinetic parameters analyzed (Fig. 5.4 and Table S5.10). Regardless of the kinetic parameter considered, the proportion of significant traits was always higher (between 1.5 and 2.6 times) for carbon source assimilation than for the chemical sensitivity assays (Table S5.9).

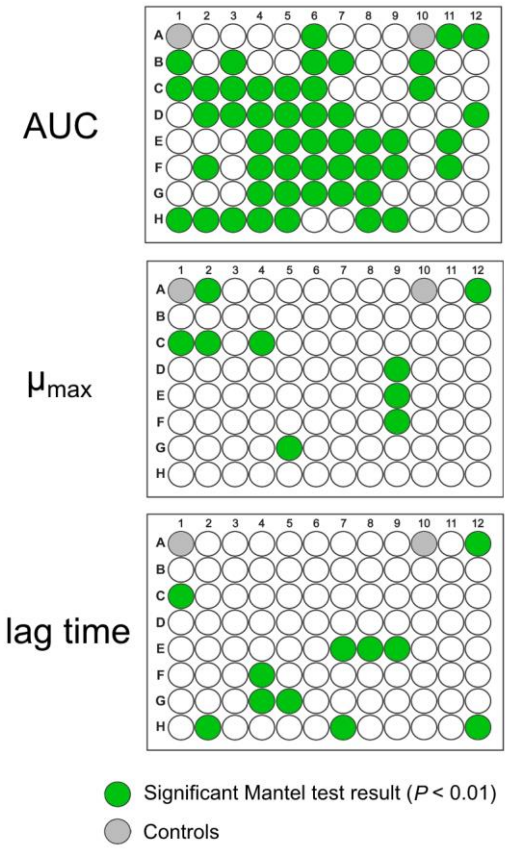


Figure 5.4: Significant associations between trait variation and phylogenetic distance of *Acinetobacter* species, as determined by Mantel test analysis. Significant associations ($p < 0.01$) for each GENIII trait and kinetic parameter (including area under the curve (AUC), slope (μ_{max}) and lag time) are indicated by green filling of wells. See Table 5.1 for the different test substrates and corresponding positions in GENIII plates. Details can be found in Tables S5.9 and S5.10 (Supporting Information).

5.3.3 Evolutionary model fitting

Finally, model fitting analyses indicated that the white noise model was the most commonly supported for all kinetic parameters (37.2 %, 58.5 % and 69.1 % of GENIII traits fitted this model when AUC, μ_{max} and lag time values, respectively, were analyzed; Fig. 5.5 and Table S5.11). Nevertheless, the kappa model provided the best fit for a 30.9 % of traits when AUC values were considered, and the importance of the lambda model ranged from 13.8 to 23.4 % of traits. In addition, there were important differences in model support between carbon source assimilation and chemical sensitivity assays (Table S5.11 and Fig. 5.5). For example, for all kinetic parameters, the white noise model was the best fit for ≥ 65.2 of the chemical sensitivity assays, while the proportion of carbon source assimilation tests that were supported by this model when AUC values were considered was 28.2 % (in this case, the kappa model, provided the best fit for 33.8 % of traits). Regardless of the kinetic parameter considered, none of the 94 GENIII traits seemed to fit the Brownian motion, Ornstein-Uhlenbeck, early-burst and drift evolutionary models.

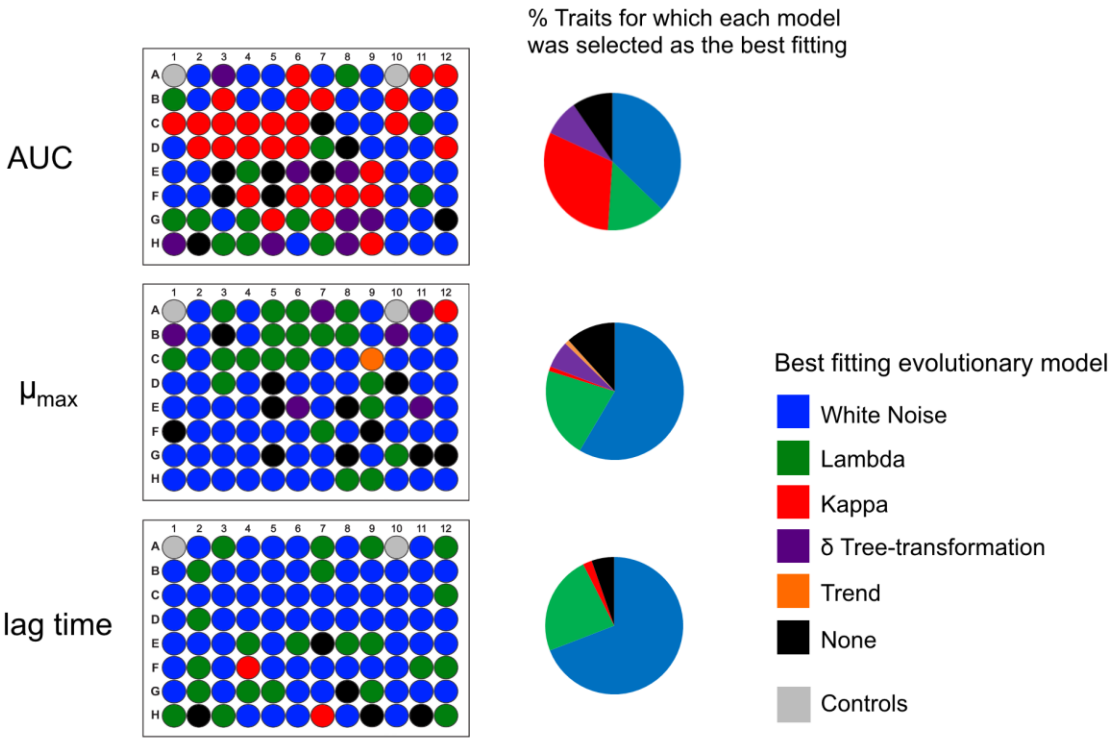


Figure 5.5: Evolutionary model fitting of GENIII trait data. Left*:* Schematic representation of GENIII plates displaying for each measured trait and kinetic parameter (including area under the curve (AUC), slope (μ_{max}) and lag time) the best fitting model. Right*:* Pie charts showing the percentage of traits for which each evolutionary model was selected as the best fit to the data. Color legend: blue, White Noise model; green, lambda model; red, kappa model; violet: δ tree-transformation model; orange, trend model; black, no choice (i.e. none of the tested models performed substantially better than the others; see Materials and Methods). See Table 5.1 for the different test substrates and corresponding positions in GENIII plates.

5.4 Discussion

Although systematic bacterial phenotyping has its roots in the publication of the first edition of the *Bergey's Manual of Determinative Bacteriology* in 1923, microbial phenotyping has not been accessible in a high-throughput format until recently, with the development of sophisticated technologies that provide a global view of different phenotypes such as Biolog's PM technology based on cell respiration (Bochner et al. 2001, Bochner, 2009). So far, metabolic and physiological characteristics of *Acinetobacter* have been mostly studied based on the system originally developed by Bouvet and Grimont (1986). Nevertheless, as this system is more labour intensive and does not allow for high-throughput phenotyping, we have chosen for Biolog's GENIII technology. It has to be noted, however, that our study could have been based on either of both systems. However, as cell respiration may occur independently of cell growth, results may differ for some tests among both systems (Bochner 2009). This may, for example, explain why certain sugars such as D-glucose which may undergo bacterial acidification but generally score negative in classic phenotypic assays for *Acinetobacter* (Baumann et al. 1968; Farrugia et al. 2013; Nemec et al. 2010), may have resulted in a positive Biolog signal in our study. A similar explanation can be given for the phenomenon why some proteolytic *Acinetobacter* species such as *A. haemolyticus*, *A. gyllenbergii* and *A. venetianus* tested negative for gelatin, while they are known to be positive in classic gelatin liquefaction tests (Krizova et al. 2015a). More specifically, in our assay strains are tested for oxidation of gelatin as sole carbon source; while a classic gelatinase assay is not performed in a minimal medium, but rather scored for production of the gelatinase enzyme itself. In line with previous phenotypic studies (Bernards et al. 1995), our Biolog results show that the *Acinetobacter* genus as a whole has a wide degree of metabolic versatility and capacity to utilize a wide range of organic compounds. Additionally, our results are in agreement with what can be predicted from recent genomic studies, suggesting huge variation in phenotypic traits (Touchon et al. 2014). Highest activity was recorded for Tween 40, amino acids and a number of carboxylic acids confirming previous findings (Krizova et al. 2015a; Mara et al. 2012; Peleg et al. 2012). Additionally, strains were found to be universally resistant against a wide diversity of stressors. Nevertheless, in general strains were sensitive to the bacteriostatic compounds fusidic acid and minocycline, for which less than 25 % of the studied strains were resistant (see Tables S5.2 and S5.4).

A common expectation in phenotyping is that phylogenetically closely related species or strains resemble each other more in phenotypic traits than compared to distant relatives; in other words, that biological similarity decreases as the evolutionary distance between species increases (Kamilar and Cooper 2013; Narwani et al. 2015). For example, in a recent investigation of the diversity of metabolic growth phenotypes at different levels of conventional taxonomic classification, Plata et al. (2015) observed that a transition from high to low phenotypic similarity occurred primarily at the genus level, but substantial phenotypic differences could be noticed even at the species level. On the contrary, phenotypic conservation was much lower for taxonomic ranks beyond the level of families (Plata et al.

2015). However, there is still debate about the ubiquitous nature of such a phylogenetically-driven pattern of variation in biological traits (Kamilar and Cooper, 2013). Fortunately, the increasing availability of robust phylogenies based on whole genome sequences has enabled scientists to assess trait variation in an evolutionary context. In this study, we provide the first investigation of the presence and degree of phylogenetic signal in phenotypic traits related to carbon source utilization and chemical sensitivity, as determined by GENIII phenotyping, within the genus *Acinetobacter*. This analysis was first applied to presence/absence (i.e. binary) data. Additionally, as growth curve features can unravel fundamental differences or similarities in the respiration behavior of distinct organisms that cannot be identified by endpoint measurements alone (Vaas et al. 2012), we subsequently focused on a number of kinetic parameters including growth rate, which is a component of microbial fitness (Blomberg, 2011). In both cases, our data supported the hypothesis that some key phenotypic traits related to carbon resource assimilation and chemical sensitivity are related to the phylogenetic placement of *Acinetobacter* species. The strongest phylogenetic signals found (regardless of the phylogenetic tree, trait data set and metric considered) were for utilization of different carbon sources such as some organic acids, amino acids and sugars, a result which was later confirmed by Mantel test analysis. Notably, these features can be considered to be 'effect traits' as they directly influence processes such as nutrient cycling that affect ecosystem functioning (Martiny et al. 2015). Overall, these results suggest that in the diversification of acinetobacters, carbon source assimilation has had a relevant role, while only a few chemical sensitivity traits (e.g. pH) have been important drivers in the evolution of the group. However, such a conclusion must be interpreted with caution. Indeed, most *Acinetobacter* species show remarkable metabolic versatility, but they also have the capacity to withstand multiple environmental stressors by mounting complex protective responses which often involve resistance to diverse chemicals (Fiester and Actis, 2013). Furthermore, interpretation of evolutionary processes or rates based only on estimates of phylogenetic signal has been discouraged by some authors (Revell et al. 2008).

Apart from analyzing the phylogenetic signal underlying interspecific variation for diverse phenotypic traits, we also used a model-fitting approach to test whether the GENIII data supported any phylogenetic model or an essentially random model of trait distribution. Notably, even when the results obtained in calculations of Blomberg's K values supported in some cases the hypothesis of significant phylogenetic signal, none of the studied traits seemed to follow a Brownian motion model of evolution. A similar result has been obtained for other microorganisms in some recent studies (e.g. Narwani et al. 2015). In contrast, model fitting results revealed that most studied traits seemed to follow a white noise model, which is a non-phylogenetic model of evolution that assumes that trait data come from a random normal distribution, and that species have no significant trait covariance (Narwani et al. 2015). Nevertheless, regardless the kinetic parameter considered, a significant proportion of carbon source assimilation traits fitted more complex models of evolution based on Brownian motion, such as lambda (which fits the extent to which the phylogeny predicts covariance among trait values for species and transform the tree by multiplying internal branches by the phylogenetic signal metric λ) and kappa (a

punctuational model in which all branch lengths are raised to an estimated power, κ, and character divergence is related to the number of speciation events between two species) (Pagel, 1999).

There are a number of potential explanations for the lack of a relationship between phylogenetic position and trait values. First, evolutionary events such as gene loss, HGT and convergent evolution may lead to random associations between phylogenetic and phenotypic relatedness and, therefore, to less phylogenetic signal (Martiny et al. 2013; 2015; Goberna and Verdú, 2016). Nevertheless, although HGT can be a relevant source of phenotypic variation in prokaryotes, detecting HGT between closely related species such as those included in this study is still challenging (but see Adato et al. 2015), and this mechanism was therefore not considered in the present study. Furthermore, phylogenies involving non-model species are usually based on one or a few genes (mainly chosen according to historical or practical criteria) that may not accurately reflect the evolutionary relationships among studied species (Aguileta et al. 2008). Fortunately, the current availability of whole genome sequences allows the construction of robust phylogenies using large numbers of genes, as it is the case for the genus *Acinetobacter* (Touchon et al. 2014). However, it is possible that the genes responsible for a particular trait are evolving at a different rate or with a different pattern of descent than the majority of the genome (Narwani et al. 2015). Finally, some traits measured in the present study are most likely determined by multiple genes in combination and by their interactions with environmental variables, so the possibility of epistasis should not be overlooked (Carlborg and Haley, 2004). Similarly, future studies could include the analysis of the accessory genome of *Acinetobacter* to inspect how genes that are only found in some *Acinetobacter* strains but not in others may have determined phenotypic variability. For example, the accessory genome has been predicted to influence many phenotypes in *Escherichia coli* (Monk et al. 2013) and *Pseudomonas aeruginosa* (Kung et al. 2010). Further research is needed to find out to which extent this is the case for *Acinetobacter*.

Carbon assimilation tests have been important biochemical assays in the discrimination of different *Acinetobacter* species (Bouvet and Grimont, 1986, Nemec et al. 2016). Although the current set of classic carbon assimilation tests used only have 9 carbon sources in common with the phenotypic assays provided by the GENIII microplate (i.e. acetic acid, L-arginine, L-aspartic acid, citric acid, D-gluconic acid, D-glucose, L-glutamic acid, L-histidine and D-malic acid), it can be derived from our study that these phenotypes are of great importance to predict phylogenetic signals as well as for taxonomic purposes, as they were considered top three traits resulting from our analyses (see Tables S5.6 and S5.8). More specifically, aspartic and citric acid were found as the characters showing the strongest phylogenetic signal (Tables S5.6 and S5.8). From a practical point of view, phylogenetic conservatism of microbial traits enables the application of phylogeny-based predictions of specific traits for microbes of which their phenotypes are presently unknown (Goberna and Verdú, 2016). This is especially appealing as phenotypic information is often lacking, e.g. due to the fact that gathering phenotypic data requires culturing of the microbial strains and classic phenotyping is often laborious and time-consuming. Moreover, dropping sequencing prices and application of massive sequencing of

environmental strains has led to phylogenetic trees of increasing size in which the percentage of species with unknown traits becomes larger and larger. This is especially true for frequently isolated or detected bacteria such as the members of the genus *Acinetobacter* that are ubiquitously present in different environments. Furthermore, focus on the phenotypic characteristics of microorganisms offers a path for interpreting the growing amount of microbiome data (Martiny et al. 2015). Indeed, an organism's set of traits governs its physiology and its interactions with other species and the environment, and the collective traits of a community interact with the environment to regulate ecosystem functioning (Martiny et al. 2015). Furthermore, in the particular case of the genus *Acinetobacter*, trait-based analyses could help to identify which attributes make some species or strains more adept at causing human disease outbreaks than others (Farrugia et al. 2013; Peleg et al. 2012), or promising candidates for bioremediation related processes (Fondi et al. 2016; Mara et al. 2012).

In conclusion, we found robust evidence for the presence of phylogenetic signal in several phenotypic traits related to carbon source assimilation and chemical sensitivity. Future work should be aimed to clarify how such traits have shaped the remarkable ability of *Acinetobacter* species to dominate in a wide variety of habitat types, a characteristic which has led some authors to consider these bacteria as 'microbial weeds' (Cray et al. 2013, Oren and Hallsworth, 2014).

Chapter VI: General conclusion and perspectives

Advances in high-throughput sequencing and phenotyping technologies have enabled new opportunities to study microbial ecology (Aw and Rose, 2011; Ercolini, 2013; Poisot et al. 2013). Next-generation sequencing (NGS) technologies are providing new insights for the assessment of microbial water quality through analysis of waterborne microbial communities for the development of improved indicators, new markers for microbial source tracking, and observation of microbially mediated processes. Furthermore, these technologies allow to monitor how microbial communities are assembled, how they adapt to external factors, how they respond to environmental changes etc. Furthermore, high-throughput phenotyping platforms such as Biolog's Phenotype Microarray technology have greatly contributed to our understanding of the functioning of microbial communities and their members. In the present doctoral thesis, these tools were used to address the following research aims:

(i) Investigation of the bacterial diversity in different water samples from the production and distribution chain of drinking water production and distribution systems using surface water or groundwater (Chapter II);

(ii) Investigation and comparison of bacterial community shifts in two parallel multi-step drinking water treatment processes using the same source of water (Chapter III).

(iii) Assessment of inter- and intraspecific genotypic and phenotypic variation within *Acinetobacter* species isolated from environmental samples (particularly water) and their counterparts isolated from clinical samples (Chapter IV);

(iv) Assessment of the link between phylogenetic and phenotypic diversification in traits related to carbon source assimilation and chemical sensitivity in *Acinetobacter* species (Chapter V).

As such, this thesis consisted of two major parts flowing into one another, i.e. a first ecological part focusing on microbial communities in drinking water production and distribution systems in which *Acinetobacter* was found as an important member (Chapter II and III), and a second, more microbiological part using *Acinetobacter* spp. as model organisms for addressing relevant ecological/evolutionary questions. Below we present the most important observations of our study and some perspectives.

Main results

Characterization of the bacterial community composition in water of drinking water production and distribution systems in Flanders, Belgium

The quality of drinking water is influenced by its chemical and microbial composition which in turn may be affected by the source water and the different processes applied in drinking water purification systems. In Chapter II, we investigated the bacterial diversity and community composition in different water samples from the production and distribution chain of thirteen drinking water production and

distribution systems (DWPDS) from Flanders that use surface water or groundwater as source water. Water samples were collected over two seasons (spring (April) and autumn (November)) from the source water, the processed drinking water within the production facility and out of the tap in houses along its distribution network. In line with previous studies (e.g. Pinto et al. 2012; Prest et al. 2014; Wu et al. 2015) phyla like *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Cyanobacteria*, *Bacteroidetes*, and *Nitrospirae* were commonly found in the water samples investigated, among which *Proteobacteria* were the most dominant. Also in other studies, *Proteobacteria* was found as the most abundant phylum in aquatic environments within the drinking water production industry (Liu et al. 2014; El-Chakhtoura et al. 2015; Bautista-de los Santos et al. 2016; Vaz-Moreira et al. 2017; Zanacic et al. 2017). Further, strong differences in bacterial community composition were found between processed drinking water originating from companies that use surface water and other that use groundwater as source water. Phyla like *Actinobacteria*, *Bacteroidetes* and *Verrumicrobia* were significantly more abundant in surface water, while *Cyanobacteria* were more abundant in surface water and processed water originating from surface water. On the other hand, no major differences were found at the stage of the tap, indicating that in general water with a similar microbial composition is delivered irrespective of the water source. A similar conclusion can be drawn when also the different sampling periods were taken into account. Comparison of the two sampling periods indicated that especially *Firmicutes* and *Gemmatimondetes* were more abundantly present in water samples taken in November versus April. At the genus level *Acinetobacter* was one of the most abundant taxa encountered, especially in April. In total, three operational taxonomic units (OTUs) defined by a 16S rRNA gene similarity threshold of 97 % were identified as *Acinetobacter*, among which one OTU even represented 4.34 % of all sequences recovered. Furthermore, members of the *Acinetobacter* genus were even found at a relative read abundance of up to 47.5% in processed water samples. Phylogenetic analysis revealed that the *Acinetobacter* sequences retrieved were closely related to a number of *Acinetobacter* species, including the most clinically important one, i.e. *A. baumannii*.

A major limitation of this study is that only a limited set of samples was investigated. Therefore, in order to draw strong conclusions on how the bacterial community composition is influenced by the source of the water as well as by seasonal influences, further investigation is needed using more samples from different DWPDS sampled over a longer period of time. Further, it is reasonable to assume that also the different treatment steps applied within the different companies may have influenced the dynamics of the microbial community composition along the distribution system. Therefore, the focus in Chapter III was on one drinking water production system that operates a parallel treatment system providing drinking water into two different distribution systems. Microbial community composition was investigated from source to tap at every stage of the production and distribution systems. Additionally, the presence of *Acinetobacter* species was determined by isolation and qPCR.

Characterization of the bacterial community composition in a parallel drinking water production and distribution system, with an emphasis on Acinetobacter *species*

Water treatment and disinfection are of utmost importance to guarantee its biological stability and to protect public health. Microorganisms play a dual role in drinking water quality and safety. They are of importance for water purification but on the other hand they can have a negative impact on drinking water quality. Especially when certain potential pathogens survive the drinking water treatment or proliferate within its distribution system and hence become a threat to public health. The goal of this study was to investigate and compare the bacterial community shifts in a full-scale drinking water production and distribution facility in Flanders (Antwerp, Belgium) that uses two series of multi-step treatment processes starting from the same source water (surface water). Furthermore, we investigated the presence and abundance of *Acinetobacter* at each step of the treatment chain using plating and isolation as well as qPCR. Finally, we studied the importance of the physicochemical characteristics of the drinking water on the bacterial community composition in the water delivered to the end user (household tap water). Quantification of total bacteria (in terms of 16S rRNA gene copies) revealed that for both production lines the amount of bacteria decreased when a treatment was applied. There was a clear difference between the bacterial community composition in both production lines. In the first line (line A), a substantial community shift was observed after slow sand filtration, resulting in a large increase in OTU richness. In the second production line (line B), OTU richness gradually increased after every treatment step. For both lines, OTU richness decreased after chlorination. Likewise, qPCR revealed the lowest amount of total bacteria after chlorination. Taxonomy assignment of the OTUs indicated that *Proteobacteria* was again the most abundant phylum. Whereas the bacterial community composition differed for both production lines, the bacterial community composition of the waters sampled at the tap (originating from line A) or in the storage tank (originating from line B) were highly similar, suggesting that the distribution of chlorinated water changes the bacterial community composition in a similar way irrespective of its composition in the production facility. In this study, seasonal effects showed to be of minor influence in shaping the bacterial community composition in comparison to spatial sampling. Seasonal differences were observed in Chapter II. This was most likely because in Chapter II the bacterial community composition was studied of drinking water production facilities using surface water as source water from different locations.

In contrast to Chapter II, *Acinetobacter* was found at low relative abundance in the water samples investigated in this study, reaching a maximum relative abundance of 2.8 %. Further research is needed to find out the factors underlying these huge differences in relative abundance. Absolute abundance of *Acinetobacter* was the lowest after chlorination for both lines. Yet, for line A, a significant increase in *Acinetobacter* presence was observed in the household tap water. Plating of the water samples revealed a total of 14 different *Acinetobacter* species based on *rpoB* (RNA polymerase beta subunit) gene similarity, among which three potential pathogenic species (i.e. *A. guillouiae*, *A. johnsonii*

and *A. lwoffii*) were found in finished drinking water or household tap water. Further research however is needed to confirm their pathogenicity and virulence. For these purposes, *Caenorhabditis elegans* worm model, the *Drosophila melanogaster* fly model, the *Galleria mellonella* caterpillar model, the *Dictyostelium discoideum* amoebic model and a number of mammalian models have been developed by which the pathogenicity and virulence of *Acinetobacter* strains can be determined (Cerqueira and Peleg, 2011). Several virulence factors have been identified in *A. baumannii* by genomic and phenotypic analyses, including outer membrane porins, phospholipases, proteases, lipopolysaccharides (LPS), capsular polysaccharides, protein secretion systems, and iron-chelating systems (Antunes et al. 2011; McConnell et al. 2013; Lin and Lan 2014). Furthermore, many reports have shown that *A. baumannii* rapidly develops resistance to antimicrobials, and multidrug-resistant strains have been isolated (McConnell et al. 2013). So far, in this regard, only very little is known for other *Acinetobacter* species. Additionally, phenotypic research can further investigate how closely related these isolates are to clinical strains from the same species. Therefore, we assessed trait differences between both groups of strains in Chapter IV, i.e. for several aquatic / environmental isolates versus clinical isolates of the same species.

Water chemical parameters were more significantly different between seasons than between sampling points with the exception of trihalomethanes. Indeed, total trihalomethanes concentration was the only parameter which was significantly different for sampling point. Total trihalomethanes concentration was much lower in one of the tap water samples originating from line A (sampling point A) in comparison to the other household tap waters. Interestingly, the bacterial community in the drinking water sampled at sampling point A was enriched in *Methylophilus* species, which are capable of utilizing chlorinated methanes (Bader and Leisinger 1994), and may therefore explain the lower trihalomethanes concentration at this sampling point.

Relative abundance of Acinetobacter *spp. and other important opportunistic premise plumbing pathogens (OPPP)*

Table 6.1 gives an overview of the average relative abundance of *Acinetobacter* spp. and other important opportunistic premise plumbing pathogens (OPPPs) found in this thesis, including *Pseudomonas*, *Mycobacterium* and *Legionella* (Chapter II and III). Although OTUs were grouped at genus level, it has to be noted that not all genus members are potentially pathogenic. Members of the genera *Acinetobacter* and *Pseudomonas* occurred at highest relative abundance in groundwater. Further, *Acinetobacter* remained at a high relative abundance throughout the entire production and distribution process when groundwater was used as source water. The genera *Mycobacterium* and *Legionella* tend to increase in relative abundance after purification and distribution. In Chapter II, a relatively high average relative abundance of *Acinetobacter* was also observed in the processed water for production facilities that use surface water as source water, yet, this was much less pronounced in Chapter III. The high average obtained in Chapter II was especially due to a high relative abundance of *Acinetobacter* in April,

reaching relative abundances from 13.5 up to 22.6 % sequences per sample. Drinking water production facilities that were sampled in November had a relative abundance of 0.12 up to 0.35 % *Acinetobacter* sequences per sample, suggesting a strong seasonal impact. However, this seasonal variation was much less pronounced in Chapter III.

Table 6.1: Average relative abundance (%) of *Acinetobacter* spp. and other important opportunistic premise plumbing pathogens found in this PhD study.

Genus	Chapter II GW[a]	Chapter II SW	Chapter III SW	Chapter II PWg	Chapter II PWs	Chapter III PWs	Chapter II HTWg	Chapter II HTWs	Chapter III HTWs
Acinetobacter	7.78	0.18	0.02	4.45	8.10	0.27	4.24	1.25	0.11
Pseudomonas	10.54	0.30	0.06	0.40	1.58	0.48	0.42	0.51	0.08
Mycobacterium	0.32	0.07	< 0.01	2.05	0.54	0.06	1.13	0.90	0.34
Legionella	0.22	< 0.01	0.02	0.32	0.18	0.44	0.22	0.92	0.47

[a] Abbreviations: GW, groundwater; SW, surface water; PW, processed water (the end product of the drinking water treatment before it is distributed), HTW, household tap water; g and s, originating from groundwater and surface water respectively.

