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MASTERARBEIT

Titel der Masterarbeit

„Influence of dietary folate uptake on the metabolic profile and gene expression of the model organism *Caenorhabditis elegans* “

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1 Introduction

In 1965, the nematode *C. elegans* was introduced as a model organism in natural science when Sydney Brenner (*1927) investigated the development of its organs and nervous system. The first genes playing a role within the process of apoptosis could be discovered in the nematode, which contributed to the clarification of the whole genetic code of *C. elegans*. [THE *C. elegans* SEQUENCING CONSORTIUM, 1998] With their achievements, Brenner and his colleagues Horvitz and Sulston earned one of the two Noble Prizes in Medicine won with investigations of the nematode *C. elegans* with their "*discoveries concerning 'genetic regulation of organ development and programmed cell death"* in 2002. The second Nobel Prize was won by Fire and Mello '*for their discovery of RNA interference- gene silencing by double-stranded RNA'* in 2006. So far, the third and last Noble Prize (in chemistry) earned through research on the nematode could be achieved by Shimomura, Chalfie and Tsien '*for their discovery and development of the green fluorescent protein, GFP'* in 2008.

C. elegans is a popular organism in natural science. The nematode is a small, free-living organism which uses bacteria as a food source and can be found in the soil of areas with temperate climate [ABADA et al., 2009]. The size of only 1 mm and the enormous number of approximately 300 progeny allow the easy cultivation of the nematode under laboratory conditions as soon as an adequate amount of food (mostly *E. coli* bacteria), a constant temperature of 20°C and either agar plates or liquid media as a space to live can be offered. Furthermore, the nematode can be stored at -180°C over a long time [RIDDL et al., 1997; BRENNER, 1974]. The lucent body of the roundworm [RIDDL et al., 1997] is interesting for studies focusing on its simple body plan [WOOD et al., 1988], the digestive system as well as the development of cells. The rapid life cycle, the small genome size (about 100 million bp), the constant number of cells and the reproducibility of its development allow investigations of genetical studies in a reasonable amount of time [RIDDL et al., 1997]. The two sexes, XO (male) and XX (hermaphrodite), allow the selective breeding of a genetically identical population through the self-fertilization of the hermaphrodites. Male mating

enables the transfer of mutations among different strains as well as the preservation and isolation of mutant strains [WORMATLAS]. On the electron microscopy level, the anatomy of *C. elegans* is completely characterized [WHITE et al., 1988]. In 1998, *C. elegans* became the first completely sequenced multicellular animal [THE *C. elegans* SEQUENCING CONSORTIUM, 1998]. Last but not least, the nematode owns a nervous system which is small enough to enable researchers to understand the role of every neuron within a given behavior. Thereby, researchers were able to comprehend how genes conduce to behavior in general [RANKIN, 2002].

2 General information and literature survey

The following pages are supposed to provide an introduction into the nematode *C. elegans*. The scientific classification, the life cycle as well as the anatomy of the nematode is given as general information. A summary of the current literature is prepared as a literature survey.

2.1 General information

Scientific classification:

Kingdom: Animalia

Phylum: Nematoda

Class: Secernentea

Order: Rhabditida

Family: Rhabditidae

Genus: Caenorhabditis

Specie: *C. elegans*

[UNIVERSITY OF MICHIGAN MUSEUM OF ZOOLOGY]

Figure 1: Picture of *C. elegans*
[Boyce Thompson Institute and Department of Chemistry and Chemical Biology]

The name *Caenorhabditis elegans* is a combination of Grecian and Latin words:

Grecian: Caeno = new, recent
Grecian: Rhabditis = rod
Latin: Elegans = nice [RIDDL et al., 1997]

The *C. elegans* life cycle:

The life cycle (**Figure 2**) of the nematode *C. elegans* is divided into different steps. It starts with adult nematodes that are able to lay eggs. At the end of the embryonic development, L1 larvae hatch with a size of about 250 µm. If there is no food available at this point, the larvae arrest in this stage. This occurrence can be useful as soon as an additional age synchronization of a population is

needed for further analysis. If the conditions stay unfavorable, e.g. the temperature is too high, the food supply too low or the area around each larva is too crowded, L1 larvae become predauer larvae (also called L2d) that turn into dauer larvae within the next 13 hours. In this stage, the nematodes can survive up to four months until better conditions can be offered. Under normal circumstances, L1 larvae develop into the L2 larval stage within twelve hours (**Figure 2**) [WORMATLAS].