Genotypic and phenotypic diversity of Acinetobacter *isolates from clinical and environmental sources, with special emphasis on (drinking) water*

The genus *Acinetobacter* comprises a large group of species that are known to flourish in diverse natural ecosystems as well as many man-made environments, including drinking water production and distribution systems. Furthermore, some *Acinetobacter* species are well adapted to human body sites, often causing opportunistic infections in certain patient populations. Despite the acknowledged importance of many *Acinetobacter* species as nosocomial pathogens, still relatively little is known about their ecology and epidemiology. It is, however, reasonable to assume that *Acinetobacter* strains find their way into clinical settings through different environmental sources, including contaminated water supplies. To test this hypothesis and to determine the level of relatedness between isolates from different environments, a comprehensive study of the relatedness of 58 isolates belonging to four *Acinetobacter* species that are associated with human infections was carried out using phenotypic and genotypic methods. Isolates were phenotyped using Biolog's GENIII microplate, which analyzes the performance of a microorganism in 94 phenotypic tests, including 71 carbon source utilization assays and 23 chemical sensitivity assays. A Spearman rank correlation analysis was performed to determine whether the ability to use different carbon sources co-vary between the different isolates. Additionally, antibiotic susceptibility profiles were generated using 15 antibiotic compounds from eight different classes. Further, isolates were genotyped by partial sequencing of the *rpoB* gene, and a Mantel test was performed to assess correlations between phenotypic and *rpoB* gene sequence similarity. When results obtained for the different carbon sources were evaluated based on the origin of the isolates (i.e. aquatic/environmental vs clinical/veterinary) (hence, irrespective of species classification), significant differences were obtained for six carbon sources. Mucic acid was utilized by several environmental/aquatic isolates (belonging to the species *A. calcoaceticus* and *A. johnsonii*), while only

one clinical isolate (belonging to *A. calcoaceticus*) was found to do so. Mucic acid, also known as galactaric acid, is a hexaric acid resulting from the oxidative ring cleavage of galactose. It is not clear so far why this compound could be particularly used by strains from aquatic environments, and not by clinical strains. By contrast, utilization of pectin, L-galactonic acid lactone and acetoacetic acid was related to isolates from clinical settings. Further, clinical isolates tend to yield a stronger signal for methyl pyruvate and L-lactic acid. Acetoacetic acid is a by-product of the partial degradation of fatty acids in the liver (Bora et al. 2019) which may explain its association with clinical strains. Such explanation could not be found for pectin and L-galactonic acid lactone, which are both plant-related compounds. L-Galactonic acid lactone is an intermediate compound in the production of L-ascorbic acid and pectin is a plant cell wall component (Harholt et al. 2010; Wheeler et al. 2015). On the other hand, 34 carbon sources and 14 chemical sensitivity assays were significantly different based on grouping by species classification, suggesting that phenotypic traits are more species- than habitat-dependent. The Spearman rank correlation test showed that several carbon sources co-varied between the different isolates. Strong significant correlations were mostly found for sugars and tend to be species dependent. Antibiotic susceptibility testing revealed that non-wild type (non-WT) phenotypes were more observed in isolates from finished drinking water (i.e. after chlorination or within the distribution network). Non-WT phenotypes were mostly found against amoxicillin, streptomycin and sulfamethoxazole/trimethoprim, which is in agreement with previous studies (Narciso-da-Rocha et al. 2013). Mantel test analysis revealed a significant relationships between the pairwise phylogenetic distance and trait differentiation among isolates for four carbon source assays (i.e. L-arginine, L-histidine, citric acid and γ-amino-butyric acid) and one chemical stressor (sodium bromate). Although these results shed more light on the inter- and intraspecific genetic and phenotypic variability within *Acinetobacter*, further research using more isolates is needed to draw strong conclusions, especially when the aim is to find specific signatures which are linked with origin. Genome analysis have already successfully been used to identify important differences between *Acinetobacter* species. Peleg and colleagues (2012) have shown that the core genome of *A. baumannii* strains contain many genes important for diverse metabolism and survival in the host. For example, *A. baumannii* was more successful in the use of nitrogen sources and was able to withstand stresses due to pH, osmotic pressure and antimicrobials in comparison to clinically less import *A. calcoaceticus* (Peleg et al. 2012). Unfortunately, this study included only one strain of *A. calcoaceticus* which makes it difficult to draw strong conclusions. Therefore, additional research is needed using multiple strains and other species, to further investigate their differences in metabolic diversity. A large set of *Acinetobacter* strains was used in Chapter V to assess whether phenotypic traits could predict the phylogenetic placement of the different *Acinetobacter* species based on their *rpoB* gene sequence as well as their core genome.

Phylogenetic signal in phenotypic traits related to carbon source assimilation and chemical sensitivity in Acinetobacter *species*

A common belief is that the phylogeny of bacteria may reflect molecular functions and phenotypic characteristics, pointing towards phylogenetic conservatism of traits. In a last experimental Chapter (Chapter V), we tested this hypothesis for a large set of *Acinetobacter* strains. Members of the genus *Acinetobacter* are widespread in nature, demonstrate a high metabolic diversity and are resistant to several environmental stressors. Notably, some species are known to cause opportunistic human infections. A total of 133 strains belonging to 33 species with validly published names, two genomic species and species of an as-yet unknown taxonomic status were analyzed using the GENIII technology of Biolog. We estimated the strength and significance of the phylogenetic signal of each trait across phylogenetic reconstructions based on partial *rpoB* and core genome sequences. Secondly, we tested whether phylogenetic distance was a good predictor of trait differentiation by Mantel test analysis. And finally, evolutionary model fitting was used to determine if the data for each phenotypic character was consistent with a phylogenetic or an essentially random model of trait distribution. The results of this study revealed that some key phenotypic traits related to substrate assimilation and chemical sensitivity are linked to the phylogenetic placement of *Acinetobacter* species. The strongest phylogenetic signals found were for utilization of different carbon sources such as some organic acids (citric acid), amino acids (L-arginine and L-histidine) and sugars (α-D-glucose and D-melibiose), thus suggesting that in the diversification of acinetobacters carbon source assimilation has had a relevant role, confirming our findings in Chapter IV. Future work should be aimed to clarify how such traits have shaped the remarkable ability of this bacterial group to dominate in a wide variety of habitats. For example, Garcia-Garcera and colleagues (2017) used metagenomics, comparative genomics of 133 *Acinetobacter* strains and a phylogenomics approach in order to assess in which environments the different *Acinetobacter* species could be isolated. They showed that strains isolated from humans and antibiotic treated bovine samples have a much less genetic diversity than the strains from untreated animals and soil samples. Furthermore, they were able to link the phylogenetic placement of the species with certain environments. However, certain species are associated with environments which are questionable to be able to isolate them from. *Acinetobacter nectaris* was linked to aquatic environments, yet, it is known that this species is accociated with floral nectar environments and require higher sugar concentrations for growth (Álvarez-Pérez et al. 2013). Additional phenotypic trait information could be useful in order to make good habitat predictions.

Perspectives

Bacterial community analysis in drinking water

Previous studies on the bacterial community composition in drinking water production and distribution systems have helped us greatly to understand how these communities are composed, formed and change during the whole chain from water catchment to the tap. Yet, several challenges still remain which are both technical and biological. Technical issues include, amongst others, sample preparation and optimization of the techniques used for assessment of the composition of the drinking water bacterial community. It has been found that sampling methodologies such as sample volume may affect the outcome of sequencing-based microbial community characterizations (Staley et al. 2015). In Staley et al. (2015), triplicate 1, 2 and 6 L volume water samples taken from a river were processed to determine variation among replicates and sample volumes. Replicate variability significantly influenced differences in the community α-diversity, while volume significantly changed β-diversity. Although it was concluded that triplicate 2 L samples allow robust microbial characterization of water samples (Staley et al. 2015), the best sampling strategy for water samples with variable bacterial numbers, as observed during the drinking water production chain, still needs to be determined. Additionally, it is known that the pore size of the filters used during sample preparation has an impact on the bacterial community composition as ultramicrobacteria might pass through larger pore sized filters (Liu et al. 2018c). Indeed, also Liu and colleagues (2018c) found clear differences in microbial community composition between water samples after filtration over a 0.22 µm filter and refiltration over a smaller pore size filter (0.10 µm). In this doctoral study, mixed cellulose ester filters, composed of cellulose acetate and cellulose nitrate, were used with a pore size of 0.45 µm, particularly to speed-up the filtration process. Although it cannot be excluded that we lost certain bacteria, our data sets contained several taxa which are considered ultramicrobacteria, suggesting loss of bacteria was probably limited. Furthermore, it is known that different DNA extraction methods, primer choice, PCR and sequencing bias, and bioinformatics sequence processing may affect the bacterial community composition (Pinto and Raskin, 2012; Brandt and Albertsen, 2018). In this PhD study, bacterial communities were characterized using standard OTUs based on a threshold of 97 % sequence similarity. This cut-off balances previous standards for defining bacterial species (Stackebrandt and Goebel, 1994) and a recognition of spurious diversity accumulated through PCR and sequencing errors (Acinas et al. 2005; Kunin et al. 2010; Rosselló-Mòra, 2011). However, due to the typical short sequence lengths obtained with second generation sequencers, a single OTU may contain closely related but distinct species. Therefore, there is a growing tendency to move towards analysis of exact sequence variants, also termed amplicon sequence variants (ASVs) (Callahan et al. 2017) or zero-radius OTUs (zOTUs) (Edgar, 2016b), increasing taxonomic resolution. However, so far these new methods have not been implemented yet in microbial ecological studies related to drinking water. Yet, this could be particularly

interesting for *Acinetobacter* species, which have highly similar 16S rRNA gene sequences (Ibrahim et al. 1997; La Scola et al. 2006). For overall ecological conclusions however, it has been shown that both methods yield similar results in terms of α-diversity and β-diversity (Glassman and Martiny, 2018), thereby reinforcing the use of any of these methods. This said, there is an urgent need for standardized approaches (from sampling up to data analysis) to study drinking water microbial communities enabling an improved way of comparison of different studies. Additionally, DNA based methods are often criticized because they do not distinguish between live, dormant and dead cells. However, there are a number of potential approaches (i.e. propidium monoazide staining or mRNA-based methods) that can be used to circumvent this issue (Li et al. 2018). Propidium monoazide is a membrane-impermeant and photo-reactive DNA binding dye which is capable of entering non-viable cells. Once it is bound to the DNA of non-viable cells, the DNA becomes unable to be multiplied by PCR. This technique has been useful to distinguish viable and non-viable cells as well as in microbial community studies (Nocker et al. 2007; Tantikachornkiat et al. 2016, Mo et al. 2019). RNA-based methods on the other hand can detect cells which produce RNA molecules. These methods have been used in several environments and have shown to be useful to define the active members of a community (Inkinen et al. 2016; Li et al. 2017b; Wuyts et al. 2018).

Further, the lack of meta data for many studies (e.g. environmental parameters, technical information of the piping system, drinking water production facility etc.) makes it often difficult to interpret and compare results from different studies. Therefore, there is not only a need for standardized methods, but it would also be advisable that researchers make their meta data (seasonal data, biological and chemical parameters) available, or even to agree on a minimum required set of parameters that needs to be measured or reported (e.g. a detailed description of the treatment steps, and information on the age, the type or the hydraulic regimes of the distribution network), as well as consistent ways of data analysis and presentation. Alternatively, one may opt to control for these parameters by studying well-defined pilot-scale drinking water treatment and distribution systems, rather than real-world systems (Regan et al. 2002; Lehtola et al. 2004b). However, it is doubtful that such pilot scale plant could realistically mimic a true drinking water treatment and distribution system.

With the introduction of NGS technologies our knowledge of the bacterial diversity and members of the drinking water environment has greatly improved. However, there is still a gap between the bacterial community composition and its impact for the drinking water industry in terms of operational hazards (e.g. deterioration of plumbing materials) and public health risks. In the last decade an increasing number of studies have become available linking microbial communities with health, disease suppression, performance and management of bioprocesses, etc. (Trivedi et al. 2016; Sommer et al. 2017). NGS has particularly revolutionized the human gut microbiome research, and links have been established between the presence of certain bacterial species and how the shape the intestinal immune response (Round and Mazmanian, 2009). By analogy with these studies, also for drinking water management, models can potentially be developed that predict microbial community compositions that

support delivery of safe water. However, for drinking water this may be more challenging as it seems not so straightforward to define an "healthy" or "unhealthy" bacterial community composition as long as water quality guidelines are based on classical indicators such as coliforms and enterococci, or OTUs can only be identified to the genus level. Until now, most studies of microbiota rely on second-generation sequencing such as 454 pyrosequencing or Illumina Miseq sequencing which target a short fragment of the 16S rRNA gene, typically around 250-300 bp. Due to the short read length associated with these sequencers, these strategies usually fail to assign taxonomy reliably at the species level (sometimes even at the genus level) (Ceuppens et al. 2017). With the launching of third-generation single-molecule technology sequencers, these short-length associated issues can be overcome by sequencing the full-length of the 16S rRNA gene (ca. 1,500 bp) or even the whole ribosomal RNA gene operon (ca. 4,500 bp), which includes the 16S rRNA gene, ITS region, and 23S rRNA gene. Recent studies have shown the great potential of third-generation sequencing technologies like MinION nanopore sequencing to profile microbial communities in detail with high identification accuracy (Cuscó et al. 2018). Alternatively, shotgun metagenomic sequencing, i.e. sequencing of all the DNA extracted from a given sample (rather than a single PCR-amplified phylogenetic marker such as 16S rRNA gene), can be performed to accurately unravel the microbial community composition (Tringe and Rubin, 2005). This method enables microbiologists to evaluate bacterial diversity and detect the abundance of microbes with great precision in various environments, without having to rely on PCR amplification. Further, in addition to detailed taxonomic information, shotgun metagenomic sequencing also provides functional information, which may also allow us to pinpoint specific microorganisms and functions that are related to important changes in the water chemistry. It is reasonable to expect that such technologies will be increasingly used in the near future in the field of microbial ecology, including studies of drinking water. Importantly, future studies should not only focus on the microbial community composition, but also on the density at which key members occur. Recent studies have shown that quantitative microbiome analysis and absolute quantification are of importance for understanding pathophysiological manifestations and interpreting disease diagnosis (Vandeputte et al. 2017; Vieira-Silva et al. 2018).

Acinetobacter in drinking water

Previous studies have shown the presence of multiple *Acinetobacter* species in drinking water, which is confirmed by the results obtained in this PhD study. Although transmission via drinking water is rarely reported, it can be assumed that *Acinetobacter* can be dispersed by drinking water. Furthermore, the increase of antibiotic resistance within the genus is frightening, especially in combination with the observation that antibiotic resistance genes and antibiotic resistant bacteria are enriched after drinking water treatment. It demands for further investigation on how these bacteria are able to survive the drinking water treatments and how this might be linked to increased antibiotic resistance. Disinfection

steps such as chlorination are presumed to attribute to increased antibiotic resistance (Jia et al. 2015), but the precise mechanisms underlying this effect still remain to be unraveled. Many antibiotic resistance genes are carried on plasmids, transposons or integrons that can act as vectors transferring these genes to other bacteria (Boucher et al. 2007; Boerlin and Reid-Smith, 2008). Replication of plasmids has been shown to be promoted by extracellular stress (Wegrzyn and Wegrzyn, 2002). It can therefore be hypothesized that chlorination acts as a stressor on bacteria surviving the chlorination step, thereby promoting the transfer and acquisition of antibiotic resistance genes. Further research is needed to confirm this scenario. In Chapter IV, we observed non-WT phenotypes for several antibiotic compounds. It still remains to be investigated whether this observation correlates with antibiotic resistance, whether these isolates are able to resist the antibiotics *in vivo* and if they possess antibiotic resistance genes. Although certain phenotypic traits were found to be predictive for the origin of the isolates further investigation is needed to determine whether isolates from drinking water could be pathogenic. In this regard, several virulence factors have been investigated in the *Acinetobacter* genus, including motility, resistance to disinfection, desiccation, biofilm formation, adherence mechanisms, iron acquisition, activities of polysaccharide membrane and outer membrane vesicles (reviewed in Wong et al. 2017). Because *A. baumannii* is the most important pathogenic member of the genus, most of the virulence factors have been studied within this species. Further research is needed to determine if the same factors also occur in other potentially pathogenic species within the genus. The metabolic versatility of the genus and the presence of several bacterial species within drinking water production and distribution system also raise questions related to their interactions with other species. *Acinetobacter* species have been shown to be able to coaggregate with other bacterial species (Chaves Simões et al. 2008; Malik et al. 2003). Coaggregation is a process by which genetically distinct bacteria become attached to one another via specific molecules (protein-saccharide interactions). Cumulative evidence suggests that such adhesion influences the development of complex multi-species biofilms. Further research is needed to understand such metabolic cooperation between strains from different genera in drinking water biofilms. It is known that the majority of bacteria occuring in drinking water pipes reside in biofilms formed on the pipe's inside or occur within loose deposits (up to 98 %) (Liu et al. 2014), by which they are better protected against desinfectants. Nevertheless, it has also been demonstrated that up to 54 % of the planktonic bacteria from the treated water reach the end user (Liu et al. 2018a). A major influence of loose deposits and biofilm has been observed on tap water planktonic and particle-associated bacteria, which was influenced by hydrolic changes (Liu et al. 2018a). Further research is required to study these mechanisms of bacterial release from loose deposits and biofilm as well as how and where regrowth occurs within the drinking water distribution systems. Furthermore, we found robust evidence for the presence of a phylogenetic signal in several phenotypic traits related to carbon source assimilation and chemical sensitivity. Future work should be aimed to clarify how such traits have shaped the remarkable ability of *Acinetobacter* species to dominate in a wide variety of habitat types. Additionally, the mathematical analysis applied in Chapter V to predict the phylogenetic placement of

the different *Acinetobacter* species based on phenotypic traits, should be further investigated for their use towards predicting the metabolic versatility of *Acinetobacter* strains based on genome information. Evolutionary placement analysis performed on the *Acinetobacter* genus has suggested that the genus may be subdivided in several, separate genera based on environmental categories (Garcia-Garcera et al. 2017). The clades defined in this study were based on whole genome sequences and were indirectly linked with their putatively natural habitat derived from 16S rRNA gene-based studies. As these studies often lack a high level of resolution, the accuracy of linking both datasets can be questioned. This said, it is generally accepted that an ecotype may represent a separate taxonomic unit, yet, subdivisions must be confirmed by clear genetic and/or phenotypic differences (Cohan, 2017). *Acinetobacter nectaris* and *A. boissieri* are two good examples of ecotype species. They are well adapted to a very specific niche namely floral nectar, yet, they both have clear different phenotypic traits. However not all *Acinetobacter* species are so niche depended. Therefore, further research is needed to get a better grip on the taxonomy of *Acinetobacter*, and the potential distinction into new genera or subdivisions. The phenotypic data generated in this PhD study may also help in this regard.

Altogether, this thesis has contributed to the knowledge of the bacterial community composition within DWPDS. However, in terms of the biology of drinking water systems, the presence of other lifeforms such as viruses, fungi, amoebae/protozoa, nematodes and copepods are often neglected when studying bacterial communities. Yet, they have been shown to also influence bacterial aquatic communities (Locas et al. 2007; Thomas and Ashbolt, 2011; Buse et al. 2013; Cram et al. 2016), for example, amoebae are grazing on bacteria and are also known to harbor and protect intracellular bacteria. There is a need for further research on these biological interactions within DWPDS. Furthermore, our study has demonstrated the presence of multiple *Acinetobacter* species within DWPDS. Additionally, we have observed that certain phenotypes can be habitat-dependent, although, most phenotypes were more species-dependent. And finally, we have discovered that the phylogenetic placement of *Acinetobacter* species can be linked to some phenotypic traits. Further research is needed to explore the ecological role of *Acinetobacter* species within DWPDS.

Supporting information

Supporting information Chapter II

Table S2.1: Bacterial community diversity indices for the different water samples investigated in Chapter II.

Sample identifier[a]	Geographical origin	Water type[b]	Sampling period	Sobs[c]	Ace[d]	Coverage [%][e]	Shannon[f]	Evenness[g]
A1Ua	Antwerp	GW	April	150	170.2	88.1	4.3	0.866
A1Wa	Antwerp	PWg	April	186	282.5	65.8	4.4	0.835
A1Ya	Antwerp	HTWg	April	140	259.7	53.9	3.6	0.716
A2Ua	Antwerp	GW	April	181	270.0	67.0	4.6	0.875
A2Wa	Antwerp	PWg	April	235	437.0	53.8	4.6	0.833
A2Ya	Antwerp	HTWg	April	183	283.8	64.5	4.3	0.818
A3Va	Antwerp	SW	April	63	77.4	81.4	3.1	0.758
A3Xa	Antwerp	PWs	April	129	150.4	85.8	3.9	0.803
A3Za	Antwerp	HTWs	April	84	116.8	71.9	3.1	0.689
A4Un	Antwerp	GW	November	122	180.5	67.6	3.7	0.773
A4Wn	Antwerp	PWg	November	192	361.1	53.2	4.3	0.817
A4Yn	Antwerp	HTWg	November	204	296.2	68.9	4.5	0.847
B1Ua	Limburg	GW	April	120	138.6	86.6	2.9	0.586
B1Wa	Limburg	PWg	April	131	159.7	82.0	3.5	0.713
B1Ya	Limburg	HTWg	April	123	123.0	100.0	3.7	0.769
C1Va	East Flanders	SW	April	68	68.0	100.0	3.7	0.869
C1Xa	East Flanders	PWs	April	69	70.5	97.9	3.1	0.741
C1Za	East Flanders	HTWs	April	58	84.1	69.0	2.3	0.558
C2Un	East Flanders	GW	November	208	416.1	50.0	4.4	0.820
C2Wn	East Flanders	PWg	November	173	285.3	60.6	3.8	0.735
C2Yn	East Flanders	HTWg	November	113	159.2	71.0	3.5	0.728
D1Ua	Flemish Brabant	GW	April	109	216.9	50.3	2.0	0.385
D1Wa	Flemish Brabant	PWg	April	188	255.5	73.6	4.5	0.847
D1Ya	Flemish Brabant	HTWg	April	132	176.9	74.6	3.2	0.655
E1Va	West Flanders	SW	April	123	186.0	66.1	3.8	0.797
E1Xa	West Flanders	PWs	April	120	196.0	61.2	3.6	0.762
E1Za	West Flanders	HTWs	April	130	189.3	68.7	3.9	0.794
E2Vn	West Flanders	SW	November	133	191.5	69.5	4.0	0.812
E2Xn	West Flanders	PWs	November	104	157.1	66.2	2.8	0.606
E2Zn	West Flanders	HTWs	November	163	238.3	68.4	4.3	0.839
E3Vn	West Flanders	SW	November	156	254.8	61.2	4.1	0.806
E3Xn	West Flanders	PWs	November	163	252.1	64.7	4.4	0.863
E3Zn	West Flanders	HTWs	November	212	339.5	62.4	4.6	0.860
E4Vn	West Flanders	SW	November	180	290.9	61.9	4.4	0.850
E4Xn	West Flanders	PWs	November	152	190.2	79.9	4.1	0.817
E4Zn	West Flanders	HTWs	November	183	278.9	65.6	4.2	0.801
E5Un	West Flanders	GW	November	133	247.0	53.8	3.5	0.718
E5Wn	West Flanders	PWg	November	212	387.9	54.7	4.5	0.833
E5Yn	West Flanders	HTWg	November	233	385.4	60.5	4.9	0.902
E6Vn	West Flanders	SW	November	164	241.6	67.9	4.1	0.806
E6Xn	West Flanders	PWs	November	158	206.9	76.4	4.1	0.812

[a] Sample identifiers "(A-E)(1-6)(U-Z)(a or n)" contain information about their origin: A-E: geographical origin (A, Antwerp; B, Limburg; C, East Flanders; D, Flemish Brabant; E, West Flanders); 1-6: studied company within a particular region; U-Z: water type (U, groundwater; V, surface water; W, processed water produced from groundwater; X, processed water produced from surface water; Y, household tap water processed from groundwater; Z, household tap water processed from surface water); a, April; n, November.
[b] GW, groundwater; PWg, processed water produced from groundwater; HTWg, household tap water processed from groundwater; SW, surface water; PWs, processed water produced from surface water; HTWs, household tap water processed from surface water.
[c] Observed richness.
[d] Abundance-based coverage estimator.
[e] Observed richness/Ace estimate * 100.
[f] Shannon-Wiener diversity index.
[g] Peilou's evenness: entropy (Shannon-Wiener diversity index) divided by the logarithm of the number of OTUs.

Table S2.2: Analysis of variance (ANOVA) of the bacterial community diversity indices.

Grouping of samples	**Number of OTUs**		**Ace**[b]		**Shannon**[c]		**Evenness**[d]	
	F value	*p* value	F value	*p* value	F value	*p* value	F value	*p* values
Water type[a] (i.e. GW, SW, PWg, PWs, HTWg and HTWg)	2.098	0.089	2.171	0.080	0.722	0.612	0.758	0.568
Sampling period (i.e. April and November)	8.642	0.006	9.576	0.004	6.738	0.013	3.381	0.074

[a] Different water types: GW, groundwater; SW, surface water; PW, processed water; and HTW, household tap water; g or s, originating from groundwater or surface water, respectively.

[b] Abundance-based coverage estimator.

[c] Shannon-Wiener diversity index.

[d] Peilou's evenness: entropy (Shannon-Wiener diversity index) divided by the logarithm of the number of OTUs.

Table S2.3: Identification[a] of operational taxonomic units (OTUs) according to the Silva database and distribution over the investigated samples.

[a] Taxonomic assignment scores are provided between brackets. In general, taxonomic assignments are considered reliable when bootstrap confidence values exceed > 80.

See excel file: PhD_Van Assche Ado_Chapter II_TableS2-3_taxonomy.xlsx

Van Assche, A. (2019, September 10). PhD Ado Van Assche. https://doi.org/10.17605/OSF.IO/W7MK3

Table S2.4: Results of the indicator species analysis for the different water types studied.

Water type[a]	OTU ID	Phylum[b]	Genus[c]	A[d]	B[e]	*p* value[f]
GW	820	***Proteobacteria* (100)**	unclassified	1.000	0.571	0.005
	448	***Proteobacteria* (100)**	unclassified	0.769	0.571	0.009
	473	***Microgenomates* (91)**	unclassified	1.000	0.429	0.019
	426	***Proteobacteria* (100)**	unclassified	1.000	0.429	0.019
	378	***Firmicutes* (100)**	unclassified	0.698	0.571	0.023
	66	***Nitrospirae* (86)**	Candidatus Magnetoovum (50)	0.927	0.429	0.036
	957	***Proteobacteria* (100)**	unclassified	0.800	0.429	0.050
	1317	Candidate division OP3(94)	unclassified	0.800	0.429	0.037
PWg	139	***Proteobacteria* (100)**	***Variibacter* (85)**	0.901	0.714	0.027
	864	***Proteobacteria* (100)**	unclassified	1.000	0.571	0.001
	801	Candidate division OP3 (29)	unclassified	0.929	0.571	0.010
	51	***Proteobacteria* (100)**	unclassified	0.788	0.571	0.016
	446	*Gracilibacteria* (56)	unclassified	1.000	0.429	0.021
	781	***Proteobacteria* (99)**	unclassified	1.000	0.429	0.019
	1159	***Proteobacteria* (100)**	***Legionella* (100)**	1.000	0.429	0.021
	597	***Parcubacteria* (99)**	unclassified	0.600	0.571	0.045
HTWg	707	***Gemmatimonadetes* (100)**	unclassified	1.000	0.429	0.022
SW	93	***Actinobacteria* (100)**	hgcI clade (89)	0.968	1.000	0.001
	56	***Actinobacteria* (100)**	hgcI clade (51)	0.943	1.000	0.001
	21	***Actinobacteria* (100)**	Candidatus Limnoluna (92)	0.934	1.000	0.001
	98	***Proteobacteria* (100)**	***Polynucleobacter* (100)**	0.914	1.000	0.001
	37	***Actinobacteria* (100)**	hgcI clade (100)	0.902	1.000	0.001
	32	***Actinobacteria* (100)**	hgcI clade (100)	0.887	1.000	0.001
	121	***Bacteroidetes* (100)**	unclassified	0.859	1.000	0.001
	191	***Actinobacteria* (100)**	Candidatus Rhodoluna (99)	0.857	1.000	0.001
	148	***Actinobacteria* (100)**	CL500-29 marine group (100)	1.000	0.857	0.001
	365	***Bacteroidetes* (100)**	***Fluviicola* (93)**	1.000	0.857	0.001
	349	***Proteobacteria* (100)**	***Arenimonas* (99)**	1.000	0.857	0.001
	25	***Bacteroidetes* (100)**	***Fluviicola* (100)**	0.971	0.857	0.001
	205	***Actinobacteria* (100)**	***Alpinimonas* (98)**	0.968	0.857	0.001
	212	***Proteobacteria* (100)**	LD28 freshwater group (100)	0.954	0.857	0.001
	95	***Actinobacteria* (100)**	hgcI clade (94)	0.952	0.857	0.001
	76	***Verrucomicrobia* (100)**	unclassified	0.933	0.857	0.001
	40	***Cyanobacteria* (100)**	unclassified	0.930	0.857	0.001
	71	***Bacteroidetes* (100)**	***Pseudarcicella* (100)**	0.929	0.857	0.001
	335	***Actinobacteria* (100)**	hgcI clade (60)	0.889	0.857	0.001
	102	***Bacteroidetes* (100)**	***Algoriphagus* (96)**	1.000	0.714	0.001
	242	***Actinobacteria* (100)**	hgcI clade (99)	1.000	0.714	0.001
	58	***Bacteroidetes* (100)**	***Sediminibacterium* (99)**	0.706	1.000	0.001
	209	***Verrucomicrobia* (100)**	unclassified	0.975	0.714	0.001
	36	***Proteobacteria* (100)**	***Polaromonas* (92)**	0.673	1.000	0.002
	134	***Actinobacteria* (100)**	Candidatus Planktoluna (96)	0.939	0.714	0.001
	967	***Bacteroidetes* (100)**	***Flavobacterium* (100)**	0.777	0.857	0.001
	34	***Bacteroidetes* (100)**	***Flavobacterium* (100)**	0.922	0.714	0.001
	1172	***Proteobacteria* (100)**	*Simplicispira* (61)	0.767	0.857	0.002

	27	***Bacteroidetes* (100)**	***Flavobacterium* (100)**	0.884	0.714	0.002
	87	***Actinobacteria* (100)**	hgcI clade (100)	0.875	0.714	0.001
	185	***Bacteroidetes* (100)**	*Flavisolibacter* (43)	0.875	0.714	0.001
	990	***Bacteroidetes* (100)**	***Flavobacterium* (100)**	0.862	0.714	0.002
	11	***Proteobacteria* (100)**	unclassified	0.857	0.714	0.003
	179	***Bacteroidetes* (100)**	***Fluviicola* (98)**	0.825	0.714	0.005
	269	***Verrucomicrobia* (100)**	unclassified	1.000	0.571	0.001
	538	***Proteobacteria* (100)**	unclassified	1.000	0.571	0.003
	308	***Bacteroidetes* (100)**	***Leadbetterella* (100)**	1.000	0.571	0.001
	450	***Bacteroidetes* (100)**	unclassified	1.000	0.571	0.001
	373	***Verrucomicrobia* (100)**	*Haloferula* (50)	1.000	0.571	0.001
	454	***Verrucomicrobia* (100)**	***Prosthecobacter* (100)**	0.928	0.571	0.006
	400	***Bacteroidetes* (100)**	*Flavisolibacter* (68)	0.905	0.571	0.003
	598	***Bacteroidetes* (100)**	unclassified	0.900	0.571	0.002
	773	***Proteobacteria* (100)**	***Aeromonas* (100)**	0.875	0.571	0.004
	414	***Bacteroidetes* (100)**	***Fluviicola* (100)**	0.833	0.571	0.009
	1394	***Bacteroidetes* (100)**	unclassified	0.778	0.571	0.008
	128	***Cyanobacteria* (100)**	unclassified	1.000	0.429	0.014
	401	***Bacteroidetes* (100)**	***Flavobacterium* (100)**	1.000	0.429	0.019
	250	***Cyanobacteria* (99)**	unclassified	1.000	0.429	0.022
	521	***Proteobacteria* (100)**	GKS98 freshwater group (97)	1.000	0.429	0.012
	918	***Planctomycetes* (100)**	***Phycisphaera* (100)**	1.000	0.429	0.015
	731	***Proteobacteria* (100)**	***Deefgea* (100)**	1.000	0.429	0.018
	752	***Verrucomicrobia* (100)**	***Haloferula* (98)**	1.000	0.429	0.023
	1076	***Bacteroidetes* (100)**	***Rheinheimera* (99)**	1.000	0.429	0.017
	1455	***Firmicutes* (91)**	Erysipelotrichaceae UCG-004 (71)	1.000	0.429	0.018
	1058	***Bacteroidetes* (100)**	***Ferruginibacter* (100)**	1.000	0.429	0.014
	1071	***Proteobacteria* (100)**	***Pseudospirillum* (100)**	1.000	0.429	0.020
	1114	*Proteobacteria* (72)	*Fretibacter* (6)	1.000	0.429	0.013
	111	***Bacteroidetes* (100)**	***Flavobacterium* (100)**	0.976	0.429	0.030
	1075	***Bacteroidetes* (100)**	***Pedobacter* (97)**	0.727	0.571	0.019
	197	***Chloroflexi* (100)**	***Roseiflexus* (100)**	0.955	0.429	0.024
	210	***Verrucomicrobia* (98)**	unclassified	0.952	0.429	0.031
	692	***Armatimonadetes* (100)**	***Armatimonas* (100)**	0.923	0.429	0.021
	983	***Proteobacteria* (100)**	*Rhodobacter* (23)	0.778	0.429	0.046
PWs	55	***Cyanobacteria* (100)**	unclassified	0.827	0.571	0.033
	49	***Cyanobacteria* (89)**	unclassified	0.942	0.429	0.036
	603	***Proteobacteria* (100)**	*Shinella* (54)	0.706	0.571	0.013
	156	***Planctomycetes* (100)**	***Singulisphaera* (92)**	0.891	0.429	0.028
	475	***Planctomycetes* (100)**	unclassified	0.833	0.429	0.045
HTWs	18	***Proteobacteria* (100)**	***Novosphingobium* (92)**	0.966	0.667	0.047
	182	***Gemmatimonadetes* (98)**	unclassified	0.933	0.500	0.004
	338	***Proteobacteria* (100)**	***Legionella* (100)**	0.896	0.500	0.019
	104	***Proteobacteria* (100)**	*Blastochloris* (28)	0.870	0.500	0.027
	309	***Proteobacteria* (100)**	unclassified	0.757	0.500	0.016
	217	***Proteobacteria* (100)**	*Ponticaulis* (49)	0.752	0.500	0.032
	343	***Proteobacteria* (100)**	unclassified	0.739	0.500	0.030
	352	***Proteobacteria* (100)**	unclassified	0.724	0.500	0.027
	375	***Proteobacteria* (100)**	unclassified	1.000	0.333	0.027

886	***Omnitrophica* (98)**	unclassified	1.000	0.333	0.027
553	***Planctomycetes* (100)**	unclassified	1.000	0.333	0.027
1471	*Proteobacteria* (70)	unclassified	1.000	0.333	0.027
915	***Proteobacteria* (82)**	unclassified	1.000	0.333	0.017
1174	***Planctomycetes* (89)**	unclassified	1.000	0.333	0.027
196	***Planctomycetes* (100)**	***Planctomyces* (99)**	0.724	0.333	0.034

[a] GW, groundwater; SW, surface water; PW, processed water; and HTW, household tap water; g or s, originating from groundwater or surface water, respectively.

[b] Phylum identification based on the Silva database; bootstrap confidence values are given within parentheses; taxonomic assignments are generally considered reliable when bootstrap confidence values exceed 80 (indicated in bold).

[c] Genus identification based on the Silva database; bootstrap confidence values are given within parentheses; taxonomic assignments are generally considered reliable when bootstrap confidence values exceed 80 (indicated in bold); when a confidence value of 0 was obtained, the OTU is considered "unclassified".

[d] Specificity score between 0 and 1; the closer to 1, the more the OTU is a robust indicator for the water type (a score of 1 indicates a unique OTU).

[e] Fidelity score between 0 and 1; the higher the score the more samples within the group contain that OTU (a score of 1 indicates that all samples of the group contain the OTU).

[f] OTUs with a *p* value less than 0.05 are considered significant indicators.

Table S2.5: Results of indicator species analysis for the two different sampling periods (April and November).

Sampling period	OTU ID	Phylum[a]	Genus[b]	A[c]	B[d]	*p* value[e]
April	1	***Proteobacteria* (100)**	***Acinetobacter* (100)**	0.992	0.667	0.004
	122	***Proteobacteria* (100)**	***Vibrio* (100)**	0.966	0.476	0.003
	62	***Proteobacteria* (100)**	***Stenotrophomonas* (98)**	1.000	0.429	0.002
	91	***Proteobacteria* (100)**	unclassified	0.816	0.524	0.018
	430	***Proteobacteria* (100)**	*Klebsiella* (46)	0.937	0.429	0.007
	84	***Cyanobacteria* (100)**	unclassified	1.000	0.381	0.001
	34	***Bacteroidetes* (100)**	***Flavobacterium* (100)**	0.982	0.381	0.023
	160	***Proteobacteria* (100)**	*Thioalkalispira* (63)	0.977	0.381	0.008
	239	***Firmicutes* (100)**	***Brevibacillus* (100)**	0.942	0.381	0.009
	246	***Bacteroidetes* (100)**	***Chryseobacterium* (99)**	1.000	0.333	0.007
	115	***Bacteroidetes* (100)**	***Hydrotalea* (99)**	1.000	0.286	0.023
	487	***Cyanobacteria* (100)**	unclassified	1.000	0.286	0.026
	208	***Proteobacteria* (100)**	***Sphingopyxis* (100)**	0.938	0.286	0.022
	238	***Proteobacteria* (100)**	***Novosphingobium* (84)**	0.935	0.286	0.047
	369	***Proteobacteria* (100)**	unclassified	1.000	0.238	0.036
	492	***Proteobacteria* (100)**	***Pseudomonas* (88)**	1.000	0.238	0.049
	742	***Nitrospirae* (100)**	unclassified	1.000	0.238	0.036
	264	***Bacteroidetes* (100)**	***Sphingobacterium* (100)**	1.000	0.238	0.044
November	10	***Firmicutes* (100)**	*Bhargavaea* (24)	0.764	0.900	0.001
	24	***Actinobacteria* (100)**	***Streptomyces* (82)**	0.879	0.750	0.001
	65	***Actinobacteria* (100)**	unclassified	0.794	0.750	0.003
	73	***Firmicutes* (100)**	***Tumebacillus* (100)**	0.850	0.700	0.002
	8	***Proteobacteria* (100)**	***Mizugakiibacter* (100)**	0.896	0.650	0.002
	172	***Actinobacteria* (100)**	***Streptomyces* (100)**	0.802	0.700	0.009
	275	***Chloroflexi* (100)**	unclassified	1.000	0.550	0.001
	194	***Proteobacteria* (100)**	***Sphingomonas* (97)**	0.816	0.650	0.004
	912	***Actinobacteria* (100)**	***Streptacidiphilus* (96)**	0.814	0.650	0.006
	165	***Bacteroidetes* (100)**	*Flavisolibacter* (48)	0.738	0.700	0.003
	50	***Proteobacteria* (100)**	***Sulfuricurvum* (100)**	0.996	0.500	0.001
	31	***Gemmatimonadetes* (100)**	unclassified	0.993	0.500	0.001
	163	***Acidobacteria* (100)**	***Bryobacter* (98)**	0.747	0.650	0.005
	42	***Acidobacteria* (100)**	***Acidobacterium* (100)**	0.766	0.600	0.005
	11	***Proteobacteria* (100)**	unclassified	0.917	0.500	0.013
	5	***Proteobacteria* (100)**	***Phreatobacter* (100)**	0.913	0.500	0.029
	192	***Proteobacteria* (100)**	***Sphingomonas* (100)**	0.820	0.550	0.008
	420	***Planctomycetes* (100)**	***Schlesneria* (100)**	0.973	0.450	0.002
	679	***Chloroflexi* (96)**	unclassified	0.957	0.450	0.003
	222	***Actinobacteria* (100)**	*Micromonospora* (56)	0.766	0.550	0.009
	46	***Actinobacteria* (100)**	unclassified	0.697	0.600	0.024
	350	***Actinobacteria* (100)**	unclassified	0.759	0.550	0.014
	7	***Proteobacteria* (100)**	***Gallionella* (97)**	0.920	0.450	0.034
	90	***Actinobacteria* (100)**	***Blastococcus* (100)**	1.000	0.400	0.001
	82	***Proteobacteria* (100)**	***Ralstonia* (100)**	0.988	0.400	0.004
	493	***Actinobacteria* (100)**	unclassified	0.876	0.450	0.013
	107	***Chloroflexi* (100)**	unclassified	0.982	0.400	0.002
	519	***Firmicutes* (100)**	***Tumebacillus* (100)**	0.856	0.450	0.006
	99	***Proteobacteria* (100)**	***Mizugakiibacter* (100)**	0.944	0.400	0.016

380	***Proteobacteria* (100)**	***Devosia* (92)**	0.817	0.450	0.025
992	***Proteobacteria* (100)**	***Gallionella* (94)**	0.897	0.400	0.006
146	***Actinobacteria* (100)**	***Patulibacter* (96)**	0.796	0.450	0.017
150	***Cyanobacteria* (100)**	unclassified	1.000	0.350	0.003
437	***Proteobacteria* (100)**	*Crenothrix* (61)	0.869	0.400	0.044
747	***Gemmatimonadetes* (100)**	unclassified	0.869	0.400	0.030
301	***Actinobacteria* (100)**	unclassified	0.963	0.350	0.003
77	***Gemmatimonadetes* (100)**	unclassified	0.920	0.350	0.027
322	***Acidobacteria* (100)**	*Acidobacterium* (76)	0.913	0.350	0.025
289	***Actinobacteria* (100)**	***Actinomadura* (100)**	0.798	0.400	0.050
653	***Actinobacteria* (100)**	unclassified	0.904	0.350	0.014
377	***Firmicutes* (100)**	***Bacillus* (83)**	0.894	0.350	0.040
600	***Proteobacteria* (100)**	***Microvirga* (100)**	0.880	0.350	0.039
201	***Proteobacteria* (100)**	unclassified	0.876	0.350	0.048
501	***Gemmatimonadetes* (100)**	***Gemmatimonas* (100)**	1.000	0.300	0.009
586	*Euryarchaeota* (40)	unclassified	1.000	0.300	0.015
262	***Proteobacteria* (100)**	*Thermomonas* (70)	0.825	0.350	0.032
764	***Actinobacteria* (100)**	***Modestobacter* (100)**	0.944	0.300	0.030
885	***Gemmatimonadetes* (100)**	*Gemmatimonas* (53)	0.794	0.350	0.040
686	***Chloroflexi* (100)**	***Sphaerobacter* (95)**	0.904	0.300	0.026
879	***Firmicutes* (100)**	*Ammoniphilus* (63)	0.904	0.300	0.026
274	***Actinobacteria* (100)**	*Actinoplanes* (48)	1.000	0.250	0.023
363	**Candidate division OP3 (100)**	unclassified	1.000	0.250	0.017
523	***Actinobacteria* (100)**	***Acidothermus* (98)**	1.000	0.250	0.025
705	***Actinobacteria* (100)**	unclassified	1.000	0.250	0.023
136	***Acidobacteria* (100)**	unclassified	1.000	0.250	0.019
931	***Actinobacteria* (100)**	*Jatrophihabitans* (22)	1.000	0.250	0.020
1112	***Proteobacteria* (100)**	*Oceanicoccus* (19)	1.000	0.250	0.022
1382	***Parcubacteria* (100)**	unclassified	1.000	0.250	0.020
87	***Actinobacteria* (100)**	**hgcI clade (100)**	0.940	0.250	0.038
479	***Proteobacteria* (100)**	***Skermanella* (100)**	1.000	0.200	0.040
308	***Bacteroidetes* (100)**	*Leadbetterella* (100)	1.000	0.200	0.050
450	***Bacteroidetes* (100)**	unclassified	1.000	0.200	0.050
481	***Actinobacteria* (100)**	***Crossiella* (100)**	1.000	0.200	0.047
893	***Firmicutes* (100)**	*Bacillus* (23)	1.000	0.200	0.044
631	***Firmicutes* (100)**	*Thalassobacillus* (46)	1.000	0.200	0.050
957	***Proteobacteria* (100)**	unclassified	1.000	0.200	0.044
1119	***Proteobacteria* (100)**	Candidatus Gigarickettsia (32)	1.000	0.200	0.048
1306	***Firmicutes* (100)**	***Terrisporobacter* (100)**	1.000	0.200	0.042
844	***Proteobacteria* (100)**	unclassified	1.000	0.200	0.042
890	***Firmicutes* (100)**	***Peptoclostridium* (93)**	1.000	0.200	0.042

[a] Phylum identification based on the Silva database; bootstrap confidence values are given within parentheses; taxonomic assignments are generally considered reliable when bootstrap confidence values exceed 80 (indicated in bold).

[b] Genus identification based on the Silva database; bootstrap confidence values are given within parentheses; taxonomic assignments are generally considered reliable when bootstrap confidence values exceed 80 (indicated in bold); when a confidence value of 0 was obtained, the OTU is considered "unclassified".

[c] Specificity score between 0 and 1; the closer to 1, the more the OTU is a robust indicator for the water type (a score of 1 indicates a unique OTU).

[d] Fidelity score between 0 and 1; the higher the score the more samples within the group contain that OTU (a score of 1 indicates that all samples of the group contain the OTU).

[e] OTUs with a *p* value less than 0.05 are considered significant indicators.

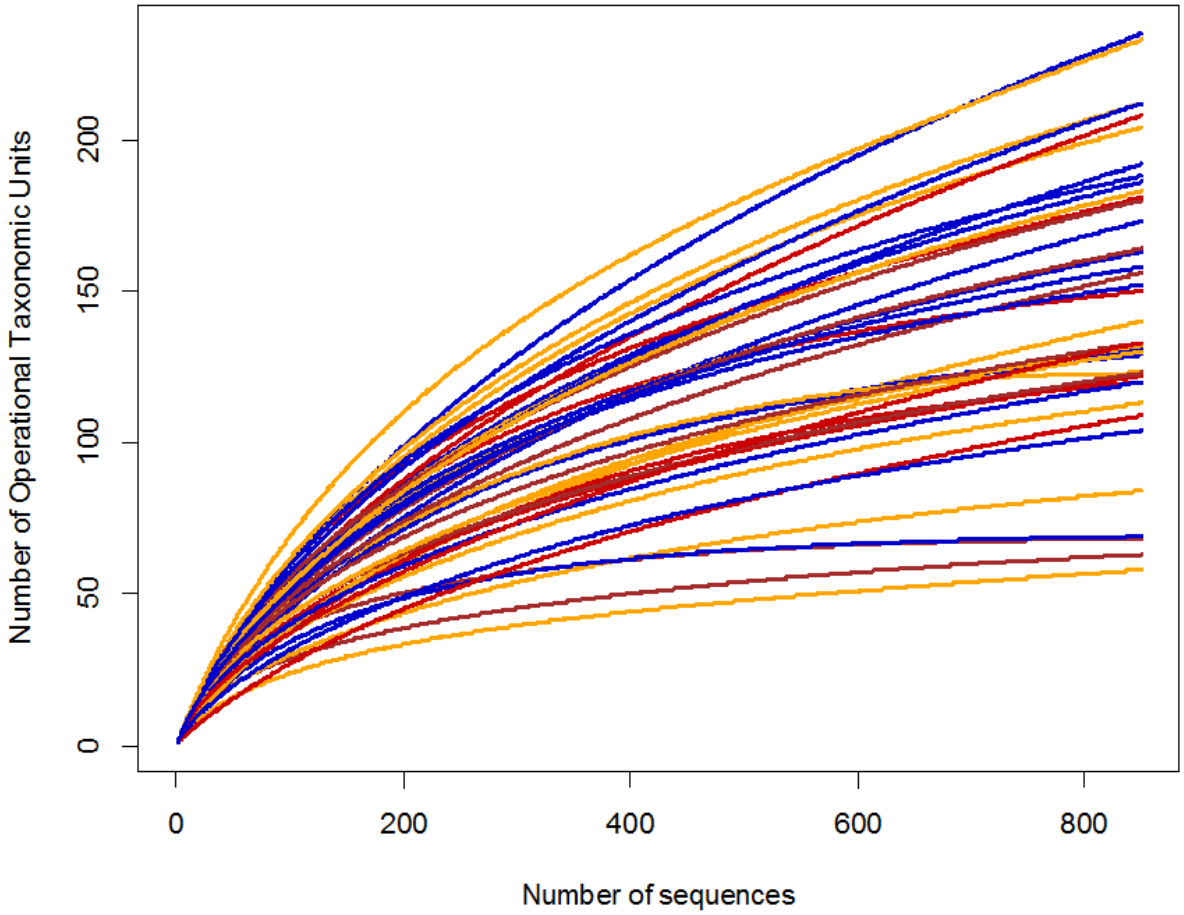


Figure S2.1: Rarefaction curves generated for each individual water sample. These curves illustrate the accumulated number of bacterial Operational Taxonomic Units (OTUs) based on a DNA dissimilarity cut-off value of 3 %. Brown lines represent surface water, red lines groundwater, blue lines processed water, and orange lines household tap water. For more information about the diversity measures for the individual samples the reader is referred to Table S2.1 (Supporting Information).

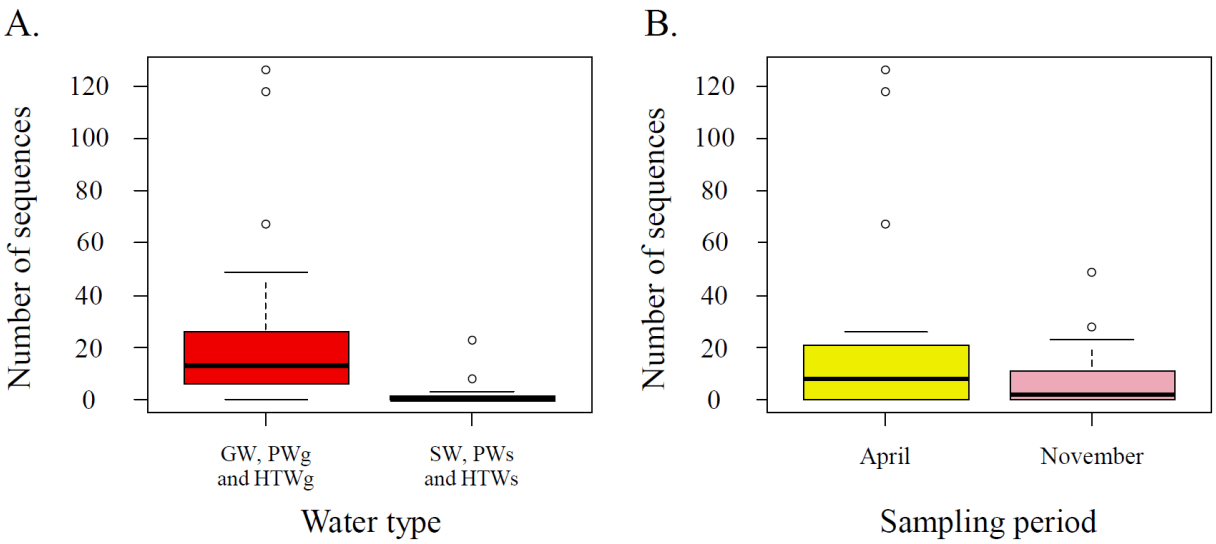


Figure S2.2: Boxplot representation of the number of *Nitrospirae* sequences in the water samples investigated in this study. Water samples were grouped based on water type (**A**) and sampling period (**B**). The boxplots show the upper and lower quartiles; the whiskers indicate variability outside the upper and lower quartiles which is no more than 1.5 times the interquartile range. Further, the median is plotted as a thick black line. GW, PWg and HTWg, groundwater, processed water produced from groundwater and household tap water processed from groundwater (n =21); SW, PWs and HTWs, surface water, processed water produced from surface water and household tap water processed from surface water (n = 20); April (n = 21); November (n = 20).

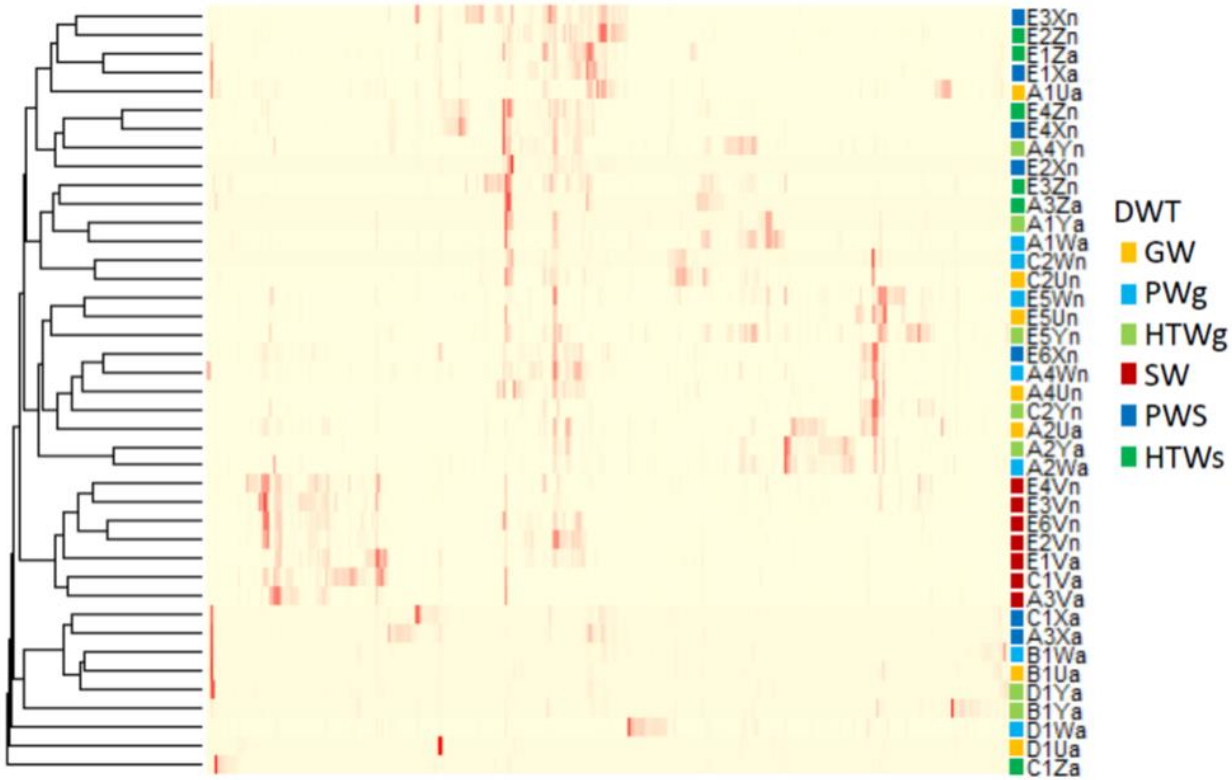


Figure S2.3: Heatmap representation showing the differences in bacterial community composition of the water samples investigated in Chapter II (columns represent different OTUs). A Bray-Curtis distance matrix was used and the samples were clustered by a UPGMA agglomeration method. GW, groundwater; PWg, processed water originating from groundwater; HTWg, household tap water originating from groundwater; SW, surface water; PWs, processed water originating from surface water; HTWs, household tap water originating from surface water. For more information about the studied samples the reader is referred to Table S2.1 (Supporting Information)

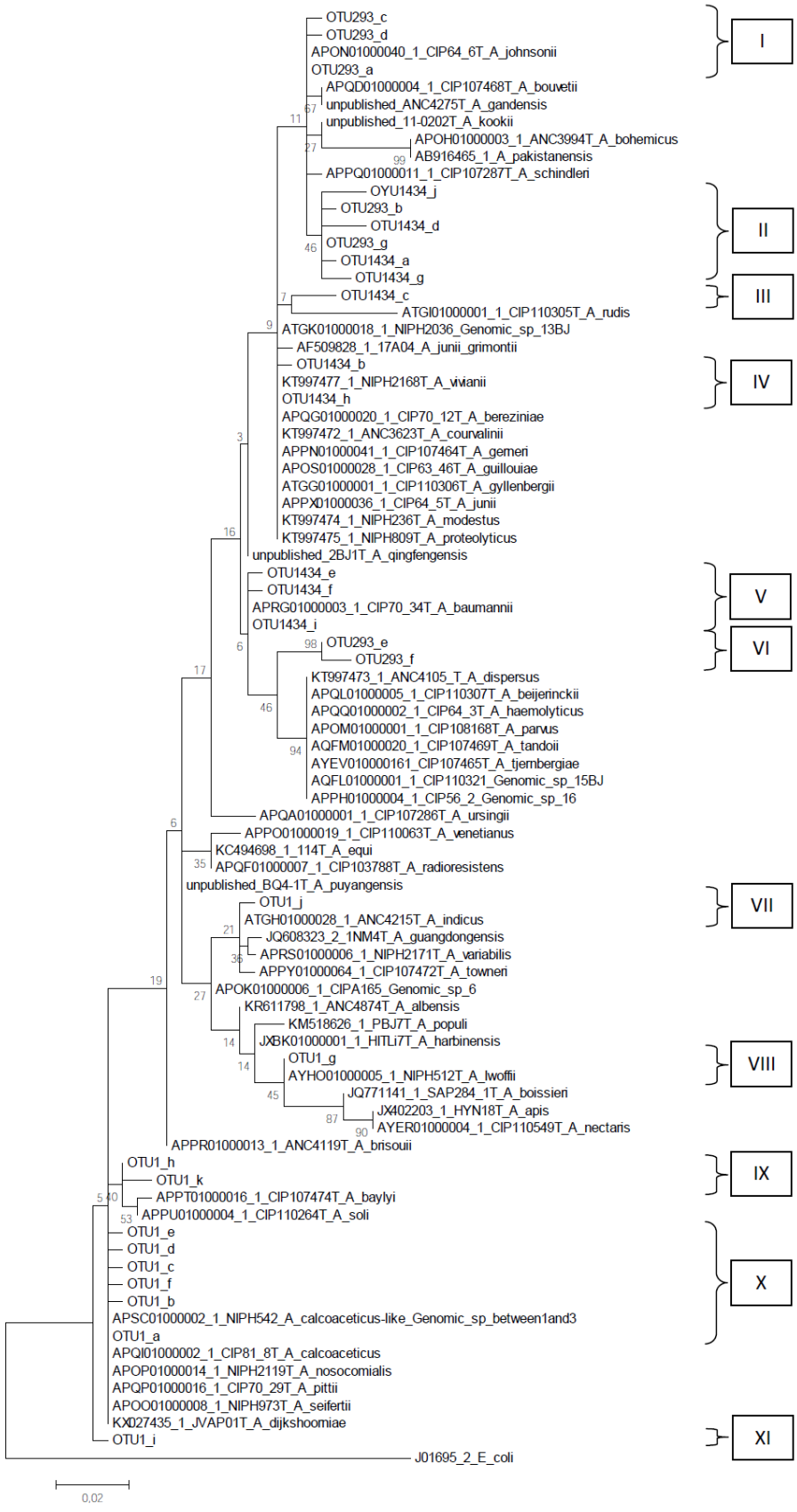


Figure S2.4: Rooted neighbor-joining tree based on the V4 region of the 16S ribosomal RNA gene (250 bp), positioning unique sequences of the three *Acinetobacter* OTUs identified in this study (OTU 1, OTU 293 and OTU 1434) among reference sequences of all known *Acinetobacter* species and a number of *Acinetobacter* genomic species. In total 11 clades can be observed housing *Acinetobacter* sequences recovered in Chapter II.