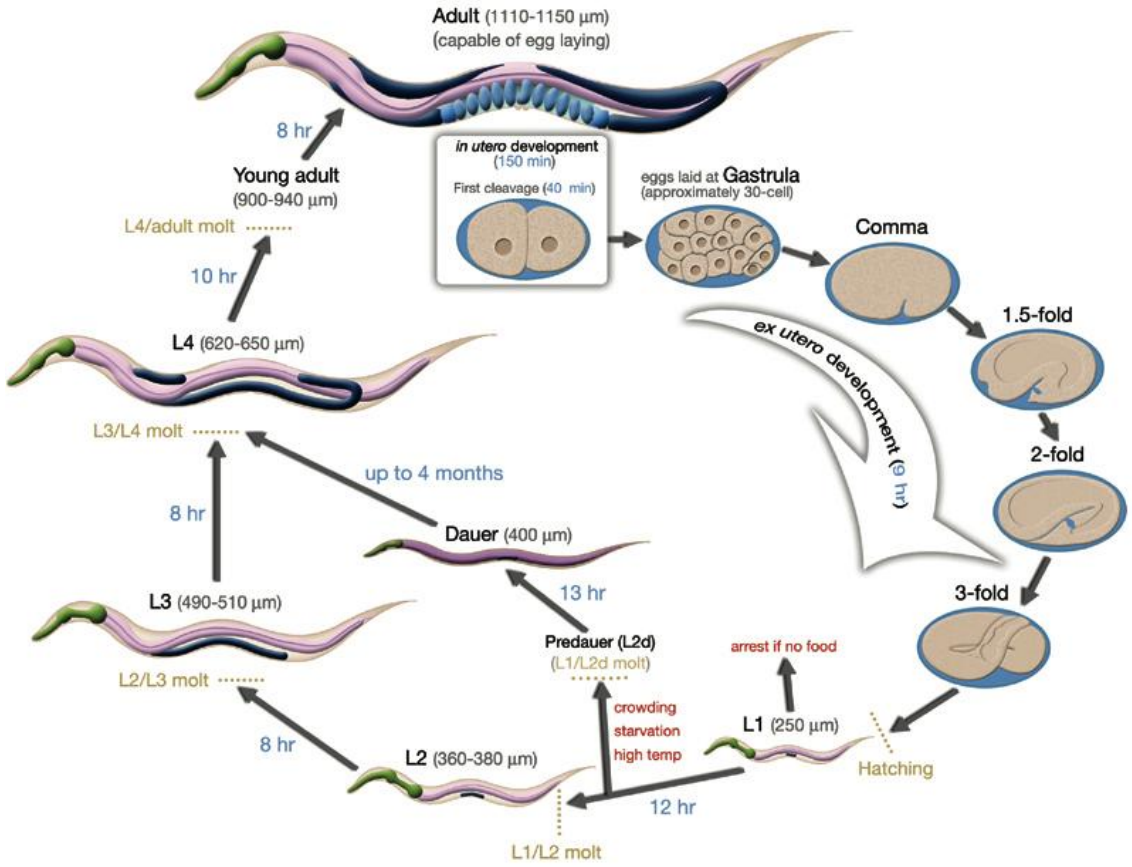


Figure 2: Life cycle of the nematode *C. elegans*. The development of nematodes starts with eggs, laid by adult worms. The following development includes hatching, L1 to L4 if needed dauer larva and young adult stage, which are again able to lay eggs. Each cycle lasts for about 50 hours at 20°C for wild type worms [WORMATLAS].

Between each new stage, the nematodes undergo molting where a new epidermis is synthesized and the old one is rejected. The development from L2 to the L3 larval stage takes about eight hours. Another eight hours are needed to reach the final L4 larval stage. To become young adult nematodes (900-940 µm), the L4 larvae need about ten hours before they reach this stage. The adult size of 1110-1150 µm is completed within the next eight hours. Therefore,

in summary, it takes about 50 hours after hatching until a newly grown wild type nematode reaches adulthood, lays its first own eggs and a new cycle begins [WORMATLAS].

Anatomy:

There is no major difference in the basic body plan of all nematodes: two concentrical tubes (inner tube and body wall (outer tube)) are divided by the pseudocoelom, a space filled with fluid. The cuticle, hypodermis, neurons, muscles and excretory system belong to the outer tube. The inner tube includes pharynx, intestine and gonads. The round shape is sustained through the internal hydrostatic pressure which is controlled through an osmoregulatory system. [RIDDL et al., 1997; WORMATLAS]

As already mentioned above, each nematode has to pass through four larval stages before it reaches adulthood. **Figure 3** shows the different sizes and appearances of all larval stages including the thinner dauer larvae.

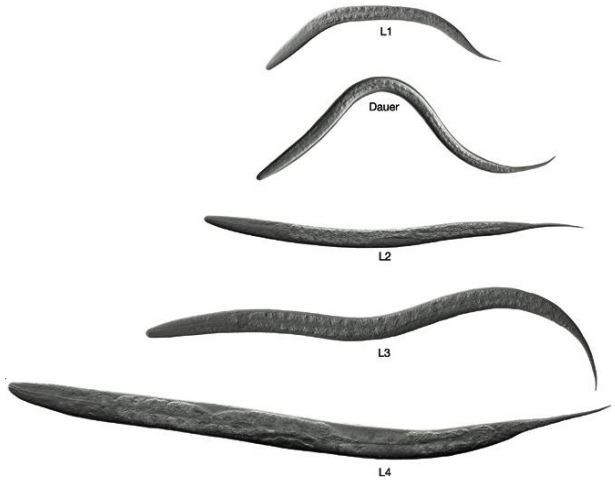
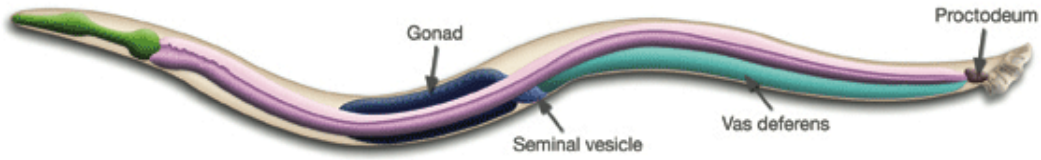


Figure 3: Physical appearance of all larvae. Different size and appearance of all larval stages (L1 to L4) including dauer larvae [modified from WORMATLAS]

With each molting between the different larval stages the nematodes grow for about one third of their own body size [RIDDL et al., 1997]. **Figure 4** shows schematically the differences between both sexes of the worm. The pharynx, excretory system and the main body muscles can be observed in hermaphrodites and males respectively. The gonads as well as the uterus and

vulva belong to the most observable distinctions. In contrast to those of the hermaphrodites, male gonads are clearly white and no uterus or vulva can be found [WORMATLAS].

A.



B.

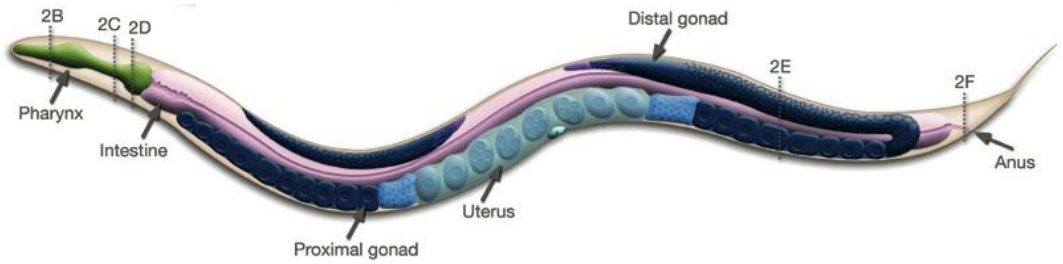


Figure 4: Schematic representation of the anatomy of an adult male and hermaphrodite nematode. A. Representation of an adult male nematode with gonads, seminal vesicle, vas deferens and proctodeum. Also visible: the different tail which is needed for the mating process **B.** Representation of an adult hermaphrodite nematode with the pharynx, intestine, proximal gonads, uterus, distal gonads and the anus. [WORMATLAS]

A hermaphrodite body contains always 959 somatic cells. After hatching, they need three days to be able to fertilize themselves. While they produce oocytes over the following four days, they pass through their fertile period. Subsequently, they live on for about 10-15 more days until they finally die. Due to the limited number of sperms within their own gonads, the number of progeny through self-fertilization is restricted to about 300 [HODGKIN, 1988; WOOD, 1988]. Through male mating this number can be expanded up to 1200-1400. Males are able to mate for six days after their last molt. The expected number of progeny fathered by only one male is about 3000 [HODGKIN, 1988]. The percentage of males within a population of *C. elegans* is normally about 0.1 and occurs because of spontaneous non-disjunction within the process of self-fertilization in the hermaphrodite germ line. Though male mating, this percentage can rise up to 50 % [WORMATLAS].

2.2 Literature survey

The following literature survey gives an overview of the current opinion about folic acid, its metabolism, *C. elegans* and folic acid, studies in which different substances have already been tested on the nematode as well as information on what is already known about metabolomics and *C. elegans.*

2.2.1 Folic acid metabolism

Folic acid, a water-soluble vitamin out of the vitamin B complex, acts as a cofactor and is part of different intracellular reactions. The cellular availability is closely regulated and well protected. Most of the folate within an organism is tightly bound to enzymes. Through the first pass metabolism, the billiary secretion, the enterohepatic recirculation as well as the recycling of aging erythrocytes, the liver is in control of the supply maintenance. A folate deficiency can be caused through an increased turn over, a decreased intake, an increased requirement or through genetic defects. Polymorphisms in different genes associated with the folate pathway have been detected and they are thought to be connected with diseases which are linked to a mild folate deficiency [reviewed by DONNELLY, 2001].

On the cellular level, three different systems are responsible for the folate uptake in mammals: The folate receptors (FOLR), the proton-coupled folate transporter (PCFT, SLC46A1) and the reduced folate carrier (FOLT, SLC19A1) which is an essential transporter in mammalian cells and possesses a major role in the growth of cells and their development [AUSTIN et al., 2010].

Once inside a cell, folic acid is reduced to dihydrofolate (DHF) with the help of the enzyme dihydrofolate reductase (DHFR) before tetrahydrofolate (THF) is formed. THF becomes 5,10-methylene-tetrahydrofolate which is processed into 5-methyl-THF by the enzyme methylene-THF-reductase (MTHFR). With the help of the methionine synthase (MS), 5-methyl-THF can then be used to remethylate homocysteine (HC) into methionine, which is needed for DNA-methylation. Once again, within this step tetrahydrofolate (THF) is produced (**Figure 16**, p. 56) [ZHAO et al., 2009].