Supporting Information Chapter III

Table S3.1: Primer design and sample specific barcodes for bacterial community composition analysis in Chapter III.
See Excel file: PhD_Ado Van Assche_Chapter III_TableS3-1.xlsx
Van Assche, A. (2019, September 10). PhD Ado Van Assche. https://doi.org/10.17605/OSF.IO/W7MK3

Table S3.2: Identification of operational taxonomic units (OTUs) according to the RDP database[a] and distribution of reads over the investigated samples.
[a] Taxonomic assignment scores are provided between brackets. In general, taxonomic assignments are considered reliable when bootstrap confidence values exceed 0.80.
See Excel file: PhD_Van Assche Ado_Chapter III_TableS3-2_taxonomy.xlsx
Van Assche, A. (2019, September 10). PhD Ado Van Assche. https://doi.org/10.17605/OSF.IO/W7MK3

Table S3.3: Relative abundance of the different genera[a] for each investigated sample (% sequences / genus).
[a] Taxonomic assignments are considered reliable when bootstrap confidence values exceed 0.80.
See Excel file: PhD_Van Assche Ado_Chapter III_TableS3-3_Percentages_sequences_per_genus.xlsx
Van Assche, A. (2019, September 10). PhD Ado Van Assche. https://doi.org/10.17605/OSF.IO/W7MK3

Table S3.4: Results of indicator species analysis for the different sampling points studied, based on the genus level after taxonomic identification[a].
[a] Taxonomic assignments are considered reliable when bootstrap confidence values exceed 0.80.
See Excel file: PhD_Van Assche Ado_Chapter III_TableS3-4_Indicator species analysis.xlsx
Van Assche, A. (2019, September 10). PhD Ado Van Assche. https://doi.org/10.17605/OSF.IO/W7MK3

Table S3.5: Physicochemical properties of the household tap waters derived from line A.

Season	Sampling location	Sample identifier	Free chlorine (mg/L)	Temperature (°C)	pH	EC[a] (µS/cm)	DO[b] (mg O2/L)	Water hardness (°F)	Nitrate (mg/L)	Ortho-phosphate (mg P/L)	Total phosphor (mg P/L)	Sulfate (mg/L)	Total organic carbon (mg C/L)	Calcium (mg/L)	Magnesium (mg/L)	Trihalomethanes (µg/L)
Winter	Ek	W_Ek	< 0.10	11.1	7.96	481	10.75	17	13	< 0.20	< 0.15	52	2.7	58.3	6.8	50
Winter	Ed	W_Ed	0.14	14.4	7.86	402	9.00	15	14	< 0.20	< 0.15	39	2.5	51.2	5.8	35
Winter	K	W_K	< 0.10	14.3	7.87	398	9.16	15	14	< 0.20	< 0.15	40	2.9	51.1	5.9	37
Winter	H	W_H	< 0.10	14.8	7.93	395	9.08	15	14	< 0.20	< 0.15	40	2.5	51.4	5.9	40
Winter	A	W_A	< 0.10	18.0	7.96	499	9.50	19	12	< 0.20	< 0.15	56	1.7	61.9	7.5	7
Spring	Ek	Sp_Ek	< 0.10	14.4	7.92	411	6.36	17	14	< 0.20	< 0.15	38	2.1	59.1	5.6	29
Spring	Ed	Sp_Ed	< 0.10	16.4	7.86	417	7.98	17	12	< 0.20	< 0.15	48	1.5	59.8	5.5	32
Spring	K	Sp_K	0.11	14,0	7.89	423	6.03	17	12	< 0.20	< 0.15	48	1.9	60.8	5.6	32
Spring	H	Sp_H	< 0.10	14.3	7.94	414	6.09	18	12	< 0.20	< 0.15	48	2.9	62.5	5.6	33
Spring	A	Sp_A	< 0.10	17.0	7.84	412	9.50	17	13	< 0.20	< 0.15	44	1.7	56.8	5.7	8
Summer	Ek	Su_Ek	0.10	21.4	7.93	406	4.22	16	9.3	< 0.20	< 0.15	40	1.4	55.3	5.7	4
Summer	Ed	Su_Ed	0.18	22.1	7.95	403	4.92	17	5.7	< 0.20	< 0.15	39	2.2	58.4	5.7	18
Summer	K	Su_K	< 0.10	20.6	7.94	405	4.61	17	5.9	< 0.20	< 0.15	39	2.1	57.8	5.7	40
Summer	H	Su_H	< 0.10	21.3	7.62	404	4.64	17	5.8	< 0.20	< 0.15	37	2.2	58.3	5.7	14
Summer	A	Su_A	< 0.10	21.4	7.96	397	8.30	15	7.6	< 0.20	< 0.15	44	1.2	52.0	5.9	5
Fall	Ek	F_Ek	< 0.10	15,0	7.82	543	5.78	21	11	< 0.20	< 0.15	62	1.6	71.6	8.6	32
Fall	Ed	F_Ed	0.11	19.9	7.88	604	7.35	24	11	< 0.20	< 0.15	79	1.2	78.6	9.8	37
Fall	K	F_K	< 0.10	15.1	7.87	606	5.73	24	11	< 0.20	< 0.15	69	3.8	78.8	9.7	37
Fall	H	F_H	0.21	15.3	7.88	606	5.89	24	11	< 0.20	< 0.15	73	1.5	78.6	9.6	40
Fall	A	F_A	< 0.10	17.0	8.00	537	9.40	21	10	< 0.20	< 0.15	62	1.0	70.4	8.4	7
p value of the ANOVA test (based on sampling location)			nd[c]	0.654	0.584	1.000	0.267	0.998	0.977	nd	nd	0.996	0.099	0.997	1.000	0.014
p value of the ANOVA test (based on season)			nd	<0.001	0.922	<0.001	0.003	<0.001	<0.001	nd	nd	<0.001	0.461	<0.001	<0.001	0.245

[a] Electrical conductivity
[b] dissolved oxygen
[c] not determined

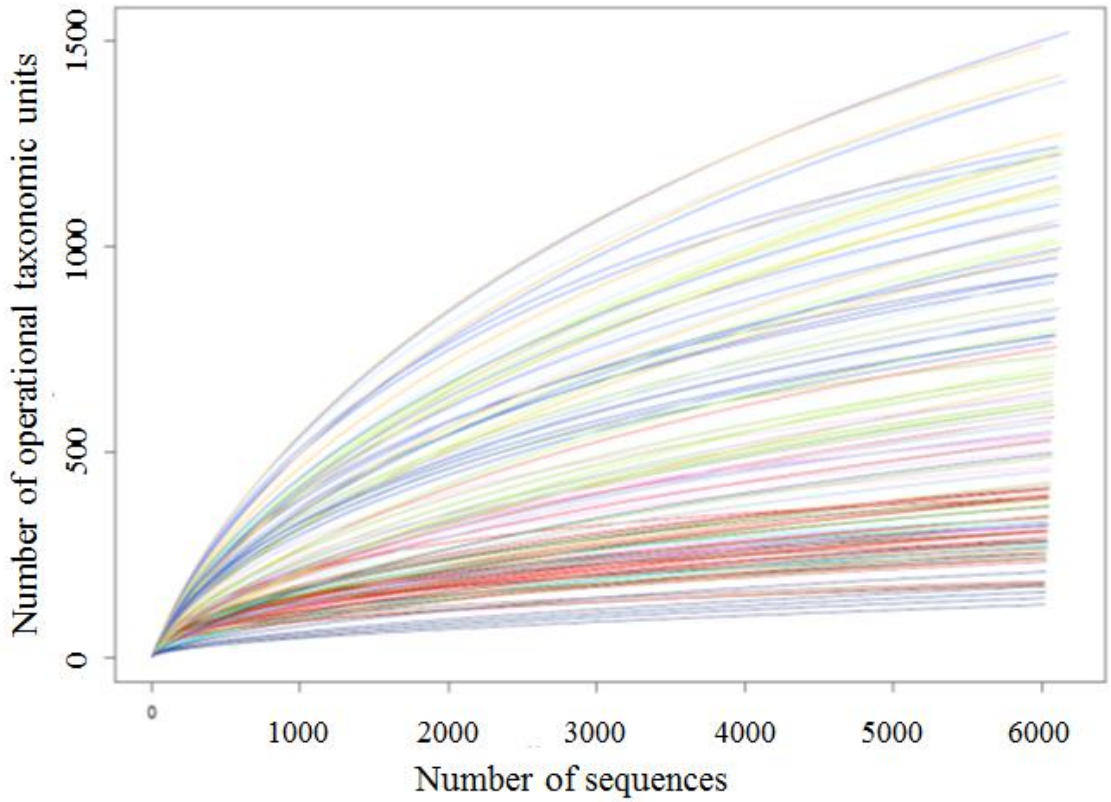
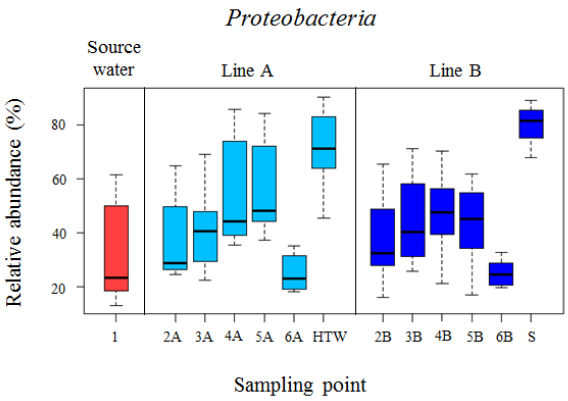
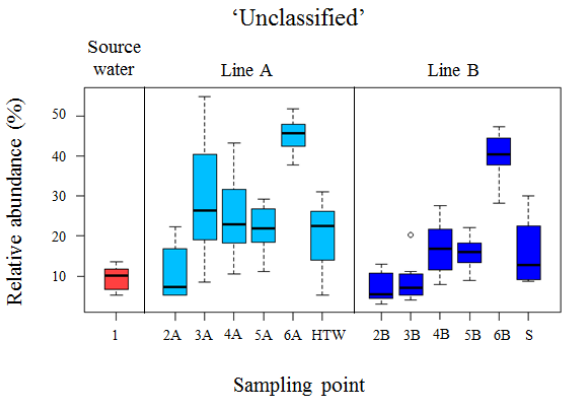
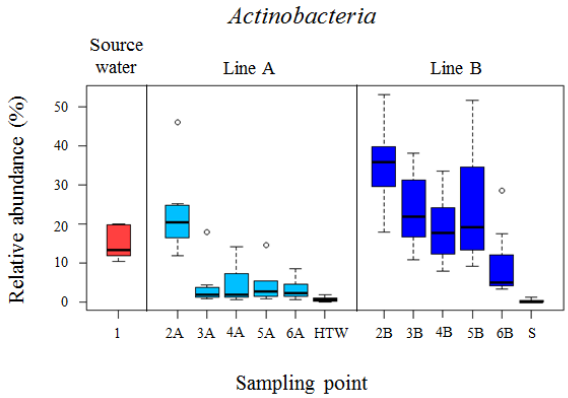
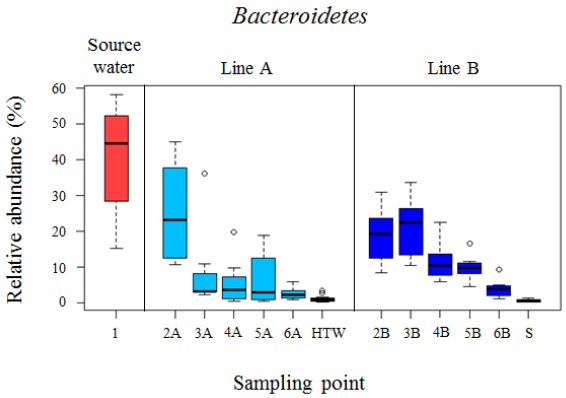


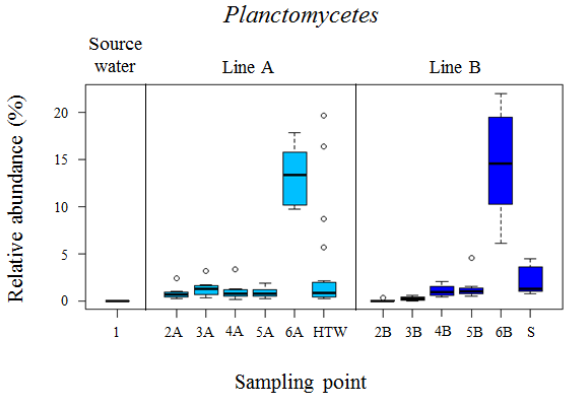
Figure S3.1: Rarefaction curves generated for each individual water sample. These curves illustrate the accumulated number of bacterial Operational Taxonomic Units (OTUs) based on a DNA dissimilarity cut-off value of 3 %. The colour of the lines represent the different sampling points (i.e. 1, 2A, 2B, 3A, 3B, 4A, 4B, 5A, 5B, 6A, 6B, S, Ek, Ed, K, H, and A,).

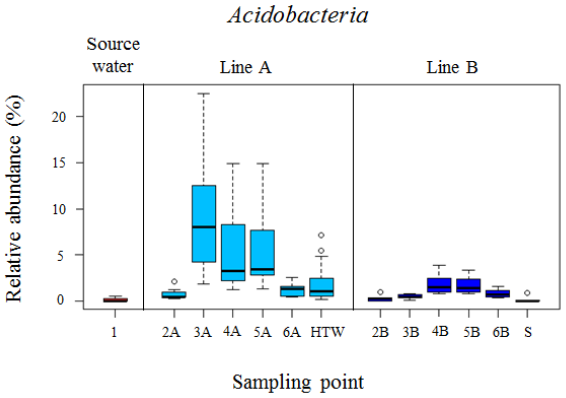

Proteobacteria
Source water
Line A
Line B
Relative abundance (%)
20
40
60
80
1
2A 3A 4A 5A 6A HTW
2B 3B 4B 5B 6B S
Sampling point

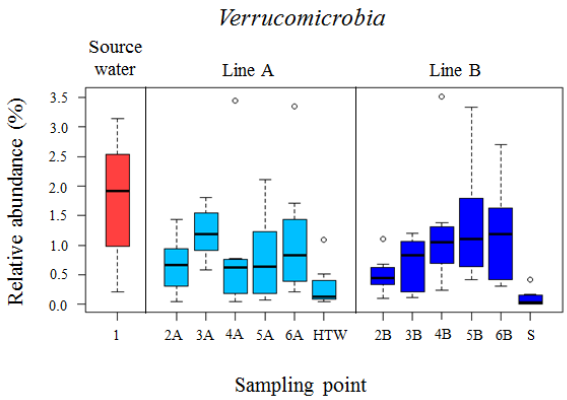

'Unclassified'
Source water
Line A
Line B
Relative abundance (%)
10
20
30
40
50
1
2A 3A 4A 5A 6A HTW
2B 3B 4B 5B 6B S
Sampling point

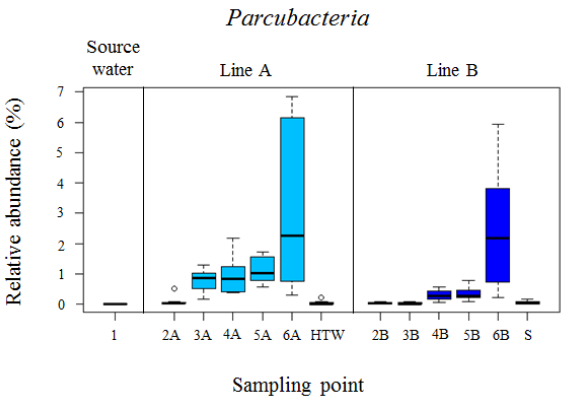

Actinobacteria
Source water
Line A
Line B
Relative abundance (%)
0
10
20
30
40
50
1
2A 3A 4A 5A 6A HTW
2B 3B 4B 5B 6B S
Sampling point


Bacteroidetes
Source water
Line A
Line B
Relative abundance (%)
0
10
20
30
40
50
60
1
2A 3A 4A 5A 6A HTW
2B 3B 4B 5B 6B S
Sampling point


Planctomycetes
Source water
Line A
Line B
Relative abundance (%)
0
5
10
15
20
1
2A 3A 4A 5A 6A HTW
2B 3B 4B 5B 6B S
Sampling point


Acidobacteria
Source water
Line A
Line B
Relative abundance (%)
0
5
10
15
20
1
2A 3A 4A 5A 6A HTW
2B 3B 4B 5B 6B S
Sampling point


Verrucomicrobia
Source water
Line A
Line B
Relative abundance (%)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
1
2A 3A 4A 5A 6A HTW
2B 3B 4B 5B 6B S
Sampling point


Parcubacteria
Source water
Line A
Line B
Relative abundance (%)
0
1
2
3
4
5
6
7
1
2A 3A 4A 5A 6A HTW
2B 3B 4B 5B 6B S
Sampling point

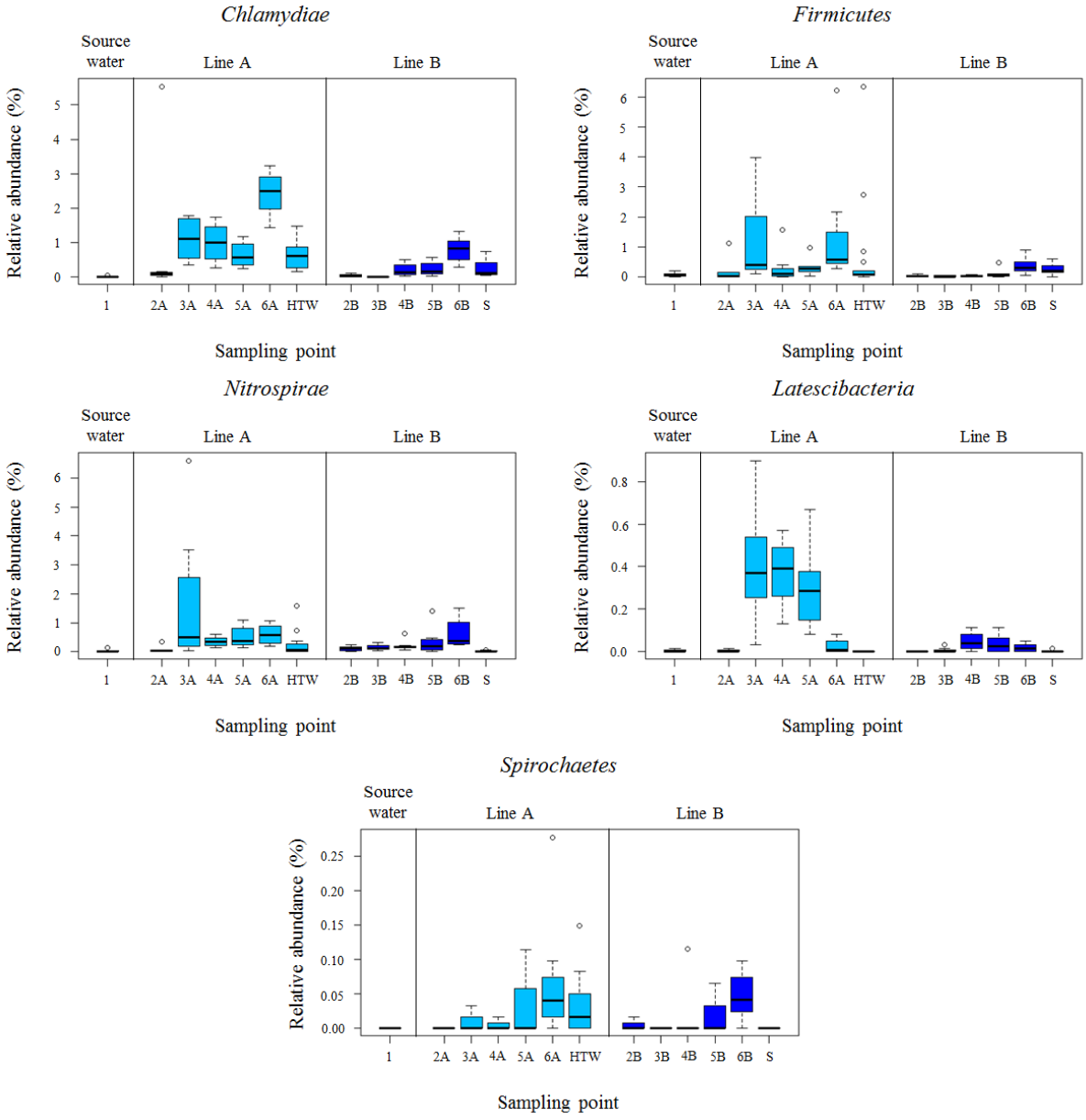


Figure S3.2: Boxplot representation of the relative abundance (%) of a selection of different phyla present in the parallel drinking water production and distribution system. The groups contain the samples taken during the different seasons. The boxplots show the upper and lower quartiles; the whiskers indicate variability outside the upper and lower quartiles which is no more than 1.5 times the interquartile range. Further, the median is plotted as a thick black line. Sampling points: 1, source water (n = 8) (red); 2A, after rapid sand filtration (n = 8); 3A, after slow sand filtration (n = 8); 4A, after activated carbon filtration (n = 8); 5A, after UV treatment (n = 8) ; 6A, after chlorination (n = 8); HTW, combined data from the different household tap waters from line A (i.e. Ek, Ed, K, H, and A) (n = 20) (light blue); 2B, after flotation (n = 8); 3B, after double layer filtration (n = 8); 4B, after activated carbon filtration (n = 8); 5B, after UV treatment (n = 8); 6B, after chlorination (n = 8); and S, storage tank with household tap water from line B (n = 8) (dark blue).

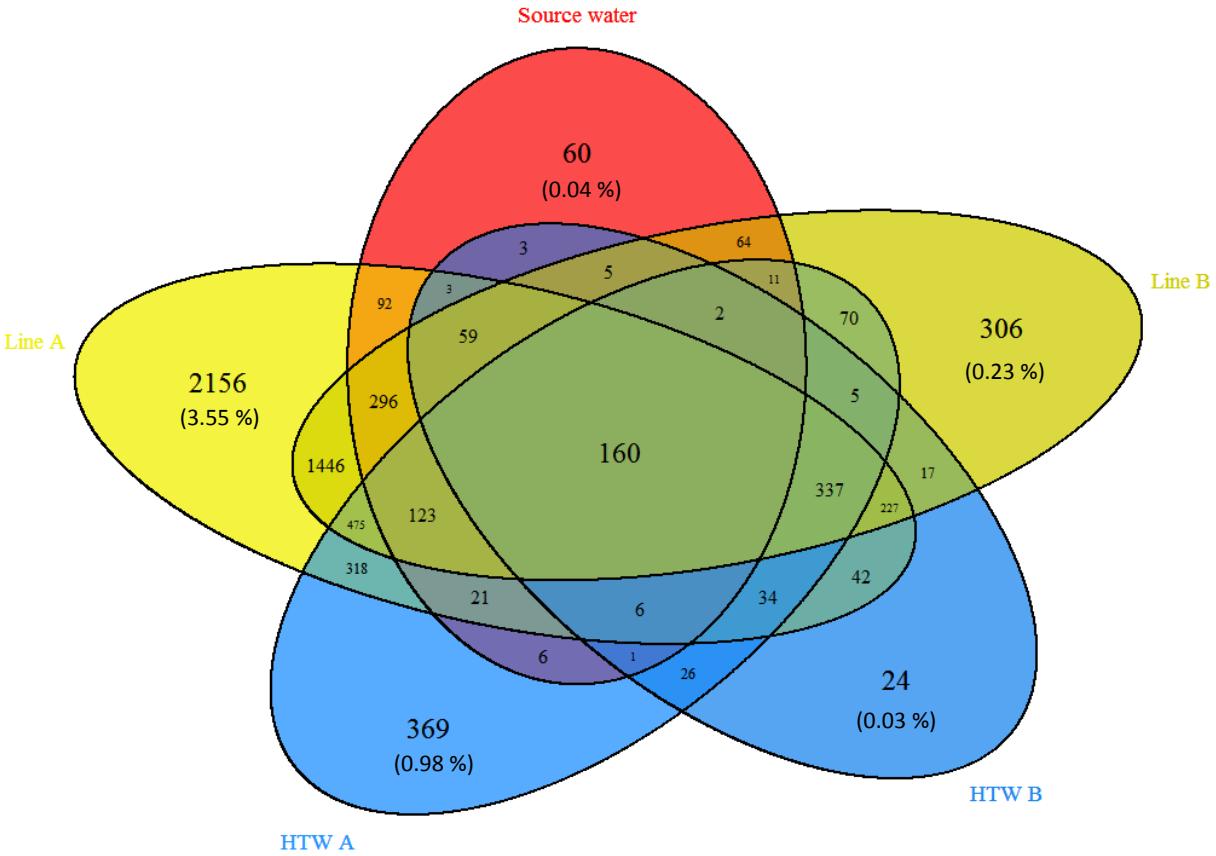


Figure S3.3: Venn diagram representation of the distribution of the number of OTUs between the different sampling groups. The different sample groups are: source water; water from the drinking water production system, i.e. line A (representing sampling points 2A, 3A, 4A, 5A and 6A) and line B (representing sampling points 2B, 3B, 4B, 5B and 6B); and water from within the distribution system, i.e. HTW A (representing sampling points A, Ed, Ek, H and K) and HTW B (storage tank water (S)).