In organisms, folates regulate the DNA synthesis, its repair and methylation and therefore maintain DNA stability. Folates donate one carbon units for the cellular metabolism. So far, deficiencies have been connected with the progression of diverse cancers (e.g. breast, ovary, pancreas, brain, and colon); whereas in his review Duthie could also find out that an excessive intake of synthetic folic acid, for example through fortified food or supplementation, is also able to increase human cancer through the increasing growth of precancerous lesions [reviewed by DUTHIE, 2011].

2.2.2 Folate metabolism and diseases

Tumor cells are able to proliferate directly to the available folic acid. This could be shown by Tomaszewski et al. who investigated the serum folate concentrations of men suffering prostate cancer. They found out that the highest proliferation rate is in men possessing the highest folate concentration [TOMASZEWSKI et al., 2011].

Because of synergistic effects, carriers of polymorphisms of the methylenetetrahydrofolate reductase (MTHFR), methylenetetrahydrofolate homocysteine methyltransferase (MTR) and the 5-methylenetetrahydrofolate homocysteine methyltranferase reductase (MTRR) are at higher risk to develop Parkinson’s disease (PD) [FONG et al., 2011].

There is an association of a severe deficiency of the enzyme methylenetetrahydrofolate reductase (MTHFR) and neurological manifestations. The deficiency of the key enzyme within the folic acid metabolism is thought to be essential for the diagnosis of infantile epileptic encephalitis [PRASAD, 2011].

A homozygous mutation of the enzyme dihydrofolate reductase (DHFR), called *p.Asp153Val*, causes a DHFR deficiency. The consequence of that is a complex, hematological and neurological disease which is treatable with folinic acid supplementation [CAIRO et al., 2011].

2.2.3 Folic acid metabolism and *C. elegans*

So far, there is not much literature giving information about *C. elegans* together with the folic acid metabolism within the nematode. Not long ago, Austin et al. have investigated the effects of a knockout of the folate transporter *folt-1* in *C. elegans*. *Folt-1* is orthologous to the human reduced folate carrier (RFC) and the FOLT-1 protein is responsible for the folate transport. Furthermore, the protein is also part of the exogenous folate uptake system. Due to a significant reduction of the germline, a reduced number of the produced sperm and the generation of only a few functional oocytes, hermaphrodite nematodes with a knockout mutation in *folt-1* become sterile. Although male nematodes stay fertile after the knockout, their spermatogenesis is reduced. Therefore, mutated males become poor maters [AUSTIN et al., 2010]. Another paper which deals with *C. elegans* and folic acid is published by Balamurugan et al. They focused on the functional characterization and cloning of a folate transporter in *C. elegans*. They identified the existence of a specialized folate uptake system (FOLT-1) in the nematode. They found out that this system seems to be working like the folate uptake process in human intestine. Furthermore, they stated that there is 40 % similarity between the human RFC and FOLT-1 found in *C. elegans*. The highest expression of *folt-1* in the nematode could be found in the pharynx and the intestine. A knock-down or a knock-out of this transporter results in a significant inhibition of the folate uptake in healthy nematodes [BALAMURUGAN et al., 2007].

Y4C6B.5, as well as the other investigated genes of the nematode *C. elegans*, is mostly unknown in current literature. BLASTP results suggest that the Y4C6B.5 gene is homologous to the human PCFT (proton-coupled folate transporter). DHFR-1 is thought to be homologues to the dihydrofolat reductase (DHFR) within mammals and MTHF-1 is supposed to be similar to the human methylentetrahydrofolat reductase (MTHFR) [WORMBASE]. It was shown previously that a gene deletion of *mthfr-1* leads to a lethal phenotype. [MAYDAN et al., 2007]. For METR-1, BLASTP results suggest a high similarity to the human methionine synthase (MS) [WORMBASE].

2.2.4 Supplementation studies with the nematode *C. elegans*

Previous studies within our working group focused on the effect of a folic acid supplementation on life span and brood size of the nematode *C. elegans*. This thesis is additionally interested in how a supplementation affects the worm, the following is supposed to provide an overview of what other groups have so far already focused on and found out.

Since his introduction to natural science, the nematode *C. elegans* has already been subject of numerous investigations. The influence of various substances on its behavior, growth, fertility, development, memory, life span and so on has already been tested and the results often contributed to the knowledge of research areas worth of further interest. The following is an excerpt of examples of substances which have yet been tested on the nematode *C. elegans*.

Cinnamomun cassia bark, ingredient of *Shi Quand Da Bu* (SQDB) and *Huo Luo Xiao Ling Dan* (HLXL), two commonly used Chinese traditional herbal formulas, were able to extend *C. elegans'* life span significantly when added to the agar plates [YU et al., 2010].

Garlic has already been famous for its beneficial effects on the human cardiovascular system. Powolny et al. demonstrated recently that diallyl trisulfide (DATS), given on top of a NGM agar plate, have favorable effects on *C. elegans*' life span by affecting the *skn-1* pathway [POWOLNY et al., 2011].

Curcumin, given as a supplement to the nematodes, is able to reduce the pathogenicity of the bacteria *pseudomonas aeroginosa* (POA1). This was discovered by measuring relevant virulence factors [RUDRAPPA and BAIS, 2008].

Iron exposure can dose-dependently cause multiple biological defects in *C. elegans* which can be passed on to the progeny [HU et al., 2008].

Vitamin E has a remarkable effect on memory behavior in the nematode *C. elegans*. High concentrations (400 µg/ml) improved the memory deficits in worms caused through a previously exposure to metal (e.g. Pb) or ultra

radiation. In both cases, vitamin E could also be proven as a preventive acting substance [YE et al., 2008].

The effect of the addition of polyunsaturated fatty acids (PUFA) into the agar of nematodes was tested by Kahn-Kirby et al. The results with strains being defective in the PUFA synthesis suggest that some PUFAs act like endogenous modulators [KAHN-KIRBY et al., 2004].

The addition of 0.01 µM or 0.05 µM selenite to the NGM-agar precipitates development and leads to a rise in the brood size. The nematodes were also more resistant to a paralysis through an acetylcholine inhibitor. In contrast to that, an addition of 20 µM retarded the development, lowered the brood size and made the worms more sensitive towards paralysis [LI et al., 2011].

Oxaloacetat is able to extend the life span of nematodes whereas glycerol showed the opposite effect. Both substances act like antagonists within the formation of methylglyoxal, a substance which is supposed to at least be partly responsible for the age-related proteotoxicity as well as organelle, molecular and cellular dysfunction. Oxaloacetat lowers the formation of methylglyoxal which leads to the extension in the life span [HIPPKISS, 2010].

C. elegans nematodes are killed when instead of the normal food source (*E. coli*) *Cryptococcus neoformans*, bacteria which are also pathogen for humans, are given as food. This was tested in 2002 by Mylonakis [MYLONAKIS, 2002].

The anticarcinogenic and antiobesity effects of epigallocatechin gallate (EGCG) for humans have already been stated. In nematodes under stress, experiments could show an extension of their longevity. This effect can be attributed to the free radical-scavenging effect of EGCG as well as to the upregulated stress-resistance-related and heat shock protein. No effects could be shown in nematodes living under normal conditions [ZHANG et al., 2009].

Behavioral effects as well as a dose-dependent stimulation of egg lying can be observed when clozapine, an antipsychotic medication for patients suffering schizophrenia, is given to *C. elegans* nematodes [KARMACHARYA, 2011].

Royal jelly could be proven to extend the life span of *C. elegans* when added into the agar before pouring the plates [HONDA et al., 2011].

A supplementation of *C. elegans* nematodes with calcium and/or cadmium led to a shorter life span and an acceleration of aging related declines [WANG et al., 2010].

2.2.5 Metabolomics and *C. elegans*

Metabolomics, also called metabonomics, is the analysis of metabolites in cells, fluids or tissues with a low (< 1000 Da) molecular weight. This can be used in order to identify the interdependency of an organism from its environment. The investigation of the conformation of metabolites within the investigated sample allows the explanation of the reactions an organism shows towards a disease, toxicity, supplementation and depletion of substances or genetical manipulation [VIANT, 2008]. Metabolomics focuses on the quantification of all metabolites within a cellular system [GOODACRE, 2007]. To be able to maximize the quantity of metabolites, different analytical techniques can be used. HNMR Spectroscopy, GC-MS, LC-MS, FT (Fourier Transform) – MS, HPLC, EC (electrochemical) – array have the capability to be such techniques [GRIFFIN, 2006]. The combination of mass spectroscopy and liquid chromatography (LC-MS) is one of the key techniques in analytics. It produces not only structural and quantitative data, but can also be employed in non-targeted as well as in targeted way. Furthermore, LC-MS is able to recognize metabolites at a level as low as a few parts-per-billion (ppb) [GRIFFITHS and WANG, 2009]. To get reliable and reproducible results the extraction of the tissue is supposed to be of high quality. Otherwise it is not possible to successfully study the metabolome of any organism. The comparison of two different extraction techniques, chloroform/methanol versus aqueous methanol, as well as different tissue disrupting techniques such as manual grinding in a cooled mortar,

homogenization and grinding media in a reciprocation and orbital tissue mill showed that both, extraction and disrupting technique, have an impact on the results, whereas the selection of the solvent is more important [GEIER et al., 2011]. As good as the opportunities of mass-spectrometry-based metabolomics may be, general applicable and standardized procedures or methods are still receivable. Furthermore, there are still problems to characterize the influence of a nutritional intervention to metabolites [SCALBERT et al., 2009].

Metabolomic investigations provide a successful opportunity to comprehend and quantify the physiological impact of a diet towards an illness. Because the metabolome is very sensitive to changes in the diet, lifestyle and genetic changes and therefore, it is also sensitive to changes of the experimental conditions, *C. elegans* is suited for being an optimal model organism. Environmental factors as well as all genetic factors can easily be controlled which make this nematode so attractive for researchers within this field [REINKE et al., 2010].