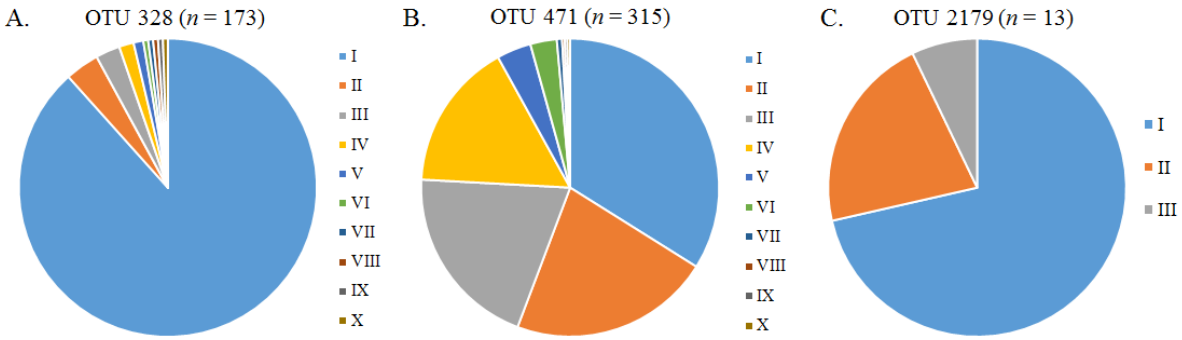


Figure S3.4: Distribution of the amplicon sequence variants within the *Acinetobacter* OTUs. The distribution represents the percentage of sequences grouped based on the closest *rpoB* gene sequence similarity with *Acinetobacter* species with a validly published name, some genomic species and isolates obtained in this study. **A**: I, *A. lwoffii* NIPH512^{T}, AVA057A2db and AVA076A2d (88.7 % of sequences within OTU 328; sequence identity of 98-100 %) ; II, *A. dijkshoorniae* JVAP01^{T}, *A. lactucae* B-41902^{T}, *A. seifertii* NIPH973^{T}, *A. pittii* CIP70.29^{T}, *A. nosocomialis* NIPH2119^{T}, *A. calcoaceticus* CIP81.8^{T}, AVA061A2d, AVA073A2d and AVA091A2d (3.7 %; 99.6-100 %); III, *A. harbinensis* HITLi7^{T} and AVA042A2d (2.6 %; 99.6-100 %); IV, *A. albensis* ANC4874^{T} andAVA042B2da (1.6 %; 99.6-100 %); V, *A. indicus* ANC4215^{T} (1.1 %; 99.6 %); VI, *A. radiorisistens* CIP103788^{T} and *A. equi* 114^{T} (0.5 %; 100 %); VII, *A. lwoffii* NIPH512^{T}, *A. towneri* CIP107472^{T}, AVA057A2db and AVA076A2d (0.5 %; 99.6 %); VIII, *A. lwoffii* NIPH512^{T}, *A. harbinensis* HITLi7^{T}, AVA042A2d, AVA057A2db and AVA076A2d (0.5 %; 99.2 %); IX, AVA080A2d (0.5 %; 100 %); X, *A. lwoffii* NIPH512^{T}, *A. towneri* CIP107472^{T}, *A. variabilis* NIPH2171^{T}, AVA057A2db and AVA076A2d (0.5 %; 99.6 %). **B**: I, *A. johnsonii* CIP64.6^{T}, AVA072A2d, AVA088A2d, AVA098A2d, AVA100B2d and AVA121A2d (33.9 %; 99.2-100 %); II, *A. beijerinckii* CIP110307^{T}, *A. dispersus* ANC4105^{T}, *A. haemolyticus* CIP64.3^{T}, *A. parvus* CIP108168^{T}, *A. tandoii* CIP107469^{T}, *A. tjernbergiae* CIP107465^{T}, genomic species 15BJ CIP110321, genomic species 16 CIP56.2, AVA075A2d and AVA123A2d (21.8; 98.8-100 %); III, *A. gandensis* ANC4275^{T} and *A. bouvetii* CIP107468^{T} (20.1 %; 98.8-100 %); IV, AVA086A3d (16.1 %; 98.8-100 %); V, *A. bereziniae* CIP70.12^{T}, *A. courvalinii* ANC3623^{T}, *A. colistiniresistens* NIPH2036^{T}, *A. gerneri* CIP107464^{T}, *A. guillouiae* CIP110306^{T}, *A. junii* CIP64.5^{T}, *A. modestus* NIPH236^{T}, *A. proteolyticus* NIPH809^{T} and *A. vivianii* NIPH2168^{T} (3.7%; 99.2-100 %); VI, *A. baumannii* CIP70.34^{T} (2.9 %; 99.2-100 %); VII, *A. bohemicus* ANC3994^{T} and AVA017A (0.6 %; 100 %); VIII, *A. gandensis* ANC4275^{T}, *A. bouvetii* CIP107468^{T} and AVA059A2d (0.3; 99.6 %); IX, *A. schindleri*, AVA093A2da and AVA093A2db (0.3 %; 100 %); X, *A. johnsonii* CIP64.6^{T}, *A. schindleri*, AVA072A2d, AVA088A2d, AVA093A2da, AVA093A2db, AVA098A2d, AVA100B2d and AVA121A2d (0.3 %; 98.4 %). **C**: I, *A. brisouii* ANC4119^{T} (71.4 %; 98-98.4 %); II, *A. ursingii* CIP107286^{T} (21.4 %; 99.6-100 %); III, *A. baumannii* CIP70.34^{T} (7.1 %; 99.2 %).

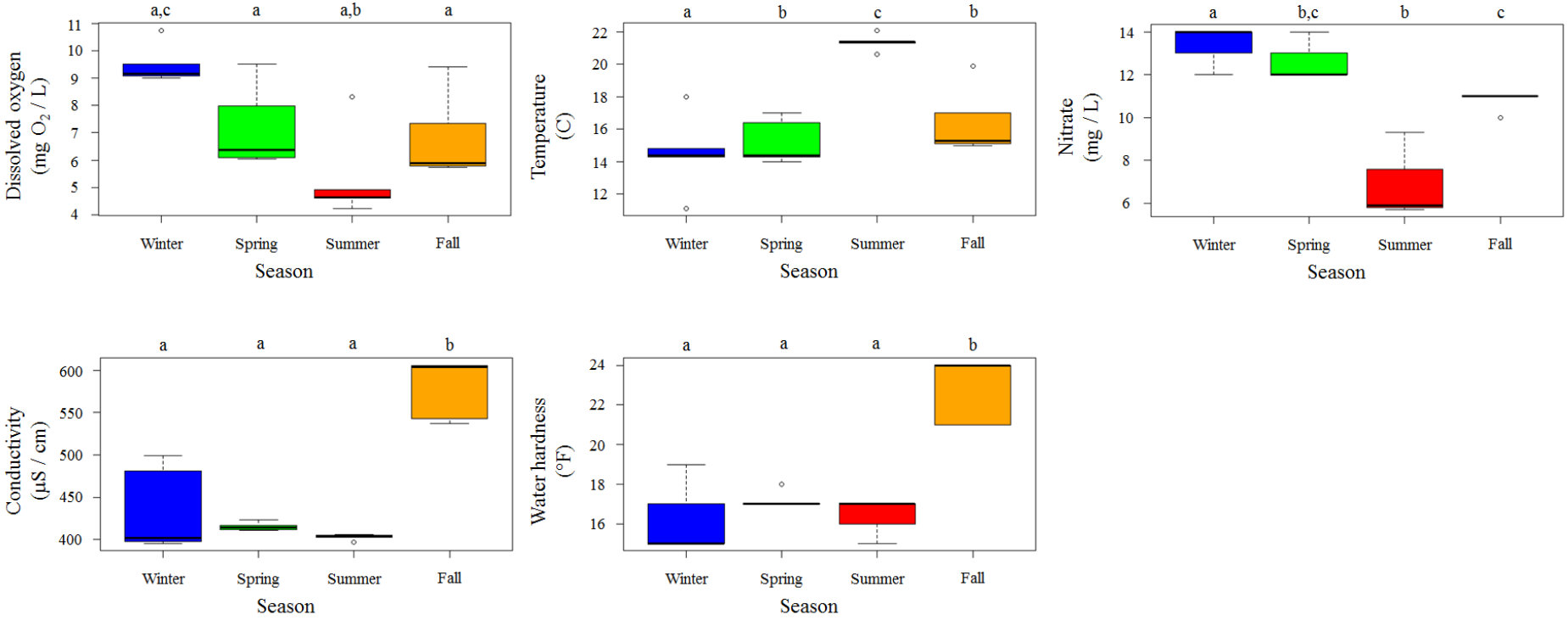


Figure S3.5: Boxplot representation of a selection of different chemical parameters measured at the household tap water locations of distribution line A. The different locations (i.e. A, Ed, Ek, H and K) are grouped based on sampling period (i.e. winter, spring, summer and fall). The boxplots show the upper and lower quartiles; the whiskers indicate variability outside the upper and lower quartiles which is no more than 1.5 times the interquartile range. Further, the median is plotted as a thick black line. The number of samples per group is 5. Significant differences are indicated at the top of each graph and are based on the Tukey's test.

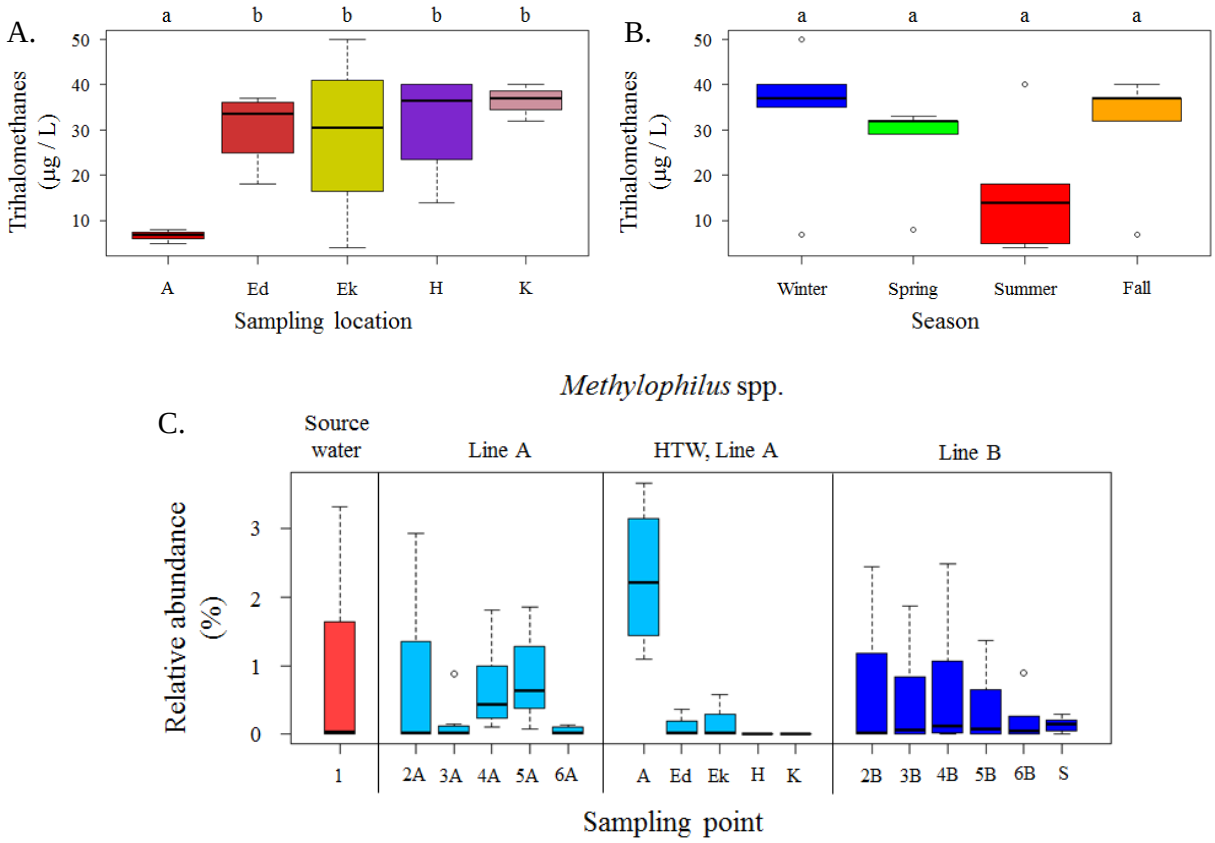


Figure S3.6: Graph A and B: Boxplot representation of the sum of trihalomethanes measured at the household tap water locations of distribution line A. The boxplots show the upper and lower quartiles; the whiskers indicate variability outside the upper and lower quartiles which is no more than 1.5 times the interquartile range. For graph A samples were grouped based on sampling location (i.e. A, Ed, Ek, H and K) and for graph B they were grouped based on sampling period (i.e. winter, spring, summer and fall). The number of samples per group are 4 and 5, respectively. Significant differences are indicated at the top of each graph and are based on the Tukey's test. **Graph C:** Boxplot representation of the relative abundance (%) of sequences identified as *Methylophilus* spp. at the different sampling locations.

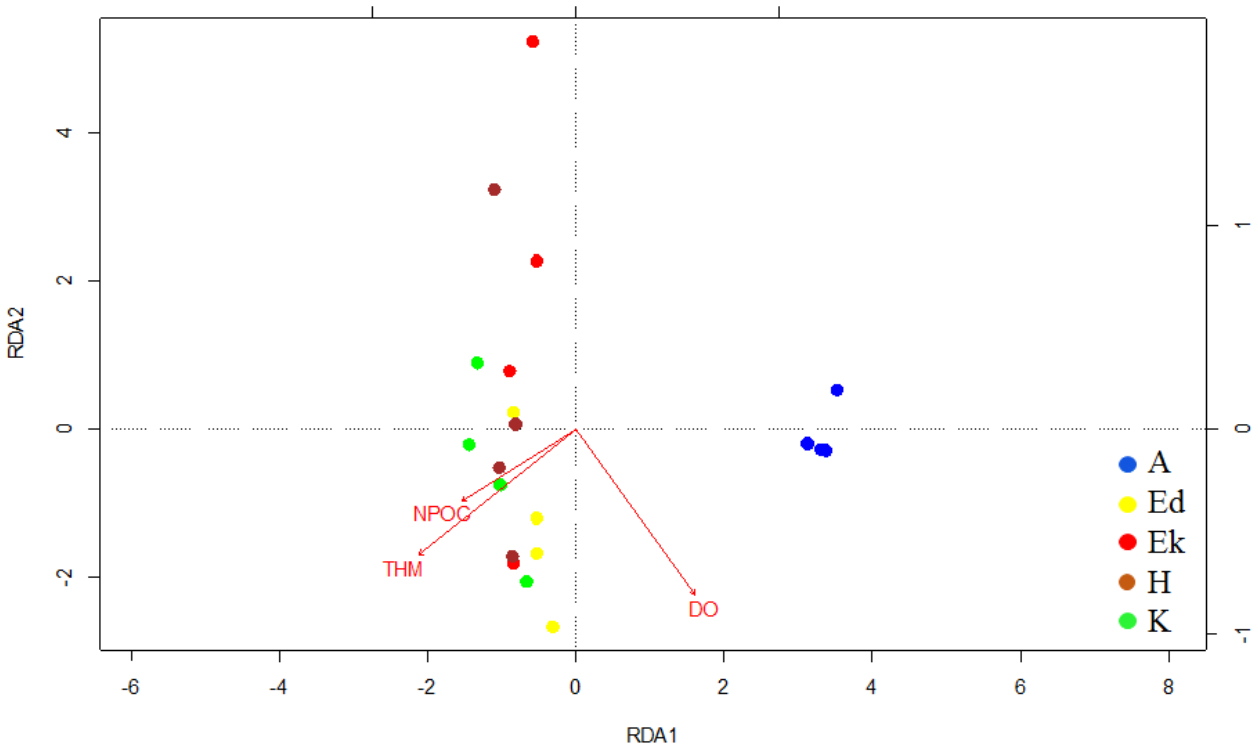


Figure S3.7: Redundancy analysis (RDA) ordination plot of the bacterial community composition of the household tap waters within the distribution system derived from line A. The sampling locations A, Ed, Ek, H and K are represented by blue, yellow, red, brown and green dots, respectively. Environmental variables explaining a significant proportion of the bacterial community composition are indicated by red arrows (NPOC, non-purgable organic carbon; THM, trihalomethanes, and DO, dissolved oxygen). The direction of the increasing gradient is represented by the arrow. RDA1 and RDA2 axis accounted for 67.1 % and 19.1 % of the total explained variation, respectively.

Supporting information Chapter IV

Table S4.1: Overview of the richness, average well color development values and the AUC value of the GENIII phenotypes.

See Excel file: PhD_Ado Van Assche_Chapter IV_ Table_S4-1_R_AWCD_AUC. Xlsx

Van Assche, A. (2019, September 10). PhD Ado Van Assche. https://doi.org/10.17605/OSF.IO/W7MK3

Table S4.2: Overview of the average inhibition zone of the different isolates against the tested antibiotics[a].

Species	Strain designation	DOR	IMI	MRP	CIP	K	AK	CN	NET	TOB	TE	AML	SXT	KF	CS	S
A. calcoaceticus	AVA 009B	27.5	30.5	26.5	25.5	22.5	22.0	21.5	21.5	20.5	20.0	13.5	22.0	0.0	15.5	14.0
A. calcoaceticus	AVA 012A	28.5	30.5	25.5	25.0	23.5	21.5	21.0	21.0	21.0	18.0	0.0	19.0	0.0	16.5	15.0
A. calcoaceticus	AVA 044A2d	26.5	30.5	27.0	28.0	23.0	23.0	22.5	22.5	22.5	19.5	12.0	22.5	0.0	16.0	17.0
A. calcoaceticus	AVA 061A2d	26.5	30.5	27.5	27.5	24.5	23.5	22.0	23.0	22.5	19.5	17.5	24.0	0.0	17.0	16.5
A. calcoaceticus	AVA 077A2d	29.0	32.0	29.0	27.0	24.5	21.5	20.0	19.5	20.5	18.5	12.0	22.0	0.0	15.5	14.0
A. calcoaceticus	DSM 30006T	40.0	40.0	40.0	40.0	40.0	38.0	35.0	36.0	30.5	38.0	37.0	37.0	21.0	20.0	24.5
A. calcoaceticus	LUH 2005	28.0	37.5	29.0	27.0	24.5	22.0	20.5	20.0	21.0	20.0	14.0	24.0	0.0	16.5	15.5
A. calcoaceticus	LUH 9144	27.0	32.5	27.5	25.0	24.0	22.5	22.0	19.5	21.0	21.5	12.5	23.5	0.0	17.0	14.5
A. calcoaceticus	LUH 12679	27.5	38.0	28.0	26.5	25.0	23.5	22.0	22.5	21.5	22.0	16.0	25.0	0.0	16.0	16.5
A. calcoaceticus	LUH 14369	27.5	36.5	29.0	26.0	24.5	23.0	23.0	23.0	22.0	22.0	15.5	23.0	0.0	16.5	16.0
A. calcoaceticus	M 23	25.5	36.5	26.5	26.0	24.0	22.0	21.5	21.5	21.5	20.5	12.0	25.0	0.0	17.0	16.0
A. calcoaceticus	RUH 944	26.0	28.0	24.0	26.0	22.0	21.5	21.0	21.0	20.5	20.5	18.5	23.0	0.0	15.5	15.5
A. calcoaceticus	RUH 2202	26.5	29.0	26.0	26.0	23.5	24.5	24.0	23.5	22.5	21.0	11.5	25.0	0.0	15.0	16.5
A. calcoaceticus	RUH 2203	26.0	34.0	26.5	26.5	23.5	24.0	23.0	22.0	22.0	20.5	14.5	23.0	0.0	16.5	15.5
A. guillouiae	AVA 011A	22.5	24.0	21.0	25.0	16.5	22.5	23.0	23.5	21.0	20.5	11.0	24.0	0.0	16.5	17.0
A. guillouiae	AVA 025A2d	24.5	28.0	23.0	27.5	20.5	24.5	23.5	24.0	23.0	20.0	12.5	22.0	0.0	16.0	17.5
A. guillouiae	AVA 059B2d	18.5	25.0	20.0	27.5	26.5	25.0	24.0	24.0	24.0	21.5	11.5	24.0	0.0	17.5	18.5
A. guillouiae	AVA 117B2d	24.0	27.0	22.5	24.5	14.0	21.0	22.0	22.0	21.0	19.0	11.0	19.0	0.0	15.0	16.0
A. guillouiae	AVA 118A2d	22.0	27.0	20.0	27.0	25.0	23.5	24.5	22.5	22.5	18.0	9.5	25.0	0.0	15.5	16.5
A. guillouiae	LUH 5606	24.5	34.5	24.0	8.5	28.0	26.5	24.0	25.0	25.0	8.5	14.5	0.0	0.0	16.5	18.0
A. guillouiae	LUH 5653	23.5	29.0	22.0	24.5	26.5	23.5	23.0	24.0	22.5	21.0	11.5	26.0	0.0	16.0	17.0
A. guillouiae	LUH 7830	22.5	25.0	20.5	26.5	27.5	25.5	25.0	25.5	23.0	21.5	10.5	25.0	0.0	16.0	18.0
A. guillouiae	M 24	23.0	26.5	24.0	28.5	27.5	27.0	26.0	26.5	25.0	23.0	13.0	28.0	0.0	17.5	19.0
A. guillouiae	RUH 2234	23.5	25.0	22.0	27.0	22.5	24.5	23.0	23.0	22.0	22.0	11.5	23.0	0.0	15.5	17.0
A. guillouiae	RUH 2861T	27.0	30.0	27.0	29.0	17.5	22.5	22.0	23.5	21.5	23.0	16.5	26.5	0.0	17.0	16.5
A. johnsonii	AVA 013A	25.5	24.5	21.5	23.0	20.5	21.5	19.5	18.5	17.0	17.0	15.5	21.0	0.0	14.5	15.0
A. johnsonii	AVA 016A	25.5	31.0	26.0	26.5	28.0	27.0	27.0	25.5	27.0	23.5	22.0	19.5	0.0	16.5	20.5
A. johnsonii	AVA 059A2d	25.5	35.0	26.0	25.5	23.5	22.5	22.5	22.5	21.5	21.0	25.0	22.5	0.0	15.5	16.5
A. johnsonii	AVA 060A2d	23.5	30.5	24.5	26.0	24.0	22.5	22.5	21.5	21.0	21.0	22.0	24.5	9.0	15.5	16.5
A. johnsonii	AVA 072A2d	25.0	29.5	25.0	26.0	22.5	21.0	20.5	21.5	19.0	20.0	20.5	19.0	0.0	16.5	14.5
A. johnsonii	AVA 074A2d	25.0	29.0	24.5	25.0	23.0	21.0	21.0	23.5	19.5	20.0	22.0	18.5	0.0	14.0	15.0
A. johnsonii	AVA 086A3d	29.5	34.0	29.0	32.0	24.0	22.5	21.5	22.0	21.0	25.0	27.0	0.0	3.5	17.0	15.5
A. johnsonii	AVA 090A2d	22.0	26.5	22.5	21.0	21.0	20.0	19.0	20.0	18.0	18.0	21.5	13.5	7.5	13.5	14.5
A. johnsonii	AVA 092A2d	24.0	28.5	23.0	22.0	23.5	22.0	20.0	21.0	20.0	20.5	19.0	18.5	3.0	14.5	15.0
A. johnsonii	AVA 098A2d	23.5	29.5	24.5	25.0	23.0	20.5	20.5	22.0	20.0	19.5	26.0	0.0	3.5	15.0	15.5

A. johnsonii	AVA 100B2d	24.0	30.5	24.5	25.0	23.0	22.0	21.0	21.5	20.0	20.5	17.5	18.0	0.0	15.0	15.0
A. johnsonii	AVA 101A2d	17.5	21.5	18.0	25.5	24.0	23.0	22.0	22.5	21.5	19.5	3.0	22.0	0.0	15.5	15.5
A. johnsonii	AVA 117A2d	21.0	23.5	21.0	27.0	24.5	23.0	22.5	22.5	21.5	21.5	19.0	22.0	0.0	15.5	16.5
A. johnsonii	AVA 119A2d	25.0	29.0	24.5	24.5	22.5	21.0	21.0	21.0	20.0	19.5	22.5	20.5	0.0	17.0	15.5
A. johnsonii	AVA 121A2d	23.5	28.5	25.5	27.0	27.0	24.0	22.5	22.0	22.5	22.0	22.5	23.0	7.0	15.0	18.0
A. johnsonii	CCUG 58904	22.5	27.5	22.0	23.0	22.0	21.0	21.5	22.0	20.5	23.5	18.0	22.5	9.0	16.0	15.0
A. johnsonii	CCUG 60467	21.0	27.0	21.0	23.5	22.5	21.5	20.5	20.5	20.5	22.5	20.0	18.0	0.0	16.0	15.5
A. johnsonii	CCUG 60882	25.5	32.5	25.5	29.5	25.0	24.5	23.0	23.5	22.5	21.5	23.0	25.5	0.0	17.0	17.0
A. johnsonii	CCUG 61200	24.0	28.0	24.5	27.5	23.0	20.5	21.5	21.5	20.5	22.0	23.0	24.5	9.0	14.5	16.5
A. johnsonii	LMG 1018	22.0	29.0	20.5	30.0	32.0	31.5	32.0	34.0	32.0	26.5	21.5	23.0	0.0	20.5	19.5
A. johnsonii	LMG 1302	24.5	36.0	25.5	24.5	22.0	21.0	21.0	21.5	20.5	21.5	23.5	24.0	10.0	15.0	0.0
A. johnsonii	RUH 2231T	26.5	35.5	24.5	27.0	22.5	23.0	21.0	22.5	21.0	22.5	23.0	20.5	9.5	14.5	13.0
A. johnsonii	RUH 2857	24.5	26.0	22.0	21.0	21.0	22.0	20.0	22.0	19.5	20.5	20.5	14.5	0.0	15.0	15.5
A. johnsonii	RUH 2859	33.5	34.0	30.0	25.5	23.0	23.5	22.0	22.0	21.5	23.0	24.5	28.5	0.0	16.5	17.0
A. lwoffii	AVA 057A2da	20.5	29.5	21.0	26.5	23.0	23.0	22.5	23.0	21.5	21.0	19.5	14.5	4.5	17.0	14.0
A. lwoffii	AVA 076A2d	30.5	30.0	30.5	27.5	27.5	27.0	25.0	25.0	24.0	23.5	24.0	27.5	16.0	15.5	19.0
A. lwoffii	AVA 080A2d	24.5	32.5	25.5	30.5	27.0	24.0	24.5	25.0	20.5	24.0	13.5	16.5	0.0	17.0	17.0
A. lwoffii	AVA 080B2d	27.0	31.5	27.5	31.5	27.5	23.0	26.0	25.5	25.0	24.0	19.0	22.5	14.5	17.0	19.0
A. lwoffii	AVA 113A3d	30.5	34.0	23.0	33.0	25.5	25.0	23.0	24.0	22.5	22.5	24.0	24.5	14.0	16.5	17.0
A. lwoffii	LMG 1136	26.0	35.0	31.0	26.0	25.0	23.5	23.0	22.5	22.5	23.5	26.0	21.0	12.0	16.0	16.0
A. lwoffii	LMG 1301	24.0	36.5	25.5	35.5	23.5	24.0	23.5	23.5	22.0	24.0	15.5	0.0	14.0	16.0	0.0
A. lwoffii	LUH 1710	36.0	35.5	37.0	28.5	26.0	25.0	23.5	24.5	23.0	27.5	33.5	25.5	6.0	16.5	16.5
A. lwoffii	RUH 2219T	34.5	36.0	35.5	28.0	25.0	24.5	23.5	23.0	23.0	24.0	25.5	15.5	11.0	15.5	16.5
	ECOFF (mm)	< 18	< 23	< 20	< 20	< 19	< 18	< 15	< 19	< 17	< 18	< 12	< 16	< 0	< 13	< 15
	Source	§	$	#	#	§	€	€	§	€	#	#	#	§	#	#
	n WT	57	57	57	57	55	58	58	57	58	56	48	50	58	58	49
	% WT	98.3 %	98.3 %	98.3 %	98.3 %	94.8%	100 %	100 %	98.3 %	100 %	96.6 %	82.8 %	86.2 %	100 %	100 %	84.5 %
	n non-WT	1	1	1	1	3	0	0	1	0	2	10	8	0	0	9
	% non-WT	1.7 %	1.7 %	1.7 %	1.7 %	5.2 %	0 %	0 %	1.7 %	0 %	3.4%	17.2%	13.8%	0 %	0 %	15.5%

[a] Average of the inhibition zone of two biological replicates. Inhibition zone diameters considered to be non-WT phenotypes are in red. Abbreviations: DOR, Doripenem; IMI, Imipenem; MRP, Meropenem; CIP, Ciprofloxacin; K, Kanamycin; AK, Amakacin; SN, Gentamicin; NET, Netilmicin; TOB, Tobramycin; TE, Tetracyclin; AML, Amoxicillin; SXT, Sulfamethoxazole/trimethoprim; KF, Cephalothin; CS, Colistin sulfate; S, Streptomycin.

b Source of the cut-off values: § Value determined in this study; $ Value determined by Hombach et al. (2012); # Value determined by Narciso-da-Rocha et al. (2013); € EUCAST value.