In their investigations, Atherton et al. focused on changes within the metabolic profile of the nematode *C. elegans* due to the deletion of the nuclear hormone receptor-49 (*nhr-49*). The measurements were performed with HNMR spectroscopy and GC-MS. The deletion of the receptor in the nematodes led to a higher ratio of unsaturated versus saturated fatty acids, a lower concentration of glucose as well as a higher amount of lactate and alanine. NHR-49 is supposed to have a high similarity to the mammalian peroxisome proliferation-activated receptors (PPARs) and therefore, the resulting metabolome was compared to the one of a PPAR-α null mouse. With the help of metabolomics, Atherton et al. were able to find out that there are commonalities in the regulation of the lipid synthesis, β-oxidation of fatty acids and changes in glycolysis and gluconeogenesis [ATHERTON et al., 2008]. Other studies investigated the influence of different diets [REINKE et al., 2010], different genetic backgrounds [FUCHS et al., 2010] and different exposure to cadmium [HUGHES et al., 2009] on the metabolic profile.

2.3 Aim of the thesis

The fact that only little is known about the folate metabolism in *C. elegans* together with the general interest in folic acid within our working group were the origin of this work which was divided into two parts.

The first project within this work deals with the establishment of a method to measure the metabolic profile of *C. elegans* via HPLC-MS. In order to validate the method, starving and non-starving nematodes were used and initial results regarding the metabolic profile of nematodes with a folic acid deficiency and supplementation could be reached. There are many publications which focus on the metabolic profile performed on the model organism *C. elegans*. Nevertheless, none could be found which deals with alterations in the profile due to a folic acid supplementation and none uses HPLC-MS for performing metabolomics.

The second project focuses on the influence of two different ways to induce a folate deficiency and their effect on the gene expression of five selected target genes within the folate metabolism in the nematode *C. elegans.* Another interest in this part was to find out what effect a folic acid supplementation would show as well as if the previously caused deficiency might be diminished through it. This and the reasons named above gave the input to do this thesis.

3 Material and Methods

3.1 Material

3.1.1 Instruments

Agilent Technologies, Vienna (Austria); **Ambion the RNA Company**, Austin (USA); **Applied Biosystems**, Carlsbad (USA); **Austr. Alco Österreich**, Spillern (Austria); Brucker, Madison (USA); **CERTOclav**, Traun (Austria); **CGC** (Caenorhabditis Genetics Center), Minneapolis (USA); **Dionex**, Germering (Germany); **Eppendorf AG**, Hamburg (Germany); **Fluka Analytical**, Munic (Germany); **Fortis Technologies Ldt**., Cheshire (United Kingdom); **Gesellschaft für Labortechnik GmbH**, Burgwedel (Germany); **Grant-bio**, Hillsborough (USA); **Greiner bio-one**, Frickenhausen (Germany); **IKA Werke** GmbH und Co. KG, Staufen (Germany); **Mettler Toledo GmbH**, Giessen (Germany); **Peqlab**, Erlangen (Germany); **Quiagen,** Hilden (Germany); **Roth**, Karlsruhe (Germany); **Sarstedt**, Nümbrecht (Germany); **Satorius AG**, Goettingen (Germany); **Stuart**, Staffordshire (United Kingdom); Sigma- Aldrich, Munic (Germany); **Thermo Electron Corperation Key**, Bonn (Germany); **Wild Heerbrugg**, Gais (Switzerland)

Table 1: Instruments

Name	**Description**	**Company**
Autoclave	Table autoclave	CERTOclav
Centrifuge	Jouan BR4i multifunction centrifuge	Thermo Electron Corporation Key Write DTM
Column	Fortis Phenyl, ϕ3µm, 150x2.1mm	Fortis Technologies Ldt.
Concentrator	Eppendorf Concentrator	Eppendorf AG
Guard column	Fortis Phenyl, ϕ3µm	Fortis Technologies Ldt.
Heating block	block heater SBH200D	Stuart
HPLC	UltiMate 3000	Dionex
Incubator	Thermomixer 5436	Eppendorf AG
Magnetic stirrer	RCT basic	IKA Werke GmbH und Co. KG
Mass Sprectrometer	micrOToF- QII	Brucker
Microscope	M3C Switzerland	Wild Heerbrugg

NanoDrop	ND-1000 Spectrophotometer	PeqLab
PCR	7300 Real Time PCR System	Applied Biosystems
pH- meter	Seven Easy TM, pH-Meter S20 with Inlab Routine per glass electrode	Mettler Toledo GmbH
PicoDrop	PicoDrop	Biozym
Pipettes	Eppendorf Reference variable (10µl, 100µl and 1000µl)	Eppendorf AG
Scale	Satorius ISO 9001 Modell KB BA 100	Satorius AG
Shaker	PMR- 30	Grant- bio
Vortex	MS 1 Minishaker	IKA Werke GmbH und Co. KG
Water quench	GFL- Inkubations-/ Inaktivierungsbad 1002	Gesellschaft für Labortechnik GmbH

3.1.2 Consumable items

Table 2: Consumable items

Name	**Company**
Falcons	Sarstedt
Glass beads (ϕ 325-600µm)	Sigma- Aldrich
HPLC veil	Agilent Technologies
Petri dish	Greiner bio-one
Pipette tip	Greiner bio-one
qPCR stripes	Peqlab
Reaction tubes	Greiner bio-one
Reaction tubes (HPLC-MS)	Eppendorf
RnaseZap	Ambion the RNA company