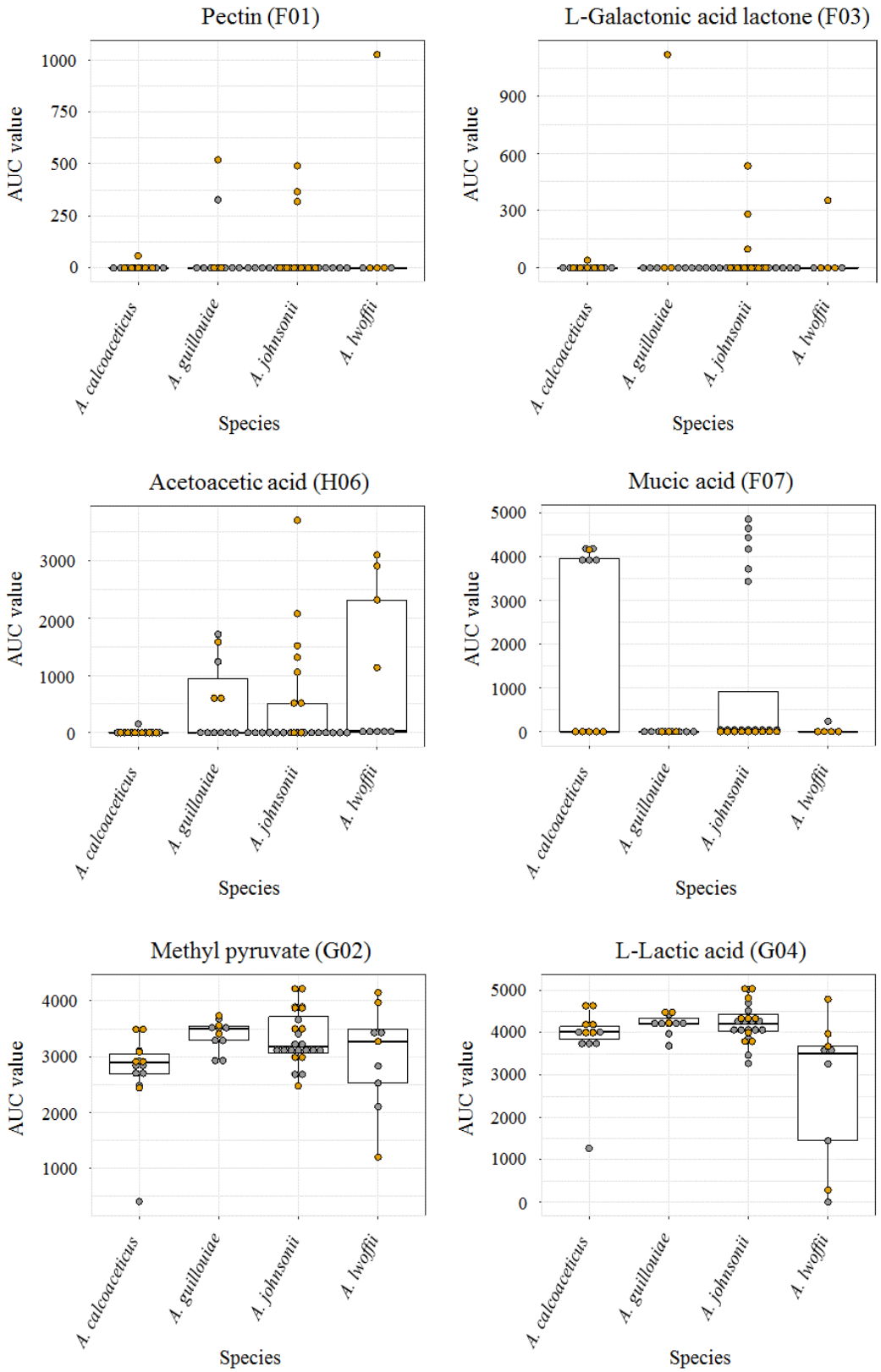


Figure S4.1: Boxplot representation of the AUC values for the GENIII phenotypes which are significantly different based on the origin of the isolates, irrespectively of the isolate's species classification. The boxplots show the upper and lower quartiles, values outside the upper and lower quartile but within 1.5 times the interquartile range are connected with a full line. Further, the median is plotted as a thick black line. The origin of isolates is indicated by color: orange, clinical/veterinary; grey, aquatic/environmental. *Acinetobacter* isolates (n = 58) were grouped per species: *A. calcoaceticus* (n = 14); *A. guillouiae* (n = 11); *A. johnsonii* (n = 24) and *A. lwoffii* (n = 9).

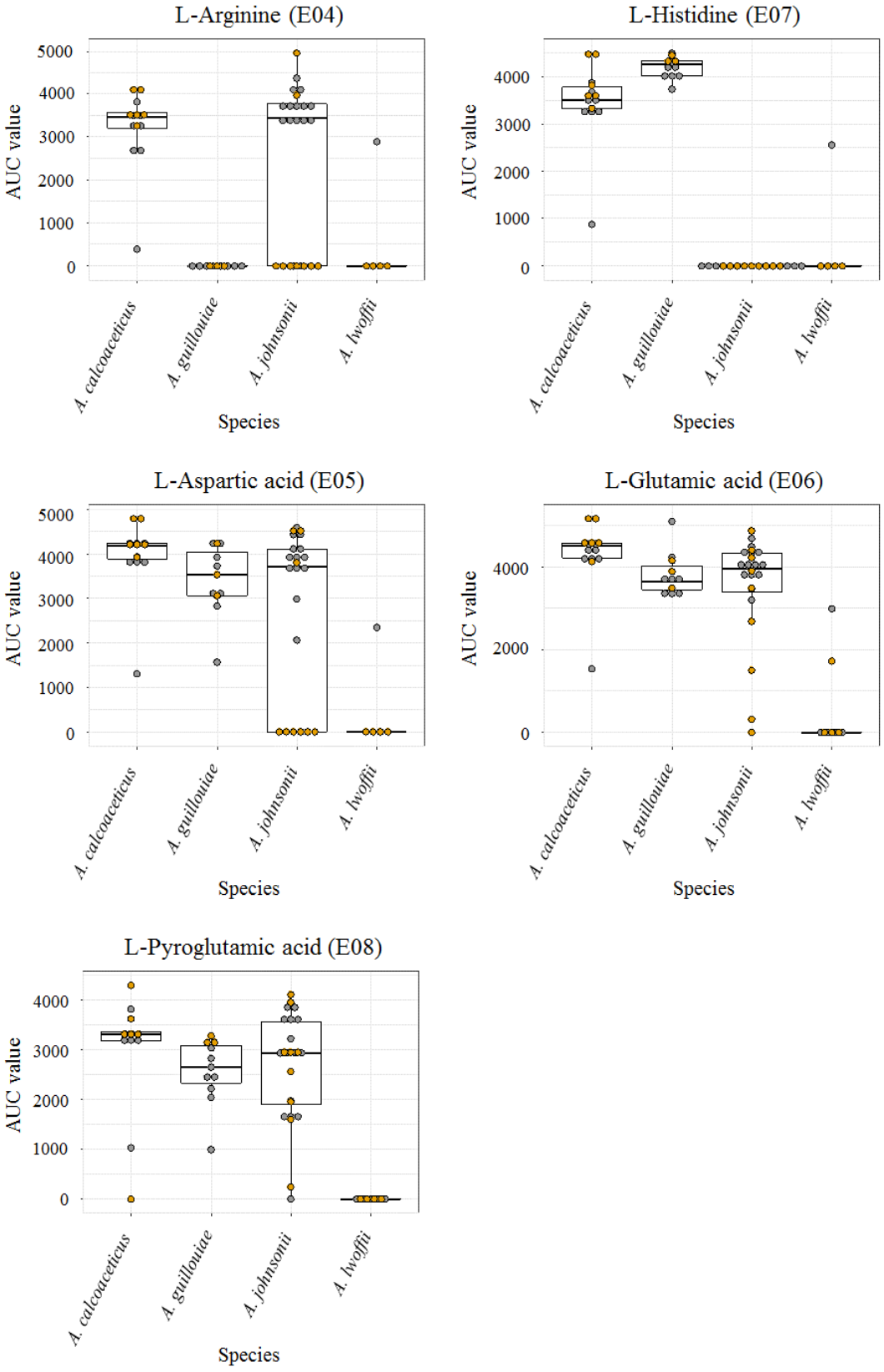


Figure S4.2: Boxplot representation of the AUC values for a selection of amino acids as sole carbon source. The boxplots show the upper and lower quartiles, values outside the upper and lower quartile but within 1.5 times the interquartile range are connected with a full line. Further, the median is plotted as a thick black line. The origin of isolates is indicated by color: orange, clinical/veterinary; grey, aquatic/environmental. *Acinetobacter* isolates (*n* = 58) were grouped per species: *A. calcoaceticus* (*n* = 14); *A. guillouiae* (*n* = 11); *A. johnsonii* (*n* = 24) and *A. lwoffii* (*n* = 9).

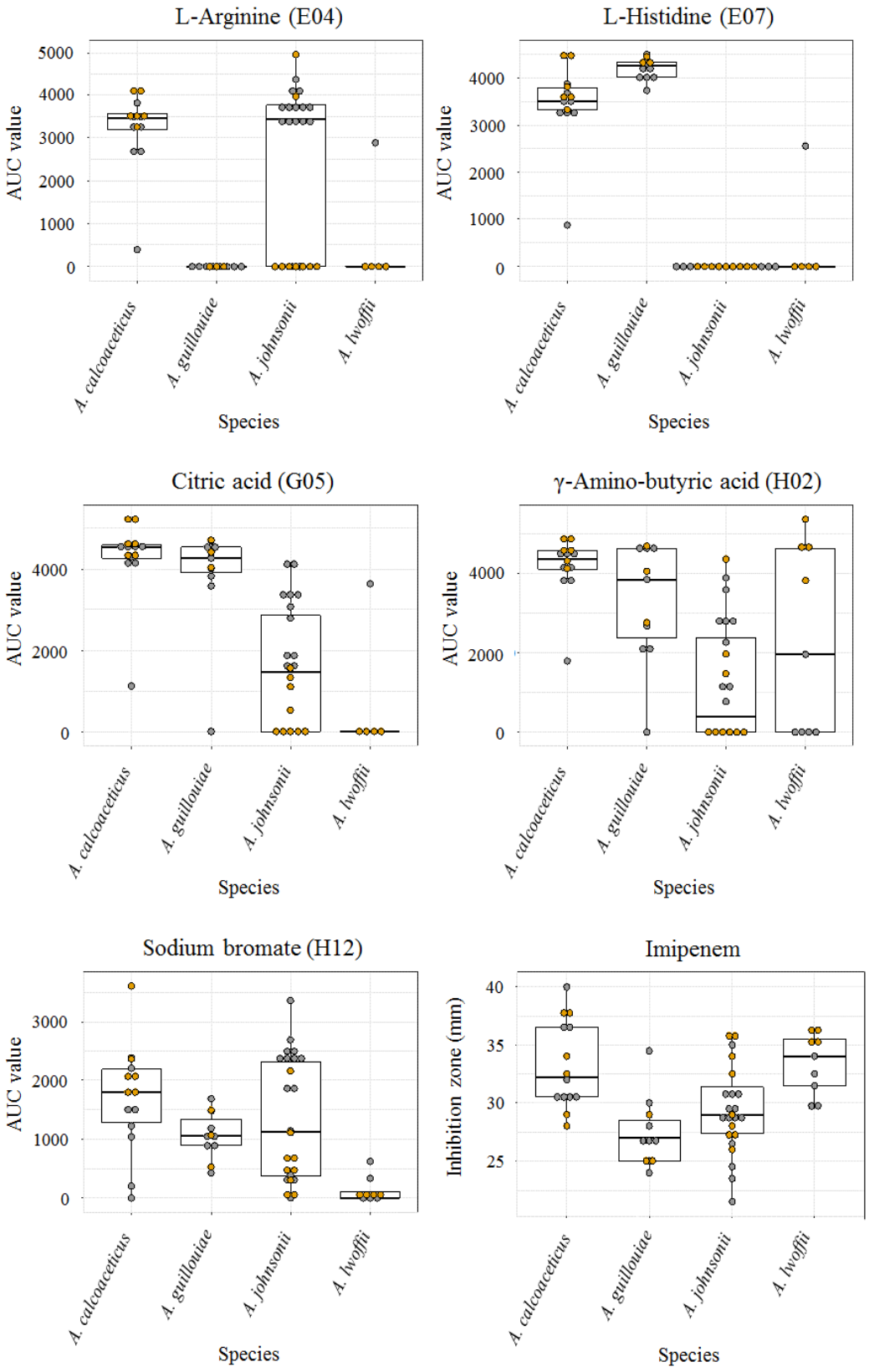


Figure S4.3: Boxplot representation of the AUC values (GENIII phenotypes) and inhibition zone of imipenem (antibiotic) which are significantly related to the pairwise genetic distance based on the partial *rpoB* gene sequence similarity. according to the Mantel test. The boxplots show the upper and lower quartiles. values outside the upper and lower quartile but within 1.5 times the interquartile range are connected with a full line. Further. the median is plotted as a thick black line. The origin of isolates is indicated by color: orange. clinical/veterinary; grey. aquatic/environmental. *Acinetobacter* isolates (n = 58) were grouped per species: *A. calcoaceticus* (n = 14); *A. guillouiae* (n = 11); *A. johnsonii* (n = 24) and *A. lwoffii* (n = 9).

Supporting information Chapter V

Table S5.1: Overview of *Acinetobacter* strains used in this study.

***Acinetobacter* species or genomic species**	**Strain designation[a]**	**AFLP cluster[b]**	***rpoB* (GenBank accession N°)**	**Genomic information[c]**	**Origin[d,e]**	**Sample[d]**	**Geographic location[d]**	**Year of isolation[d]**	**Reference[f]**	**Received from**
'Genomic species 6'	LUH 286 (Gerner-Smidt 39, MGH 97923, NIPH 1852)	22	KU961595		Cli	Urine	Sweden		1, 2	I. Tjernberg
'Genomic species 6'	LUH 4717 (NIPH 298)	22	KU961596	+	Cli	Wound	Czech Republic	1994		A. Nemec
'Genomic species 6'	RUH 2867 (ATCC 17979)	22	EU477115	+	Cli	Throat		1956 or before	1, 3	
'Genomic species NB14' (recently described as *A. dijkshoorniae*)	LUH 7351 (NIPH 2230)	14	KU961599		Cli	Urinary tract	The Netherlands	2001	4, 5	
Genomic species NB14' (recently described as *A. dijkshoorniae*)	LUH 10243	14	KU961597		Cli	Sputum	The Netherlands	2005	4, 5	
Genomic species NB14' (recently described as *A. dijkshoorniae*)	LUH 13626 (MOD3)	14	KU961598		Cli	Wound	Italy	2004	4, 5, 6	E. Carretto < APSI study group
Genomic species NB14' (recently described as *A. dijkshoorniae*)	RUH 53 (LMD 71.43, ATCC 13809, NIPH 814)	14	KJ956458		Env	Soil	The Netherlands	Before 1960	4, 5	J. van der Toorn
'Genomic species NB21'	LUH 8557 (013-4)	6	KU961600		Vet	Faeces cow	The Netherlands	2003		Animal health service
'Genomic species NB28'	LUH 5603 (RecB)	29	KU961601		Vet/Aq	Fish pond	Denmark			L. Guardabassi
'Genomic species NB28'	LUH 5605 (F2-36)	29	KU961602		Vet/Aq	Fresh water plus sediment	Denmark		7	L. Guardabassi
'Genomic species NB33'	LUH 5611 (F4-37)	30	KU961603		Vet/Aq	Fish pond	Denmark	1997-98	7	L. Guardabassi
'Genomic species NB4'	LUH 8917 (LMG V68)	38	KU961604		Env		Vietnam			G. Huys
'Genomic species NB53'	LUH 13549 (153A)	37	KU961605		Env		Germany	2008		H. Seifert
'Genomic species NB54'	LUH 14563 (NIPH 3789)	39	KU961606	+	Env/Aq	Pond	Czech Republic	2009		A. Nemec
'*A. calcoaceticus*-like'[g]	LUH 1469 (Gerner-Smidt 10095, CCUG34786)	11	EU477122	+	Cli	Abscess	Sweden		8	I. Tjernberg
'*A. calcoaceticus*-like'[g]	LUH 7045 (NIPH 2226)	11	KU961607		Vet	Nose dog	The Netherlands	2000		J. Wagenaar
'*A. calcoaceticus*-like'[g]	LUH 10726	12	KU961608		Cli	Wound	USA	2005	9	K. Petersen
'A. oleivorans'	JCM 16667 (KCTC 23045)	N.T.	GU292310	+	Env	Rice paddy/soil	South Korea			

A. baumannii	LUH 4708 (NIPH 70)	10	HQ123413		Cli	Trachea secretion	Czech Republic	1992	10	A. Nemec
A. baumannii	LUH 5875[h] (NIPH 1669)	10	HQ123411		Cli	Blood	The Netherlands	1997	10	
A. baumannii	RUH 134[h] (LMG 10541)	10	HQ123410		Cli	Urine	The Netherlands	1982	1	
A. baumannii	RUH 875[h] (LMG 10543)	10	HQ123409		Cli	Urine	The Netherlands	1984	1	
A. baumannii	RUH 3023[T] (ATCC 19606[T], LMG 1041[T])	10	EU477108	+	Cli	Urine			1	
A. baylyi	LUH 4836 (BD4, ATCC 33304)	16	EU477155		Env	Soil	USA	1998	11	
A. baylyi	LUH 5822 (93A2, NIPH 2313)	16	FJ754446				USA		11	L.N. Ornston
A. baylyi	LUH 9341[T] (B2[T], CCM7195[T])	16	FJ754445	+	Env	Activated sludge	Australia		11, 12	E. Carr
A. baylyi	LUH 9557 (A7, DSM 14959)	16	FJ754447		Env	Activated sludge	Australia		11, 12	E. Carr
A. baylyi	LUH 9558 (C5, DSM 14963)	16	FJ754448		Env	Activated sludge	Australia		11, 12	E. Carr
A. beijerinckii	LUH 4561 (Aci 509, NIPH 770)	17	EU477120		Env	Soil footpath	Greece	1993-94	13	H. Seifert
A. beijerinckii	LUH 4759[T] (Tjernberg 58A[T],LMG 25324[T])	17	EU477124	+	Cli	Wound	Sweden	1980-81	13, 14	I. Tjernberg
A. beijerinckii	LUH 4771 (Tjernberg 190, NIPH 850)	17	EU477125		Env	Plastic foam	Sweden	1980-81	13	
A. beijerinckii	LUH 6214 (NIPH 1453, CCUG 56139)	17	EU477130		Vet	Airbag horse	Belgium		13	L.A. Devriese
A. beijerinckii	RUH 2371 (NIPH 2011)	17	EU477137		Cli	Sputum	The Netherlands	1987	13	
A. bereziniae	LUH 7438 (118FFC, NIPH 2535)	25	FJ754456		Cli	Blood	Portugal	1998	15	G. da Silva
A. bereziniae	LUH 7832 (V0112893, NIPH 2537)	25	FJ754458		Vet	Wound seal	The Netherlands	2001	15	J. Wagenaar
A. bereziniae	LUH 8524 (130380-2, NIPH 2539)	25	FJ754459		Cli	Human clinical specimen	Netherlands	2003	15	H. Wagenvoort
A. bereziniae	LUH 9667 (8630, NIPH 2542)	25	FJ754460		Vet	Eye rabbit	Ireland	2003	15	S. Fanning
A. bereziniae	RUH 2224[T] (ATCC 17924[T], LMG 1003[T])	25	EU477116	+	Cli	Wound		Before 1960	15	
A. bohemicus	CCUG 63842[T] (ANC 3994[T])	N.T.	KJ124834	+	Env	Deciduous forest soil	Czech Republic	2011	16	

A. bouvetii	LUH 9342^{T} (4B02^{T}, DSM14964^{T})	28	EU477150	+	Env	Activated sludge	Australia		12	E. Carr
A. brisouii	DSM 18516^{T} (CIP 110357^{T})	N.T.	KC510989	+	Env	Peat layer	Korea			
A. calcoaceticus	LUH 2005 (ANC 3804)	13	HQ123420		Cli	Amputation stump	The Netherlands	1994	17	
A. calcoaceticus	LUH 12679 (NIPH 2706)	13	HQ123423		Cli	Sputum	Czech Republic	2005	17	A. Nemec
A. calcoaceticus	LUH 14369 (ANC 3680)	13	KU961609	+	Env/Aq	Water	Czech Republic	2008		A. Nemec
A. calcoaceticus	RUH 582 (LMG 10516)	13	HQ123424		Env	Soil	The Netherlands	1984	17	
A. calcoaceticus	RUH 2201^{T} (ATCC 23055^{T})	13	EU477149	+	Env	Soil	The Netherlands	Before 1911	17	
A. gandensis	DSM 28097^{T} (LMG 27960^{T})	N.T.	KJ569689		Vet	Dung from horse	Belgium		18	
A. gandensis	LUH 5725	21	KU961610		Vet	Uterus horse	Belgium	1999		M. Vaneechoutte
A. gandensis	LUH 8494	21	KU961611		Vet	Faeces cow	The Netherlands	2003		J. Wagenaar
A. gerneri	LUH 9343^{T} (9A01^{T}, DSM 14967^{T})	26	EU477151	+	Env	Activated sludge	Australia		12	
A. junii / 'A. grimontii'	LUH 9344^{T} (DSM 14968^{T})	2	EF611390	+	Env	Activated sludge	Australia		12, 19	
A. guillouiae	LUH 5606 (F4-7, NIPH 2525)	20	FJ754451		Vet/Aq	Fresh water and sediment	Denmark	1997	7, 15	L. Guardabassi
A. guillouiae	LUH 5653 (NIPH 2529)	20	FJ754453		Cli	Blood	The Netherlands	1999	15	
A. guillouiae	LUH 7830 (NIPH 2536)	20	FJ754457		Vet	Eye cat	The Netherlands	2001	15	J. Wagenaar
A. guillouiae	LUH 13178 (ANC 3626)	20	FJ754429		Env	Soil	Czech Republic	2007	15	A. Nemec
A. guillouiae	RUH 2861^{T} (CIP63.46^{T}, ATCC11171^{T})	20	EU477117	+	Env	Sewage		Before 1951	15	I. Tjernberg
A. gyllenbergii	LUH 1737 (80, SEIP 14.83, NIPH 802)	34	EU477121		Cli	Blood	France		13, 20	P. Bouvet
A. gyllenbergii	LUH 1742^{T} (CCUG 51248^{T}, RUH 422^{T}, NIPH 2150^{T})	34	EU477148	+	Cli	Urine	The Netherlands		13, 20	P. Bouvet < L. Dijkshoorn
A. gyllenbergii	LUH 4712 (CCUG 56138, NIPH 230)	34	EU477106	+	Cli	Vagina	Czech Republic	1994	13	A. Nemec
A. gyllenbergii	LUH 5809 (3268, NIPH 2202)	34	EU477131		Cli	Trachea	Hong Kong, China	1998	13	E.T. Houang

A. gyllenbergii	RUH 3064 (NIPH 2246)	34	EU477145		Cli	Sputum	The Netherlands	1989	13	
A. haemolyticus	RUH 406 (Gilardi 2890)	4	KU961612		Cli		USA	1984	1	A. Lampe < B. Vogel
A. haemolyticus	RUH 415 (NIPH 1871)	4	KU961613		Cli	Pus	The Netherlands	1984	1	
A. haemolyticus	RUH 2215^{T} (ATCC 17906^{T})	4	EU477109	+	Cli	Sputum	USA	Before 1963	1	
A. indicus	DSM 25388^{T} (CCM 7832^{T})	N.T.	JF772169	+	Env	Dump site	India			
A. indicus	LUH 5836 (NIPH 2206)	32	KJ847274		Cli	Ear	The Netherlands	1999		
A. indicus	LUH 8511	N.T.	KJ847275		Vet	Faeces cow	The Netherlands	2003		J. Wagenaar
A. indicus	LUH 9179 (LMG M151)	32	KU961614		Env	Fish farm	Malaysia	2005		G. Huys
A. indicus	LUH 10524	32	KU961615		Cli	Wound	The Netherlands	2005		
A. johnsonii	RUH 2231^{T} (ATCC 17909^{T})	35	EU477113	+	Cli	Duodenum		Before 1963		
A. johnsonii	RUH 2857 (Tjernberg 112)	36	KU961616		Cli	Urine	Sweden	1980-81	1, 14	I. Tjernberg
A. johnsonii	RUH 2859 (Tjernberg 137)	35	KU961617		Cli	Urine	Sweden	1980-81	1, 14	I. Tjernberg
A. junii	RUH 204 (NIPH 1855)	1	KU961618		Cli	Blood	The Netherlands	1983	1	
A. junii	RUH 2228^{T} (ATCC 17908^{T})	1	EU477110	+	Cli	Urine		Before 1962	1	
A. junii	RUH 2230 (Tjernberg 178, NIPH 1856)	1	KU961619		Env/Aq	Water	Sweden	1980-81	14	I. Tjernberg
A. kookii	DSM 29071^{T} (11-0202^{T}, KCTC 32033^{T})	N.T.	JX844152		Env	Soil	South Korea		21	
A. kookii	LUH 8638 (053-2)	31	KU961620		Env	Soil	The Netherlands	2003	21	
A. kookii	LUH 10268 (M014)	31	KU961621		Env	Fish farm	Malaysia	2005	21	G. Huys
A. kookii	LUH 10288 (TH120)	31	KU961622		Env	Fish farm	Malaysia	2003	21	G. Huys
A. kookii	LUH 13522 (102a)	31	KU961623		Env	Beet field	Germany		21	H. Seifert
A. lwoffii	LUH 1710 (ATCC 9957)	41	KU961624	+	Cli	Gangrenous lesion	Italy	Before 1945		
A. lwoffii	RUH 2219^{T} (ATCC 15309^{T})	41	EU477111	+			France	Before 1940		

A. nosocomialis	LUH 7150 (NIPH 3806)	9	KU961625		Cli	Aspirate	UK	2000	17	
A. nosocomialis	LUH 7430 (NIPH 3803)	9	HQ123400		Cli	Skin	Hong Kong, China	2001	17	E.T. Houang
A. nosocomialis	RUH 2210 (ATCC 17903)	9	EU477118					Before 1963		
A. nosocomialis	RUH 2376^{T} (LMG 10619^{T})	9	HQ123389	+	Cli	Sputum	The Netherlands	1987	17	
A. nosocomialis	RUH 3417 (NIPH 2812)	9	HQ123397		Cli	Sputum	Denmark	1984-85	17	P. Gerner-Smidt
A. parvus	LUH 3313 (LMG 21766)	40	KU961626	+	Cli	Skin	The Netherlands	1995	22	
A. parvus	LUH 4616^{T} (LMG 21765^{T})	40	EU477107	+	Cli	Ear outpatient	Czech Republic	1994	22	A. Nemec
A. parvus	LUH 7036	40	KU961627		Vet	Ear dog	The Netherlands	2001	22	J. Wagenaar
A. parvus	LUH 9635	40	KU961628		Cli	Blood	Netherlands	2004		
A. pittii	LUH 3538 (NIPH 789)	8	HQ123382		Cli	Trachea	Hungary	1994	17	L. Kiss
A. pittii	LUH 14366 (NIPH 336)	8	HQ123387		Cli	Urine	Czech Republic	1993	17	A. Nemec
A. pittii	RUH 502 (LMG 10554)	7	HQ123374		Cli	Drain	The Netherlands	1984	1, 17	
A. pittii	RUH 1163 (LMG 10555)	7	HQ123378		Cli	Toe web	The Netherlands	1985	1, 17	
A. pittii	RUH 2206^{T} (ATCC19004^{T}, LMG 1035^{T})	7	EU477114	+	Cli	Cerebrospinal fluid		Before 1967	1,17	
A. radioresistens	RUH 2225 (Tjernberg 109, NIPH 2271)	42	EU445667		Cli	Urine	Sweden	1980-81	1, 14	I. Tjernberg
A. radioresistens	RUH 2863 (Tjernberg 73)	42	KU961629		Cli	Wound	Sweden	1980-81	1	I. Tjernberg
A. radioresistens	RUH 2865^{T} (FO-1^{T}, IAM 13186^{T})	42	EU477112	+	Env	Cotton	Japan		1	I. Tjernberg < Y. Nishimura
A. schindleri	LUH 5677 (1B805)	27	KU961630		Vet	Trachea python	The Netherlands	1989		J. Wagenaar
A. schindleri	LUH 5832^{T} (NIPH 1034^{T})	27	EU477128	+	Cli	Urine	Czech Republic	1998	23	A. Nemec
A. schindleri	LUH 7428 (CUHK 4313)	27	KU961631		Cli	Blood	Hong Kong, China	2001		E. T. Houang
A. seifertii	LUH 1471 (Gerner-Smidt 5804, NIPH 826)	5	HQ123426		Cli	Blood	Denmark	1990-91	24	P. Gerner-Smidt

A. seifertii	LUH 1472^{T} Gerner-Smidt 10090^{T}, CCUG 5804^{T}, NIPH 973^{T})	5	EU477126	+	Cli	Ulcer	Denmark	1990-91	8, 24	P. Gerner-Smidt
A. seifertii	LUH 8128	5	KU961632		Cli	Catheter	The Netherlands	2002		
A. seifertii	RUH 1139 (NIPH 806)	5	KU961633		Cli	Throat	The Netherlands	1985	24	
A. soli	LUH 5786 (CUHK 4321, NIPH 2197)	23	KU961634		Cli	Blood	Hong Kong, China	1997		E.T. Houang
A. soli	LUH 5787 (CUHK 7044, NIPH 2198)	23	KU961635		Env	Vegetables	Hong Kong, China	1997		E.T. Houang
A. soli	LUH 7287 (NIPH 2229)	23	KU961636		Cli	Sputum	The Netherlands	2001		
A. soli	LUH 11756 (NIPH 2899)	23	KU961637	+	Cli	Sputum	Czech Republic	2005		A. Nemec
A. soli	LUH 14692^{T} (CCUG 59023^{T}, KCTC 22184^{T})	23	HQ148175	+	Env	Forest soil	Korea			
A. tandoii	LUH 5617	19	KU961638		Env	Sewage	Denmark		7	L. Guardabassi
A. tandoii	LUH 9345^{T} (4N13^{T}, DSM 14670^{T})	19	EU477122	+	Env	Activated sludge	Australia		12	E. Carr
A. tandoii	LUH 13385 (CCUG 47563, NIPH 3629)	19	KU961639		Env	Sediment	India	2003		A .Nemec
A. tjernbergiae	LUH 9346^{T} (DSM 14971^{T})	33	EU477153	+	Env	Activated sludge	Australia		12	E. Carr
A. tjernbergiae	LUH 9559 (7B02)	33	KU961640		Env	Activated sludge	Australia		12	E. Carr
A. towneri	LUH 8636 (52-2)	24	KU961641		Env	Soil	The Netherlands	2003		
A. towneri	LUH 09347^{T} (DSM 14962^{T})	24	EU477154	+	Env	Activated sludge	Australia		12	E. Carr
A. towneri	LUH 10282 (M142)	24	KU961642		Env	Fish farm	Malaysia	2005		G. Huys
A. towneri	LUH 13865 (CCUG 56484, NIPH 3700)	24	KU961643		Aq	Seawater	Korea			A. Nemec
A. ursingii	LUH 3792^{T} (NIPH 137^{T})	18	EU477105	+	Cli	Blood	Czech Republic	1993		A. Nemec
A. ursingii	LUH 7426 (CUHK 4308, NIPH 2232)	18	KU961644		Cli	Blood	Hong Kong, China	2001		E. T. Houang
A. ursingii	LUH 9820	18	KU961645		Cli	Wound	The Netherlands	2004		
A. ursingii / 'A. septicus'	LUH 13238 (NIPH 3649)	18	EF611383				Czech Republic		25, 26	A. Nemec

A. ursingii	RUH 1501	18	KU961646		Cli	Hairy skin	Netherlands	1985	23	
A. variabilis	CCUG 26390^{T} (NIPH 2171^{T})	N.T.	EU477119	+	Cli	Urine	Sweden		27	
A. variabilis	LUH 1091 (Tjernberg 118)	3	KU961647		Cli	Faeces	Sweden	1993	14	I. Tjernberg
A. variabilis	LUH 6307	3	KU961648		Cli	Post mortum wire	The Netherlands	2000		
A. venetianus	LUH 3904^{T} (RAG-1^{T}, ATCC 31012^{T})	15	EU477136	+	Env	Seawater			28	
A. venetianus	LUH 4379 (VE-C3, NIPH 1924)	15	EU496378		Env	Oil in lagoon			28	A. Vaneechouttte
A. venetianus	LUH 5627 (S1-2, NIPH 2310)	15	EU496381		Env	Aquaculture pond	Denmark		28	L. Guardabassi
A. venetianus	LUH 7437 (CUHK 7025)	15	KU961649		Env	Vegetables	Hong Kong	2001	28	E. T. Houang
A. venetianus	LUH 8758 (T4, MBIC 1332)	15	EU496379		Env	Seawater	Japan		28	M. Vaneechoutte < S. Yamamoto

[a] ATCC = American Type Culture Collection, USA; ANC and NIPH = collection A. Nemec, Prague, CZ; CCUG = Culture Collection University of Gothenburg, SE; CUHK = The Chinese University of Hong Kong, CN; DSM = German Collection of Microorganisms and Cell Cultures, DE; IAM = Institute of Molecular and Cellular Biosciences, The University of Tokyo, JP; KCTC = Korean Collection for Type Cultures, KR; LMD = Laboratory of Microbiology and Enzymology, Delft University of Technology, NL; LUH and RUH = collection of Leiden University Medical Center, NL; MBIC = Marine Biotechnology Institute Co. Ltd., JP. T = type strain.

[b] AFLP clustering based on a cut-off of 50 % similarity (N. T. = not tested)

[c] + = strain included in the phylogenetic analysis of Touchon et al. (2014; Genome Biol Evol 6:2866-2882), based on protein encoding genes of the core genome of *Acinetobacter* strains.

[d] Empty fields represent unknown data.

[e] Cli = clinical sample; Vet = veterinary sample; Aq = aquatic sample; and Env = environmental sample.

[f] 1, Janssens et al. (1997) Int J Bacteriol, 47:1179-1187; 2, Gerner-Smidt et al. (1991) J Clin Microbiol, 29:277-282; 3, Smith et al. (2007) Genes Div, 21:601-614; 4, Espinal et al. (2015) Antimicrob Agents Chemother, 59: 6657-6660; 5, Cosgaya et al. (2016) Int J Syst Evol Microbiol, doi:10.1099/ijsem.0.001318; 6, Carretto et al. (2011) Infect Genet Evol, 11:1319-1326; 7, Guardabassi et al. (2000) J Med Microbiol, 49:929-936; 8, Gerner-Smidt & Tjernberg (1993) APMIS, 101:826-832; 9, Petersen et al. (2011) J Clin Microbiol, 49:159-166; 10, Nemec et al. (2011) Res Microbiol, 162:393-404; 11, Vaneechoutte et al. (2006) Appl Environ Microbiol, 72:932-936; 12, Carr et al. (2003) Int J Syst Evol Microbiol, 53:953-963; 13, Nemec et al. (2009) Int J Syst Evol Microbiol, 59:118-124; 14, Tjernberg & Ursing (1989) APMIS, 97:595-605; 15, Nemec et al. (2010) Int J Syst Evol Microbiol, 60:896-903; 16, Krizova et al. (2014) Syst Appl Microbiol, 37:467-473; 17, Nemec et al. (2011) Res Microbiol, 162:393-404; 18, Smet et al. (2014) Int J Syst Evol Microbiol, 64:4007-4015; 19, Vaneechoutte et al. (2008) Int J Syst Evol Microbiol, 58:937-940; 20, Bouvet & Jeanjean (1989) Res Microbiol, 140:291-299; 21, Choi et al. (2013) Int J Syst Evol Microbiol, 63:4402-4406; 22, Nemec et al. (2003) Int J Syst Evol Microbiol, 53:1563-1567; 23, Nemec et al. (2001) Int J Syst Evol Microbiol, 51:1891-1899; 24, Nemec et al. (2015) Int J Syst Evol Microbiol, 65:934-924; 25, Kilic et al. (2008) J Clin Microbiol, 46:902-908; 26, Nemec et al. (2008) J Clin Microbiol, 46:2828-2827; 27, Krizova et al. (2015) Int J Syst Evol Microbiol, 65:857-863; 28, Vaneechoutte et al. (2009) 59:1376-1381.

[g] '*A. calcoaceticus*-like' strains are also known as 'genomic species between 1 and 3' (Gerner-Smidt & Tjernberg (1993; APMIS, 101:826-832); Touchon et al. (2014; Genome Biol Evol 6:2866-2882)).

[h] LUH 5875, RUH 134, RUH 875: reference strains of European (international) clone III, II and I, respectively.

Table S5.2: Kinetic parameter values obtained in this study (the average of two independent repeats is shown) (3 excel worksheet pages).

See excel file: PhD_Ado Van Assche_Chapter V_TableS5-2_Kinetic parameters.xlsx

Van Assche, A. (2019, September 10). PhD Ado Van Assche. https://doi.org/10.17605/OSF.IO/W7MK3

Table S5.3: Carbon source assimilation assays on GENIII plates for the differentiation of different *Acinetobacter* species (GENIII rows A until F)[a].

Species	(*n* =)	A02 (Dextrin)	A06 (b-Gentiobiose)	B03 (D-Melibiose)	C01 (D-Glucose)	C02 (D-Mannose)	C03 (D-Fructose)	C04 (D-Galactose)	C05 (3-O-Methyl-D-Glucose)	C06 (D-Fucose)	C07 (L-Fucose)	C08 (L-Rhamnose)	D07 (D-Fructose-6-Phosphate)	D08 (D-Aspartic Acid)	D09 (D-Serine #1)	E02 (Gly-Pro)	E03 (L-Alanine)	E04 (L-Arginine)	E05 (L-Aspartic Acid)	E06 (L-Glutamic Acid)	E07 (L-Histidine)	E08 (L-Pyroglutamic Acid)	E09 (L-Serine)	F01 (Pectin)	F02 (D-Galacturonic Acid)	F03 (L-Galactonic Acid-g-Lactone)	F04 (D-Gluconic Acid)	F05 (D-Glucuronic Acid)	F06 (Glucuronamide)	F07 (Mucic Acid)	F08 (Quinic Acid)	F09 (D-Saccharic Acid)
'Genomic species 6'	3	v	v	+	+	+	-	+	-	+	v	-	v	-	-	-	+	v	+	+	+	-	+	v	v	v	-	v	+	-	v	-
'Genomic species NB14' (recently described as *A. dijkshoorniae*)	4	v	-	-	+	v	-	+	v	+	v	v	v	-	v	-	+	+	+	+	+	+	+	-	-	v	-	v	+	+	+	+
'Genomic species NB21'	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
'Genomic species NB28'	2	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	+	-	-	-	+	v	-	-	-	+	-	-	-
'Genomic species NB33'	1	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-
'Genomic species NB4'	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	-	-	-	-	-	+	-	-	-
'Genomic species NB53'	1	+	-	-	-	-	-	+	-	+	+	+	+	-	-	-	+	+	+	+	-	+	-	+	+	+	-	+	+	+	+	-
'Genomic species NB54'	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-
A. calcoaceticus-like ('genomic species between 1 and 3')	3	+	-	-	+	+	-	+	v	+	-	-	+	-	-	-	+	+	+	+	+	+	v	v	-	-	v	-	+	v	+	v
'A. oleivorans'	1	+	-	-	+	+	-	+	-	+	+	-	+	-	+	-	+	+	+	+	+	+	-	-	-	-	-	-	+	+	+	+
A. baumannii	5	v	-	v	+	v	-	+	v	+	-	-	v	-	-	v	+	+	+	+	+	+	v	v	-	-	v	-	+	+	+	+
A. baylyi	5	+	v	v	+	v	v	+	v	+	v	v	v	+	v	-	+	v	+	+	-	v	-	v	v	v	+	v	+	+	+	+
A. beijerinckii	5	v	-	-	v	v	-	v	-	v	v	-	v	-	-	-	+	-	+	+	+	-	+	v	v	v	-	-	+	-	v	-
A. bererziniae	5	+	-	-	v	v	-	v	v	v	v	v	v	-	+	-	+	-	+	+	v	+	-	v	-	-	-	-	+	-	v	-
A. bohemicus	1	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-
A. bouvetii	1	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	-	-	-	-	+	-	-	-
A. brisouii	1	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-

A. calcoaceticus	5	v	-	v	v	v	-	+	v	+	-	-	v	+	-	v	+	v	+	+	+	v	v	v	-	v	-	-	+	v	v	v
A. gandensis	3	+	-	-	v	v	-	v	v	+	v	v	v	-	-	-	-	-	-	v	-	-	-	v	v	v	-	v	+	-	-	-
A. gerneri	1	+	-	-	-	-	-	+	-	+	-	-	+	-	-	+	+	-	+	+	-	+	-	+	+	+	-	-	+	-	-	-
A. guillouiae	5	+	-	-	-	-	-	v	v	v	v	v	+	v	v	-	+	-	+	+	+	+	-	v	v	v	-	v	+	-	v	-
A. gyllenbergii	5	v	-	-	-	-	-	v	-	v	v	-	+	-	-	-	v	v	v	+	+	+	-	-	-	-	-	-	+	-	v	-
A. haemolyticus	3	+	v	v	+	+	-	+	-	+	v	v	v	-	v	-	+	+	v	+	v	-	+	-	-	v	-	v	+	-	+	-
A. indicus	5	+	-	-	-	-	-	v	-	v	v	v	v	-	-	-	+	-	-	+	-	-	-	v	v	v	-	v	v	-	-	-
A. johnsonii	3	+	-	-	-	-	-	-	-	-	v	-	+	-	-	-	+	v	v	+	-	+	-	+	v	+	-	v	+	-	v	-
A. junii	4	+	-	-	-	-	-	v	v	v	v	v	v	-	-	-	v	+	-	+	v	-	v	v	v	v	-	v	+	-	-	-
A. kookii	5	v	-	-	-	-	-	v	-	v	v	v	v	-	-	-	v	-	-	-	-	-	-	v	v	v	-	v	+	-	v	-
A. lwoffii	2	+	-	-	-	-	-	-	-	-	-	-	v	-	-	-	+	-	-	v	-	-	-	v	v	v	-	-	v	-	-	-
A. nosocomialis	5	+	-	-	+	v	-	+	v	+	-	-	v	-	-	v	+	+	+	+	+	+	v	v	-	v	v	-	+	-	+	-
A. parvus	4	-	-	-	-	-	-	v	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. pittii	5	v	-	v	+	+	-	+	+	+	v	-	v	-	v	v	+	+	+	+	+	+	v	v	v	v	-	v	+	v	v	v
A. radioresistens	3	v	-	-	-	-	-	v	-	v	v	-	v	-	-	-	+	+	-	+	-	-	-	v	v	v	-	v	+	-	-	-
A. schindleri	3	v	-	-	-	-	-	v	v	v	v	v	v	-	-	-	+	-	-	v	-	-	-	v	v	v	-	v	+	-	-	-
A. seifertii	4	v	-	v	+	+	-	+	v	+	v	-	+	+	v	v	+	+	+	+	+	+	v	v	-	v	-	-	+	-	+	-
A. soli	5	+	v	+	+	v	+	+	v	+	+	v	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	v	+	v
A. tandoii	3	+	-	-	-	-	-	v	-	+	+	v	+	v	v	-	+	+	+	+	+	-	-	+	+	+	-	v	+	-	-	-
A. tjernbergiae	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	v	-	+	+	-	-	-	-	-	-	-	v	-	-	-
A. towneri	4	v	-	-	-	-	-	-	-	-	-	-	v	-	-	-	-	-	-	-	-	-	-	v	-	-	-	-	+	-	-	-
A. ursingii	5	v	-	-	-	-	-	v	-	v	-	-	-	-	-	-	v	-	v	+	-	-	-	v	-	-	-	-	v	-	v	-
A. variabilis	3	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	v	-	-	-	-	-	-	-	-	-	-	-	-
A. venetianus	5	v	-	-	v	v	-	v	-	v	v	-	v	-	-	-	+	+	-	+	+	-	+	v	v	v	-	v	+	-	v	-

[a] Based on area under the curve (AUC). Strains were considered positive when the average value of two independent experiments exceeded 1.5 times that for the blank. + = all strains positive; - = all strains negative; v = variable phenotype (No signal was observed for the following carbon sources: D-maltose, D-trehalose, D-cellobiose, sucrose, turanose, stachyose, D-raffinose, α-D-lactose, β-methyl-D-glucoside, D-salicin, N-acetyl-glucosamine, N-acetyl-β-D-mannosamine, N-acetyl-D-galactosamine, N-acetyl-neuraminic acid, inosine, D-sorbitol, D-mannitol, D-arabitol, myo-inositol, glycerol, D-glucose-6-phosphate, and gelatin).

Table S5.3 (continued): Carbon source assimilation assays on GENIII plates for the differentiation of different *Acinetobacter* species (GENIII rows G and H)[a].

Species	(*n* =)	G01 (p-Hydroxy-Phenylacetic Acid)	G02 (Methyl Pyruvate)	G03 (D-Lactic Acid Methyl Ester)	G04 (L-Lactic Acid)	G05 (Citric Acid)	G06 (a-Keto-Glutaric Acid)	G07 (D-Malic Acid)	G08 (L-Malic Acid)	G09 (Bromo-Succinic Acid)	H01 (Tween 40)	H02 (g-Amino-n-Butyric Acid)	H03 (a-Hydroxy-Butyric Acid)	H04 (b-Hydroxy-Butyric Acid)	H05 (a-Keto-Butyric Acid)	H06 (Acetoacetic Acid)	H07 (Propionic Acid)	H08 (Acetic Acid)	H09 (Sodium Formate)
'Genomic species 6'	3	-	+	-	-	+	+	+	+	+	+	-	-	+	v	v	+	+	-
'Genomic species NB14' (recently described as *A. dijkshoorniae*)	4	-	+	-	+	+	v	v	+	+	+	+	+	+	+	v	+	+	-
'Genomic species NB21'	1	-	+	-	+	-	-	+	+	+	+	-	+	+	+	+	+	+	-
'Genomic species NB28'	2	-	-	-	v	-	v	v	v	-	+	-	-	-	-	+	-	+	-
'Genomic species NB33'	1	-	-	-	+	-	-	-	+	+	+	+	-	-	+	+	+	+	-
'Genomic species NB4'	1	-	+	-	+	-	+	+	+	+	+	+	+	-	+	+	+	+	-
'Genomic species NB53'	1	-	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-
'Genomic species NB54'	1	+	+	-	+	-	+	+	+	+	+	+	+	-	+	+	+	+	-
A. calcoaceticus-like ('genomic species between 1 and 3')	3	-	+	-	+	+	+	v	+	+	+	+	+	+	+	v	+	+	v
'A. oleivorans'	1	-	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-
A. baumannii	5	-	+	-	+	+	+	v	+	+	+	+	+	+	+	v	+	+	-
A. baylyi	5	v	+	v	+	+	+	+	+	+	+	+	+	+	+	+	+	+	v
A. beijerinckii	5	-	+	-	-	+	+	v	+	+	+	+	-	+	+	+	+	+	-
A. bererziniae	5	-	+	-	+	+	v	+	+	+	+	+	+	+	+	v	+	+	-
A. bohemicus	1	-	+	-	+	-	-	-	-	-	+	+	-	-	-	+	-	+	-
A. bouvetii	1	+	+	-	+	-	+	-	+	+	+	-	-	+	+	+	+	+	-
A. brisouii	1	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-
A. calcoaceticus	5	-	v	v	+	+	v	-	+	v	+	+	v	+	v	v	+	+	v
A. gandensis	3	-	+	-	+	v	v	-	+	+	+	-	v	v	+	+	+	+	-
A. gerneri	1	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
A. guillouiae	5	-	+	-	+	v	+	v	+	+	+	+	+	+	+	v	+	+	-

A. gyllenbergii	5	-	+	-	+	+	+	+	+	+	+	-	+	+	+	v	+	+	-
A. haemolyticus	3	-	+	-	-	+	+	+	+	+	+	+	-	+	+	+	+	+	v
A. indicus	5	-	+	-	+	-	v	v	v	v	+	-	v	+	v	+	+	+	-
A. johnsonii	3	-	+	-	+	v	v	-	+	+	+	v	v	+	v	+	v	+	-
A. junii	4	-	+	-	v	+	v	v	v	v	+	v	v	+	v	+	+	+	-
A. kookii	5	-	+	-	+	-	+	v	v	v	+	v	+	v	+	v	+	+	-
A. lwoffii	2	-	+	-	+	-	-	-	v	v	+	+	v	+	v	+	v	+	-
A. nosocomialis	5	-	+	v	+	+	v	v	+	v	+	+	v	+	+	v	+	+	-
A. parvus	4	-	+	-	-	-	-	-	-	-	+	-	-	-	-	v	v	+	-
A. pittii	5	-	+	-	+	+	v	+	+	+	+	+	+	+	+	v	+	+	-
A. radioresistens	3	-	+	-	+	-	+	-	v	v	+	+	v	+	-	v	+	+	-
A. schindleri	3	-	+	-	+	v	v	-	v	v	+	-	v	+	+	+	+	+	-
A. seifertii	4	-	+	-	+	+	+	v	+	+	+	+	+	+	+	v	+	+	v
A. soli	5	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A. tandoii	3	-	+	-	+	v	+	+	+	+	+	+	+	+	+	+	+	+	-
A. tjernbergiae	2	-	+	-	-	-	+	-	v	v	+	-	-	-	-	+	+	+	-
A. towneri	4	-	+	-	+	-	-	v	v	v	+	-	v	v	v	v	v	v	-
A. ursingii	5	-	+	-	+	v	+	v	+	+	+	-	v	+	v	+	+	+	-
A. variabilis	3	-	+	-	v	-	v	v	+	+	+	v	-	v	v	+	v	+	-
A. venetianus	5	-	+	-	-	+	+	v	+	+	+	v	-	+	+	v	+	+	v

[a] Based on area under the curve (AUC). Strains were considered positive when the average value of two independent experiments exceeded 1.5 times that for the blank. + = all strains positive; - = all strains negative; v = variable phenotype.

Table S5.4: Chemical sensitivity assays on GENIII plates for the differentiation of different *Acinetobacter* species (GENIII columns 10, 11 and 12)[a].

Species	(*n* =)	Osmotic stressors				Acidic stressors				Toxins					Chaotropes		Inhibitors with unknown mechanism of inhibition						Dye	
		B10 (1% NaCl)	B11 (4% NaCl)	B12 (8% NaCl)	C12 (D-Serine #2)	A11 (pH 6)	A12 (pH 5)	C11 (Fusidic Acid)	G10 (Nalidixic Acid)	D10 (Troleandomycin)	D11 (Rifamycin SV)	E10 (Lincomycin)	F10 (Vancomycin)	H10 (Aztreonam)	E11 (Guanidine Hydrochloride)	G11 (Lithium Chloride)	C10 (1% Sodium Lactate)	D12 (Minocycline)	E12 (Niaproof)	G12 (Potassium Tellurite)	H11 (Butyric Acid)	H12 (Sodium Bromate)	F11 (Tetrazolium Violet)	F12 (Tetrazolium Blue)
'Genomic species 6'	3	+	+	v	+	+	-	v	v	v	v	+	v	+	+	+	+	-	v	v	+	+	+	+
'Genomic species NB14' (recently described as *A. dijkshoorniae*)	4	+	+	+	+	+	+	v	v	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
'Genomic species NB21'	1	+	-	-	+	+	-	-	+	-	+	-	+	+	+	+	+	-	+	+	+	-	+	+
'Genomic species NB28'	2	v	-	-	+	+	-	-	+	-	v	-	-	+	-	-	v	-	-	v	-	-	+	+
'Genomic species NB33'	1	+	-	-	-	+	-	-	+	-	-	-	-	+	-	-	+	-	-	+	-	-	+	+
'Genomic species NB4'	1	+	-	-	+	+	+	-	+	-	+	+	+	+	+	-	+	-	+	+	+	+	+	+
'Genomic species NB53'	1	+	+	-	+	+	-	-	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+
'Genomic species NB54'	1	+	-	-	+	+	+	-	+	-	+	+	+	+	-	-	+	-	-	+	-	-	+	+
A. calcoaceticus-like ('genomic species between 1 and 3')	3	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
'A. oleivorans'	1	+	+	-	+	+	+	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
A. baumannii	5	+	+	v	+	+	+	v	v	+	+	+	+	+	+	+	+	v	+	+	+	+	+	+
A. baylyi	5	+	+	v	+	+	v	-	+	v	+	+	+	+	+	+	+	-	+	+	+	v	+	+
A. beijerinckii	5	+	v	-	+	+	-	-	v	v	+	+	+	+	+	+	+	-	+	v	v	+	+	+
A. bererziniae	5	+	+	v	+	+	+	v	v	+	+	+	+	+	+	+	+	v	+	+	+	+	+	+
A. bohemicus	1	+	-	-	+	+	-	-	+	-	+	-	+	+	-	-	+	-	-	-	+	+	+	+
A. bouvetii	1	+	-	-	-	+	-	-	+	-	+	+	+	+	+	-	+	-	+	-	+	-	+	+
A. brisouii	1	+	-	-	+	+	+	-	+	-	+	+	+	+	+	-	+	-	-	+	+	-	+	+
A. calcoaceticus	5	v	v	-	v	+	v	-	v	v	v	v	v	v	v	v	+	-	v	v	v	v	v	v
A. gandensis	3	+	+	-	+	+	-	-	+	-	+	+	+	+	+	-	+	-	+	+	+	+	+	+
A. gerneri	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
A. guillouiae	5	+	v	-	+	+	v	-	+	v	+	+	+	+	+	v	+	-	+	+	+	+	+	+
A. gyllenbergii	5	+	v	-	+	+	v	-	v	v	+	v	+	v	+	v	+	-	v	v	v	v	+	+

A. haemolyticus	3	+	+	+	+	+	v	v	v	v	+	+	+	+	+	+	+	-	+	v	+	+	+	+
A. indicus	5	+	+	v	+	+	-	-	+	v	+	+	+	+	+	v	+	-	+	+	+	-	+	+
A. johnsonii	3	+	v	-	+	+	v	-	v	-	+	+	+	+	+	+	+	v	+	+	+	+	+	+
A. junii	4	+	v	-	+	+	-	-	-	v	+	v	+	+	+	+	+	v	+	+	v	v	+	+
A. kookii	5	+	v	-	v	+	v	-	v	v	+	v	+	+	+	-	+	v	+	+	+	-	+	+
A. lwoffii	2	+	+	-	v	+	-	-	+	-	+	v	v	+	+	+	+	-	v	+	+	-	+	+
A. nosocomialis	5	+	+	-	v	+	+	v	+	+	+	+	+	+	+	+	+	-	+	v	+	+	+	+
A. parvus	4	v	-	+	+	+	+	-	v	v	+	v	v	+	-	v	v	+	+	+	+	+	+	+
A. pittii	5	+	+	-	+	+	+	v	v	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
A. radioresistens	3	+	+	v	+	+	-	-	v	-	v	+	v	v	+	v	+	-	v	v	v	v	+	+
A. schindleri	3	+	v	v	+	+	v	v	v	-	+	v	v	+	+	v	+	v	+	+	+	-	+	+
A. seifertii	4	+	+	v	+	+	+	v	v	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
A. soli	5	+	+	v	+	+	+	-	v	v	+	+	+	+	+	v	+	-	+	v	v	v	+	+
A. tandoii	3	+	+	-	+	+	-	-	v	v	+	+	+	+	+	+	+	-	+	+	+	+	+	+
A. tjernbergiae	2	+	-	+	+	+	+	-	-	-	-	+	-	+	v	-	+	v	-	+	-	-	+	+
A. towneri	4	+	v	-	+	+	v	-	v	-	+	-	v	+	+	+	+	-	v	v	v	-	+	+
A. ursingii	5	+	+	v	+	+	+	v	v	v	+	+	+	+	+	+	+	v	+	+	+	+	+	+
A. variabilis	3	+	+	-	+	+	-	-	+	-	+	+	v	+	+	+	+	-	+	+	+	-	+	+
A. venetianus	5	+	+	+	+	+	v	v	v	+	+	+	+	+	+	+	+	-	+	v	+	+	+	+

[a] Based on area under the curve (AUC). Strains were considered positive when the average value of two independent experiments exceeded 1.5 times that for the blank. + = all strains positive; - = all strains negative; v = variable phenotype.

Table S5.5: Comparison of the results obtained in tests of phylogenetic signal for presence/absence of carbon source assimilation and chemical sensitivity traits determined using GENIII plates.