3.1.3 Chemicals

Table 3: Chemicals

Name	**Company**
Acetonitrile	Roth
DEPC H_2O	Ambion INC.
Ethanol	Austr. Alco Österreich
Glucose (20 %)	Roth
Kanamycine (3 mg/ml)	Roth
Mercaptoethanol	Sigma-Aldrich
Methanol	Roth
Sulfamethoxazole stock solution (1 mM)	Fluka
Thymine (5 mg/ml)	Sigma-Aldrich

3.1.4 Kits and primer

Table 4: Kits

Name	**Use**	**Company**
Gene expression Assay Mix	PCR	Applied Biosystems
QuantiTect Reverse Transcription Kit	cDNA transcription	Qiagen
Rneasy MiniKit (50)	RNA extraction	Qiagen
TaqMan universal Master Mix	PCR	Applied Biosystems

Table 5: Primer

Cosmid number	**Gene name**	**Function/ Assay ID Bestellnumer von Applied**	**Reference or target gene**	**Company**
C06A8.1		Methylenetetrahydrofolate reductase (NADPH) activity/ Ce02406138_g1	Target gene	Applied Biosystems
C06H2.4	*folt-1*	Reduced folate carrier activity/ Ce02481435_g1	Target gene	Applied Biosystems
C36B1.7	*dhfr1*	Dihydrofolate Reductase/ Ce02411259_g1	Target gene	Applied Biosystems

C54G10.3	*pmp3*	Acyl-CoA transporter/ Ce02485188_m1	Reference gene	Applied Biosystems
F20H11.3	*mdh-2*	Malatdehydrogenase/ Ce02448105_g1	Reference gene	Applied Biosystems
F40E10.3	*csq-1*	Calcium ion binding protein/ Ce02402596_m1	Reference gene	Applied Biosystems
R03D7.1		Cobalamin binding, homocysteine S-methyltransferase activity, metal ion binding, methionine synthase activity, zinc ion binding/ Ce02438802_g1	Target gene	Applied Biosystems
Y45F10D.4		Iron binding protein; involved in Fe- S cluster formation/ Ce02467252_g1	Reference gene	Applied Biosystems
Y4C6B.5		Predicted proton-coupled folate transporter/ Ce02463760_g1	Target gene	Applied Biosystems

3.1.5 Bacteria and Nematodes

Table 6: Bacteria

Bacteria	**Phenotype**	**Description**	**Provided by**
MH828 *E. coli*	thy A (ts) argE3 rna	Arginin auxotroph, thy A deficient 'parents' of MH951	gift from M. Herrington lab
MH951 *E. coli*	thy A (ts) argE3 rna ∆ fol A :: kan3	*fol A*- knocked out (replaced by kan resistence) *E. coli* strain	gift from M. Herrington lab
OP50 *E. coli*	*ura*$^-$	Uracil auxotroph strain of *E. coli*	CGC

Table 7: Nematodes

Name	**Description**	**Genotype**	**Company**
N2 var. Bristol		Wild type	CGC
RB 755	Methionine synthase deficient strain	R03D7.1(ok521) II	CGC

3.1.6 Buffers and media

Table 8: Bleach solution

Chemical	Quantity	Company
5 M KOH	2.0 ml	Roth
NaOCl	3.0 ml	Roth
ad 25 ml dH_2O		

Table 9: DYT-media

Chemical	Quantity	Company
Bacto Yeast Extract	10.0 g	Fluka Analytical
NaCl	5.0 g	Roth
Peptone	16.0 g	Roth
ad 1000 ml dH_2O, autoclave		

Table 10: Folic acid (2mM)

Chemical	Quantity	Company
Folic acid	0.04414 g	Roth
dissolve in 2.5 ml 5 M KOH, ad 50 ml dH_20		

Table 11: LB-media

Chemical	Quantity	Company
LB- Broth	20.0 g	Roth
ad 100 ml dH_2O, autoclave		

Table 12: M9-buffer

Chemical	**Quantity**	**Company**
KH_2PO_4	3.0 g	Roth
Na_2HPO_4	6.0 g	Roth
NaCl	5.0 g	Roth
ad 1000 ml dH_2O, autoclave		
1 M $MgSO_4$	1.0 ml	Roth

Table 13: M9 Minimal medium with glucose

Chemical	**Quantity**	**Company**
1 M $CaCl_2$	50.0 µl	Roth
1 M $MgSO_4$	1.0 ml	Roth
20% Glu- Solution	10.0 ml	
M9- salt solution	100.0 ml	
ad 500ml dH_20, mix until all substances are fully dissolved		

Table 14: M9 salt solution

Chemical	**Quantity**	**Company**
KH_2PO_4	15.0 g	Roth
Na_2HPO_4	64.0 g	Roth
NaCl	2.5 g	Roth
NH_4Cl	5.0 g	Roth
ad 1000 ml dH_2O, autoclave		