GENIII assays	Index values[a]	Phylogenetic tree (*n*)[b]	
		***rpoB* (133)**	**Genomic (40)**
Carbon source assimilation	Range	-0.044 – 0.877	-0.749 – 0.953
	Mean	0.387	0.156
	D < 0[c]	1 (1.4 %)	13 (18.3 %)
Chemical sensitivity	Range	0.237 – 0.990	0.236 – 1.159
	Mean	0.533	0.634
	D < 0[c]	0 (0%)	0 (0%)
Overall	Range	-0.044 – 0.990	-0.749 – 1.159
	Mean	0.425	0.285
	D < 0[c]	1 (1.1 %)	13 (13.8 %)

[a] In each case, the range of D values and the mean D value obtained for the selection of GENIII traits is indicated. Additionally, the number (and percentage) of traits for which D values were < 0 (i.e. highly phylogenetically clustered) is presented.
[b] Phylogenetic tree used for computation of phylogenetic signal. *n*, number of *Acinetobacter* strains included in the analyses.
[c] See details in Materials and Methods.

Table S5.6: Overview of the top three traits[a] based on the phylogenetic signal tests for presence/absence of phenotypic traits determined using GENIII plates.

Phylogenetic tree (*n*)[b]	Top three traits (AUC)
rpoB (133)	L-Pyroglutamic acid (-0.044)
	L-Arginine (0.005)
	L-Histidine (0.013)
Genomic (40)	Citric acid (-0.749)
	α-D-Glucose (-0.748)
	D-Melibiose (-0.537)

[a] In each case, the three traits with the lowest values of D (whose values are provided within parentheses) and thus, displaying more phylogenetic signal, are shown.
[b] Phylogenetic tree used for computation of phylogenetic signal. *n*, number of *Acinetobacter* strains included in the analyses.

Table S5.7: Comparison of the results obtained in tests of phylogenetic signal for different kinetic parameters of carbon source assimilation and chemical sensitivity traits determined using GENIII plates.

Phylogenetic tree (*n*)[a]	Kinetic parameter	Index	Carbon source assimilation			Chemical sensitivity			Overall		
			Range	Mean	Significant traits[b]	Range	Mean	Significant traits[b]	Range	Mean	Significant traits[b]
rpoB (133)	AUC	K	$3.5\cdot10^{-7}$ – $1.9\cdot10^{-5}$	$3.4\cdot10^{-6}$	32 (45.1 %)	$5.8\cdot10^{-7}$ – $1.1\cdot10^{-5}$	$2.5\cdot10^{-6}$	6 (26.1 %)	$3.5\cdot10^{-7}$ – $1.9\cdot10^{-5}$	$3.2\cdot10^{-6}$	38 (40.4 %)
		I	-0.096 – 0.775	0.468	68 (95.8 %)	0.151 – 0.635	0.399	22 (95.7 %)	-0.096 – 0.775	0.451	90 (95.7 %)
		C_{mean}	-0.091 – 0.777	0.472	68 (95.8 %)	0.159 – 0.638	0.403	22 (95.7 %)	-0.091 – 0.777	0.455	90 (95.7 %)
		λ	0.073 – 0.983	0.781	30 (42.3 %)	0.242 – 0.979	0.686	6 (26.1 %)	0.073 – 0.983	0.758	36 (38.3 %)
	μ_{max}	K	$9.1\cdot10^{-8}$ – $2.5\cdot10^{-4}$	$1.4\cdot10^{-5}$	0 (0 %)	$2.6\cdot10^{-7}$ – $1\cdot10^{-5}$	$2.1\cdot10^{-6}$	0 (0 %)	$9.1\cdot10^{-8}$ – $2.5\cdot10^{-4}$	$1.1\cdot10^{-5}$	0 (0 %)
		I	-0.058 – 0.393	0.023	3 (4.2 %)	-0.048 – 0.211	0.034	1 (4.3 %)	-0.058 – 0.393	0.025	4 (4.3 %)
		C_{mean}	-0.051 – 0.396	0.029	2 (2.8 %)	-0.033 – 0.214	0.038	0 (0 %)	-0.051 – 0.396	0.031	2 (2.1 %)
		λ	0.023 – 0.809	0.127	0 (0 %)	0.031 – 0.349	0.123	0 (0 %)	0.023 – 0.809	0.126	0 (0 %)
	lag time	K	$3.2\cdot10^{-7}$ – $6.2\cdot10^{-6}$	$1.5\cdot10^{-6}$	8 (11.3 %)	$2.2\cdot10^{-7}$ – $3.2\cdot10^{-5}$	$2.3\cdot10^{-6}$	1 (4.3 %)	$2.2\cdot10^{-7}$ – $3.2\cdot10^{-5}$	$1.7\cdot10^{-6}$	9 (9.6 %)
		I	-0.097 – 0.597	0.186	32 (45.1 %)	-0.042 – 0.510	0.159	11 (47.8 %)	-0.097 – 0.597	0.179	43 (45.7 %)
		C_{mean}	-0.081 – 0.601	0.193	32 (45.1 %)	-0.031 – 0.512	0.165	10 (43.5 %)	-0.081 – 0.601	0.186	42 (44.7 %)
		λ	0.057 – 0.871	0.388	13 (18.3 %)	0.078 – 0.696	0.358	6 (26.1 %)	0.057 – 0.871	0.381	19 (20.2 %)
Genomic (40)	AUC	K	0.057 – 0.848	0.255	18 (25.4 %)	0.056 – 0.415	0.135	0 (0 %)	0.056 – 0.848	0.226	18 (19.1 %)
		I	-0.209 – 0.670	0.309	47 (66.2 %)	-0.104 – 0.536	0.160	7 (30.4 %)	-0.209 – 0.670	0.273	54 (57.4 %)
		C_{mean}	-0.204 – 0.677	0.320	43 (60.6 %)	-0.090 – 0.543	0.177	6 (26.1 %)	-0.204 – 0.677	0.285	49 (52.1 %)
		λ	0 – 1	0.476	26 (36.6 %)	0 – 0.956	0.207	3 (13 %)	0 – 1	0.410	29 (30.9 %)
	μ_{max}	K	0.020 – 0.571	0.143	1 (1.4 %)	0.035 – 0.351	0.178	0 (0 %)	0.020 – 0.571	0.152	1 (1.1 %)
		I	-0.167 – 0.358	0.034	7 (9.9 %)	-0.102 – 0.500	0.014	1 (4.3 %)	-0.167 – 0.500	0.029	8 (8.5 %)
		C_{mean}	-0.137 – 0.371	0.056	6 (8.5 %)	-0.090 – 0.507	0.024	1 (4.3 %)	-0.137 – 0.507	0.048	7 (7.4 %)
		λ	0 – 1	0.199	8 (11.3 %)	0 – 0.737	0.039	1 (4.3 %)	0 – 1	0.160	9 (9.6 %)
	lag time	K	0.025 – 0.551	0.123	3 (4.2 %)	0.019 – 0.268	0.091	0 (0 %)	0.019 – 0.551	0.115	3 (3.2 %)
		I	-0.200 – 0.434	0.065	6 (8.5 %)	-0.227 – 0.341	0.040	1 (4.3 %)	-0.027 – 0.434	0.059	7 (7.4 %)
		C_{mean}	-0.187 – 0.459	0.082	6 (8.5 %)	-0.165 – 0.357	0.057	1 (4.3 %)	-0.187 – 0.459	0.076	7 (7.4 %)
		λ	0 – 0.947	0.130	3 (4.2 %)	0 – 0.565	0.117	1 (4.3 %)	0 – 0.947	0.127	4 (4.3 %)

[a] Phylogenetic tree used for computation of phylogenetic signal indices. *n*, number of *Acinetobacter* strains included in the analyses.
[b] Number (and percentage) of traits for which the corresponding phylogenetic signal index is statistically significant ($p < 0.01$).

Table S5.8: Overview of the top three traits[a] based on the phylogenetic signal tests for different kinetic parameters of phenotypic traits determined using GENIII plates.

Phylogenetic tree (*n*)[b]	Kinetic parameter	Top three traits			
		Blomberg's K	**Moran's I**	**Abouheif's C_{mean}**	**Pagel's λ**
rpoB (133)	AUC	L-Histidine ($1.9 \cdot 10^{-5}$), L-Lactic acid ($1.8 \cdot 10^{-5}$), p-Hydroxy-phenylacetic acid ($1.3 \cdot 10^{-5}$)	L-Aspartic acid (0.775), L-Pyroglutamic acid (0.770), L-Arginine (0.718)	L-Aspartic acid (0.777), L-Pyroglutamic acid (0.771), L-Arginine (0.720)	D-Aspartic acid (0.983), 1% Sodium lactate (0.979), Citric acid (0.974)
	μ_{max}	Sucrose ($2.5 \cdot 10^{-4}$), L-Serine ($2.3 \cdot 10^{-4}$), p-Hydroxy-phenylacetic acid ($8.3 \cdot 10^{-5}$)	D-Aspartic acid (0.393), D-Galactose (0.240), Troleandomycin (0.211)	D-Aspartic acid (0.396), D-Galactose (0.251), Troleandomycin (0.214)	D-Aspartic acid (0.809), D-Galactose (0.538), Citric acid (0.412)
	lag time	Tetrazolium blue ($3.2 \cdot 10^{-5}$), γ-Amino-butyric acid ($6.2 \cdot 10^{-6}$), L-Histidine ($4.5 \cdot 10^{-6}$)	L-Histidine (0.597), L-Pyroglutamic acid (0.595), Sodium bromate (0.510)	L-Histidine (0.601), L-Pyroglutamic acid (0.598), Sodium bromate (0.512)	L-Lactic acid (0.871), L-Pyroglutamic acid (0.857), D-Serine [D09] (0.840)
Genomic (40)	AUC	L-Aspartic acid (0.848), L-Histidine (0.848), γ-Amino-butyric acid (0.633)	D-Mannose (0.670), D-Galactose (0.668), D-Fucose (0.664)	D-Mannose (0.677), D-Galactose (0.677), D-Fucose (0.674)	Acetic acid (1), γ-Amino-butyric acid (1), L-Histidine (1)
	μ_{max}	L-Malic acid (0.571), Sucrose (0.513), Inosine (0.502)	pH 5 (0.500), α-D-Glucose (0.358), D-Saccharic acid (0.354)	pH 5 (0.507), α-D-Glucose (0.371), D-Saccharic acid (0.364)	Inosine (1), Sucrose (1), L-Malic acid (0.997)
	lag time	L-Histidine (0.551), γ-Amino-butyric acid (0.333), L-Pyroglutamic acid (0.278)	D-Gluconic acid (0.434), L-Pyroglutamic acid (0.391), L-Histidine (0.366)	D-Gluconic acid (0.459), L-Pyroglutamic acid (0.404), L-Histidine (0.382)	L-Histidine (0.947), γ-Amino-butyric acid (0.892), L-Pyroglutamic acid (0.846)

[a] In each case, the three traits scoring best for the corresponding phylogenetic signal index (whose values are provided within parentheses) are shown.
[b] Phylogenetic tree used for computation of phylogenetic signal indices. *n*, number of *Acinetobacter* strains included in the analyses.

Table S5.9: Comparison of Mantel test results for carbon source assimilation and chemical sensitivity assays.

Kinetic parameter	**GENIII assays**	**No. (%) of significant tests[a]**
AUC	Carbon source assimilation	42 (59.2 %)
	Chemical sensitivity	7 (30.4 %)
	Overall	49 (52.1 %)
μ_{max}	Carbon source assimilation	8 (11.3 %)
	Chemical sensitivity	1 (4.3 %)
	Overall	9 (9.6 %)
lag time	Carbon source assimilation	9 (12.7 %)
	Chemical sensitivity	2 (8.7 %)
	Overall	11 (11.7 %)

[a] Significant associations between trait variation and phylogenetic distance of *Acinetobacter* species; $p < 0.01$.

Table S5.10: Overview of the z statistic of the Mantel test for each GENIII phenotype (Significant *p* values (i.e. < 0.01) are shown with green background)

GENIII test	AUC		μ		Lambda	
	z statistic	***p* value**	**z statistic**	***p* value**	**z statistic**	***p* value**
A01 (Negative Control)						
A02 (Dextrin)	105.13	0.053	43884.29	0.003	1560.18	0.719
A03 (D-Maltose)	24.25	0.037	18644.34	0.421	3128.12	0.947
A04 (D-Trehalose)	21.04	0.661	4861.60	0.255	1936.59	0.269
A05 (D-Cellobiose)	26.18	0.099	41652.06	0.117	1995.95	0.145
A06 (Gentibiose)	110.66	0.001	41652.06	0.111	1995.95	0.155
A07 (Sucrose)	24.50	0.321	89900.42	0.297	2468.14	0.607
A08 (D-Turanose)	29.91	0.231	36999.07	0.751	1735.29	0.879
A09 (Stachyose)	19.54	0.407	4202.29	0.537	2094.73	0.827
A10 (Positive Control)						
A11 (pH 6)	78.48	0.005	59730.32	0.841	982.63	0.295
A12 (pH 5)	277.80	0.001	48170.34	0.001	4294.79	0.003
B01 (D-Raffinose)	29.46	0.009	28583.11	0.443	1842.22	0.571
B02 (α-D-Lactose)	35.91	0.033	10949.79	0.237	3287.84	0.199
B03 (D-Melibiose)	152.34	0.001	76888.54	0.105	1924.04	0.035
B04 (β-Methyl-D-Glucoside)	36.26	0.029	3311.03	0.143	1612.73	0.139
B05 (D-Salicin)	33.26	0.109	52359.41	0.261	1294.18	0.161
B06 (N-Acetyl-D-Glucosamine)	33.15	0.009	19383.21	0.551	1665.58	0.927
B07 (N-Acetyl-β-D-Mannosamine)	30.84	0.001	13985.28	0.935	2178.86	0.347
B08 (N-Acetyl-D-Galactosamine)	29.29	0.079	10815.96	0.369	1701.07	0.193
B09 (N-Acetyl-Neuraminic Acid)	22.57	0.391	49218.53	0.821	1663.15	0.851
B10 (1% NaCl)	146.60	0.007	156616.50	0.725	1056.96	0.037
B11 (4% NaCl)	244.56	0.143	41880.61	0.349	1426.98	0.673
B12 (8% NaCl)	121.21	0.033	26113.06	0.573	1147.33	0.287
C01 (α-D-Glucose)	207.28	0.001	12036.89	0.001	2113.98	0.007
C02 (D-Mannose)	172.28	0.001	14951.10	0.001	1842.02	0.469
C03 (D-Fructose)	127.33	0.001	14272.05	0.237	1978.83	0.067
C04 (D-Galactose)	233.90	0.001	7672.22	0.003	1756.05	0.051
C05 (3-Methyl Glucose)	131.15	0.001	20084.18	0.241	1316.96	0.439
C06 (D-Fucose)	252.91	0.001	18709.30	0.139	1538.50	0.025
C07 (L-Fucose)	125.15	0.015	12925.17	0.185	1308.42	0.577
C08 (L-Rhamnose)	80.40	0.029	55174.53	0.317	1446.78	0.487
C09 (Inosine)	35.48	0.649	65094.09	0.325	1346.27	0.879
C10 (1% Sodium Lactate)	153.71	0.001	103741.30	0.309	1044.95	0.015
C11 (Fusidic Acid)	112.78	0.405	348064.30	0.735	3006.79	0.573
C12 (D-Serine #2)	205.11	0.615	45414.63	0.147	795.14	0.965
D01 (D-Sorbitol)	24.73	0.181	4740.37	0.255	1140.57	0.359
D02 (D-Mannitol)	36.72	0.009	24346.23	0.381	1653.42	0.777
D03 (D-Arabitol)	48.42	0.001	6790.26	0.377	1256.94	0.017
D04 (myo-Inositol)	49.97	0.003	5927.20	0.423	1562.42	0.417
D05 (Glycerol)	60.85	0.001	12439.07	0.245	1964.25	0.399
D06 (D-Glucose-6-Phosphate)	82.32	0.001	5773.29	0.273	1770.55	0.961
D07 (D-Fructose-6-Phosphate)	110.94	0.009	5494.86	0.567	1092.72	0.511
D08 (D-Aspartic Acid)	167.41	0.023	9545.88	0.069	1974.18	0.247
D09 (D-Serine #1)	114.55	0.129	8486.08	0.001	2829.80	0.945
D10 (Troleandomycin)	300.54	0.055	24411.39	0.399	1034.52	0.025
D11 (Rifamycin SV)	193.41	0.733	61949.47	0.667	1319.63	0.159
D12 (Minocycline)	95.14	0.005	7457.66	0.667	905.21	0.649
E01 (Gelatin)	44.09	0.087	7294.92	0.155	1303.03	0.525
E02 (Glyl-L-Proline)	90.70	0.019	5661.24	0.149	2384.65	0.267
E03 (L-Alanine)	248.63	0.253	71100.77	0.733	2265.94	0.281
E04 (L-Arginine)	362.85	0.001	67963.15	0.373	4004.78	0.157

E05 (L-Aspartic Acid)	382.46	0.001	79778.60	0.035	3614.57	0.021
E06 (L-Glutamic Acid)	258.77	0.001	308578.90	0.317	2071.18	0.325
E07 (L-Histidine)	393.66	0.001	172477.40	0.961	2656.39	0.001
E08 (L-Pyroglutamic Acid)	366.45	0.001	41400.99	0.119	3376.36	0.001
E09 (L-Serine)	223.76	0.001	6341.34	0.001	2930.57	0.007
E10 (Lincomycin)	277.03	0.321	177827.40	0.193	1248.20	0.615
E11 (Guanidine Hydrochloride)	239.04	0.003	33856.21	0.147	1177.64	0.761
E12 (Niaproof 4)	289.81	0.085	176043.60	0.681	1558.95	0.291
F01 (Pectin)	77.87	0.281	11758.63	0.577	1165.23	0.919
F02 (D-Galacturonic Acid)	89.46	0.005	12677.30	0.221	1333.05	0.741
F03 (L-Galactonic Acid Lactone)	105.77	0.013	9237.20	0.975	1055.15	0.221
F04 (D-Gluconic Acid)	154.62	0.001	56617.13	0.155	1686.95	0.001
F05 (D-Glucuronic Acid)	101.95	0.005	21412.43	0.489	1069.58	0.459
F06 (Glucuronamide)	217.97	0.001	39053.45	0.381	923.17	0.463
F07 (Mucic Acid)	249.75	0.005	17578.96	0.029	2224.27	0.099
F08 (Quinic Acid)	317.43	0.001	22293.24	0.015	1791.77	0.017
F09 (D-Saccharic Acid)	230.75	0.001	16331.50	0.005	2022.18	0.031
F10 (Vancomycin)	318.06	0.015	89492.07	0.385	1057.48	0.035
F11 (Tetrazolium Violet)	370.76	0.005	179669.80	0.507	622.94	0.937
F12 (Tetrazolium Blue)	272.47	0.047	65037.04	0.457	719.30	0.487
G01 (p-Hydroxy-Phenylacetic Acid)	133.09	0.439	5991.03	0.963	1278.90	0.587
G02 (Methyl Pyruvate)	184.22	0.589	23506.26	0.875	2541.47	0.181
G03 (D-Lactic Acid Methyl Ester)	98.45	0.023	11237.46	0.973	2500.11	0.621
G04 (L-Lactic Acid)	352.85	0.001	38849.88	0.145	3119.94	0.001
G05 (Citric Acid)	403.00	0.001	70863.43	0.001	2800.04	0.005
G06 (α-Keto-Glutaric Acid)	311.18	0.001	78199.33	0.779	3747.05	0.237
G07 (D-Malic Acid)	248.19	0.001	11623.63	0.143	4436.38	0.023
G08 (L-Malic Acid)	268.85	0.001	59707.76	0.075	3045.67	0.755
G09 (Bromo-Succinic Acid)	256.21	0.015	23820.62	0.065	2851.38	0.087
G10 (Nalidixic Acid)	150.74	0.811	6761.30	0.709	215.17	0.305
G11 (Lithium Chloride)	282.54	0.107	26926.41	0.243	1492.62	0.395
G12 (Potassium Tellurite)	345.00	0.019	210691.30	0.465	4499.09	0.467
H01 (Tween 40)	257.23	0.009	38039.03	0.049	1989.91	0.243
H02 (γ-Amino-Butyric Acid)	356.04	0.001	106841.60	0.023	3567.37	0.009
H03 (α-Hydroxy-Butyric Acid)	212.93	0.001	15148.66	0.055	3409.85	0.037
H04 (β-Hydroxy-D,L-Butyric Acid)	271.19	0.001	73156.18	0.201	2725.43	0.847
H05 (α-Keto-Butyric Acid)	208.54	0.001	10966.80	0.143	2610.00	0.911
H06 (Acetoacetic Acid)	192.60	0.481	37641.18	0.545	2488.16	0.623
H07 (Propionic Acid)	188.13	0.881	27281.18	0.979	2669.72	0.007
H08 (Acetic Acid)	333.91	0.005	196997.10	0.485	1733.24	0.029
H09 (Formic Acid)	96.86	0.005	8881.37	0.401	1542.95	0.017
H10 (Aztreonam)	204.41	0.169	13654.42	0.647	389.39	0.211
H11 (Sodium Butyrate)	229.97	0.737	25266.44	0.269	2915.60	0.117
H12 (Sodium Bromate)	175.65	0.193	12999.88	0.357	2858.58	0.005

Table S5.11: Comparison of model fitting results for carbon source assimilation and chemical sensitivity assays.

Kinetic parameter	GENIII assays	Evolutionary model[a]					
		White noise	Lambda	Kappa	Delta	Trend	None
AUC	Carbon source assimilation	20 (28.2 %)	11 (15.5 %)	24 (33.8 %)	8 (11.3 %)	0 (0 %)	8 (11.3 %)
	Chemical sensitivity	15 (65.2 %)	2 (8.7 %)	5 (21.7 %)	0 (0 %)	0 (0 %)	1 (4.3 %)
	Overall	35 (37.2 %)	13 (13.8 %)	29 (30.9 %)	8 (8.5 %)	0 (0 %)	9 (9.6 %)
μ_{max}	Carbon source assimilation	40 (56.3 %)	19 (26.8 %)	0 (0 %)	3 (4.2 %)	1 (1.4 %)	8 (11.3 %)
	Chemical sensitivity	15 (65.2 %)	1 (4.3 %)	1 (4.3 %)	3 (13 %)	0 (0 %)	3 (13 %)
	Overall	55 (58.5 %)	20 (21.3 %)	1 (1.1 %)	6 (6.4 %)	1 (1.1 %)	11 (11.7 %)
lag time	Carbon source assimilation	48 (67.6 %)	17 (23.9 %)	2 (2.8 %)	0 (0 %)	0 (0 %)	4 (5.6 %)
	Chemical sensitivity	17 (73.9 %)	5 (21.7 %)	0 (0 %)	0 (0 %)	0 (0 %)	1 (4.3 %)
	Overall	65 (69.1 %)	22 (23.4 %)	2 (2.1 %)	0 (0 %)	0 (0 %)	5 (5.3 %)

[a] Number (and percentage) of traits for which each evolutionary model was selected as the best fit to the data.

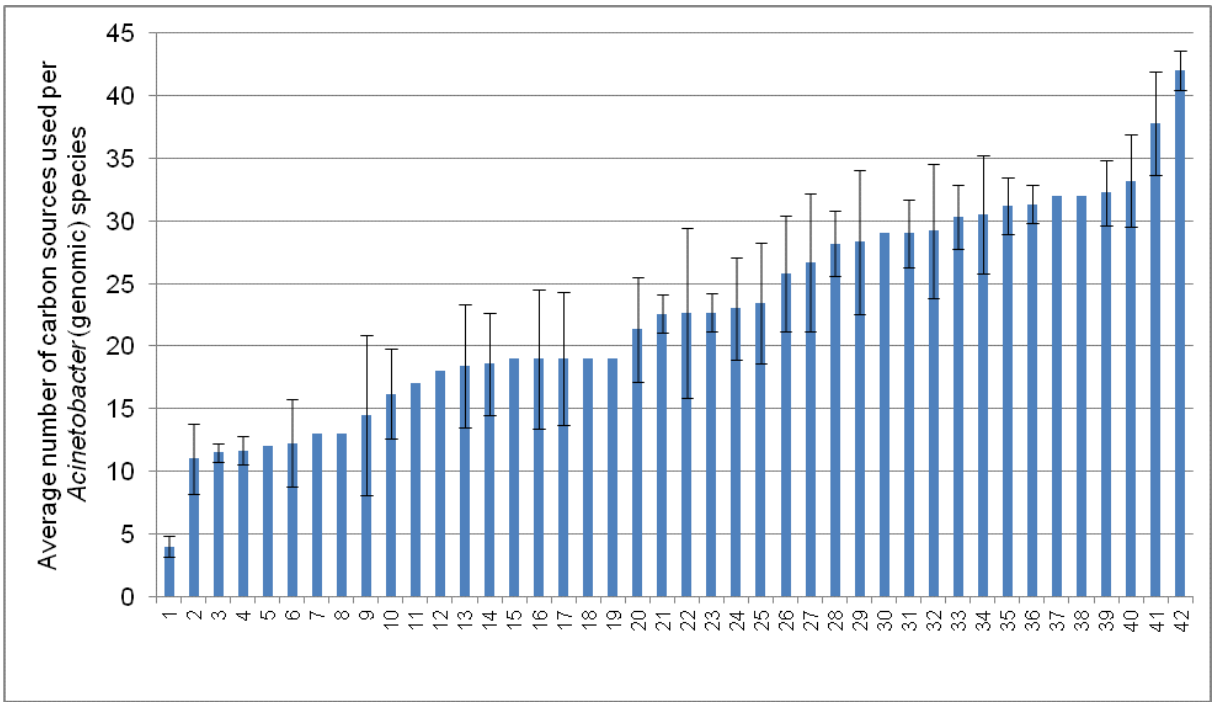


Figure S5.1. Average number of carbon sources used per *Acinetobacter* species. For each *Acinetobacter* species studied the number of the common carbon sources used by all strains within the species is indicated as well as the number of tested strains (*n*). Error bars represent standard deviation.
Acinetobacter species or genomic species: 1, *A. parvus* (3, *n* = 3); 2, *A. tjernbergiae*, (9, *n* = 2); 3, 'genomic species NB28', (9, *n* = 2); 4, *A. variabilis*, (8, *n* = 3); 5, *A. bohemicus*, (12, *n* = 1); 6, *A. towneri*, (4, *n* = 4), 7, 'genomic species NB33', (13, *n* = 1); 8, 'genomic species NB21', (13, *n* = 1); 9, *A. lwoffii*, (9, *n* = 2); 10, *A. ursingii*, (11, *n* = 5); 11, *A. bouvetii*, (17, *n* = 1); 12, *A. grimonti* (*A. junii*), (18, *n* = 1); 13, *A. indicus*, (10, *n* = 5); 14, *A. kookii*, (9, *n* = 5); 15, 'genomic species NB54', (19, *n* = 1); 16, *A. radioresistens*, (12, *n* = 3); 17, *A. schindleri*, (10, *n* = 3); 18, *A. brisouii*, (19, *n* = 1); 19, 'genomic species NB4', (19, *n* = 1); 20, *A. gandensis*, (12, *n* = 3); 21, *A. gyllenbergii*, (18, *n* = 5); 22, *A. junii*, (13, *n* = 3); 23, *A. johnsonii*, (16, *n* = 3); 24, *A. beijerinckii*, (18, *n* = 5); 25, *A. venetianus*, (16, *n* = 5); 26, *A. guillouiae*, (20, *n* = 5); 27, 'genomic species 6', (21, *n* = 3); 28, *A. bereziniae*, (20, *n* = 5); 29, *A. haemolyticus*, (24, *n* = 3); 30, *A. gerneri*, (*n* = 1); 31, *A. nosocomialis*, (22, *n* = 5); 32, *A. calcoaceticus*, (16, *n* = 5); 33, *A. tandoii*, (27, *n* = 3); 34, *A. dijkshoorniae* ('genomic species NB14'), (26, *n* = 4); 35, *A. baumannii*, (26, *n* = 5); 36, *A. calcoaceticus*-like ('genomic species between 1 and 3'), (27, *n* = 3); 37, '*A. oleivorans*', (32, *n* = 1); 38, 'genomic species NB53', (32, *n* = 1); 39, *A. seifertii*, (27, *n* = 4); 40, *A. pittii*, (25, *n* = 5); 41, *A. baylyi*, (28, *n* = 5); 42, *A. soli*, (38, *n* = 5).

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