Table 15: NGM (nematode growth media)-agar

Chemical	**Quantity**	**Company**
Agar	17.0 g	Roth
NaCl	3.0 g	Roth
Pepton	2.5 g	Roth
ad 1000 ml dH_2O and autoclave		
1 M $CaCl_2$	0.5 ml	Roth
1 M $MgSO_4$	1.0 ml	Roth
Cholesterol (0.5 g/ 100ml)	1.0 ml	Roth
Nystatine	3.0- 4.0 ml	
Potassium- phosphate-buffer	25.0 ml	
add 10 ml Thymine if the agar is used for MH951or MH828 *E. coli* bacteria; in the case of MH951 bacteria kanamycine is needed on each plate (c= 0,03 mg/ml)		

Table 16: Nystatine

Chemical	**Quantity**	**Company**
Solution A:		
Ammoniumacetate	115.62 g	Roth
ad 200 ml dH_2O		
Solution B:		
EtOH (96 % absolut)	200.0 ml	Roth
Nystatine	4.0 g	Roth
add solution A and B slowly and by turns to the Nystatine; use a magnetic stirrer at 50°C		

Table 17: Potassium phosphate buffer

Chemical	**Quantity**	**Company**
K_2HPO_4	35.28 g	Roth
KH_2PO_4	108.6 g	Roth
ad 1000 ml dH_2O, adjust to pH 6.0 with 5 M KOH		

3.2 Methods

3.2.1 General *C. elegans* handling

The following is supposed to give an insight in how the nematodes were cultivated and how the folate deficiency in wild type nematodes was induced. Furthermore, the chosen way in which the worm population was synchronized as well as the supplementation study is shown.

3.2.1.1 Cultivation of *C. elegans*

In these projects two *C. elegans* strains were used. N2 var. Bristol wild type and RB755 a methionine synthase deficient strain. N2 and RB755 were kept on 6 cm agar plates with OP50 *E. coli* as food source. The maintenance consisted of the transfer of about five nematodes once a week onto new plates, which were prepared with the suitable food source the day before their use. The backup line was used as soon as a high number of worms were needed for further analysis.

3.2.1.2 Different feeding of N2 var. Bristol wild type to induce a folate deficiency within the nematodes

Wild type control worms were fed with OP50 *E. coli* on NGM agar plates. To induce folate deficiency they were on the one hand fed with OP50 *E. coli* with inhibited folate biosynthesis and on the other hand with MH828 and MH951 *E. coli* bacteria.

Bacteria with inhibited *de novo* biosynthesis of folic acid

As prokaryotes, bacteria are able to synthesize folic acid, which makes this vitamin non-essential for them. They become dependent on folic acid as soon as their *de novo* biosynthesis is inhibited [ZHAO et al., 2009]. The unavailability of folic acid can be achieved by a minimal media, which contains glucose and various salts to give a source of nitrogen. Sulfamethoxazole (SMX), a sulfonamid, was added to the minimal media in order to inhibit the formation of folic acid within the bacteria. Normally, the enzyme dihydropteridinsynthase combines dihydropteridin and p-amino-benzoic acid (pABA) to form

dihydropteroinacid, which is the first step of the *de novo* biosynthesis of folic acid. SMX competes against pABA (**Figure 5**). Therefore, the higher the concentration of SMX is, the lower is the biosynthesis rate of folic acid and folates. In this thesis, 1 ml OP50 bacteria over night culture grew in 25 ml minimal media with glucose in which either 0 µl (0 µM) or 250 µl (10 µM) SMX were used to induce a folate deficiency in bacteria and further on in the nematodes when they were fed with these bacteria. Previous studies in this group have shown that the addition of 10 µM SMX was sufficient to induce a folate deficiency in worms in the end.

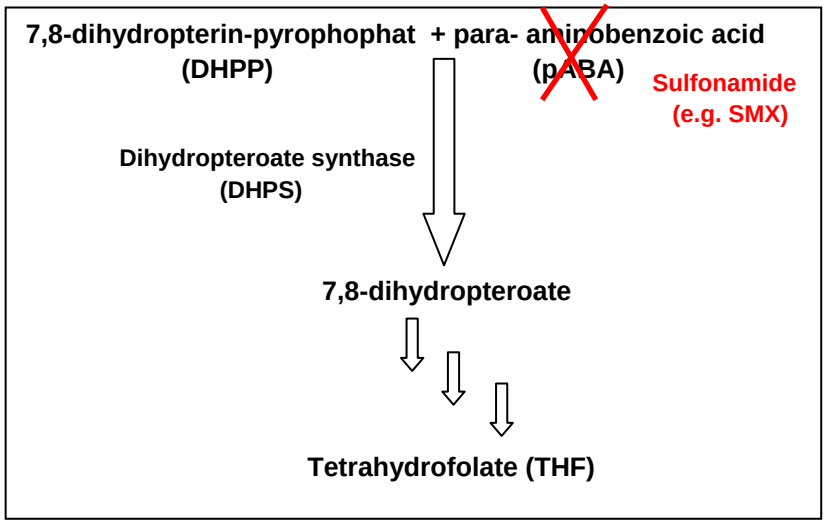


Figure 5 Biosynthesis of folic acid. Dihydropteroate diphosphate plus pABA form dihydropteroic acid which becomes tetrahydofolic acid. The figure also shows where the sulfonamides interact in the biosynthesis. [Modified from ACHARI et al., 1997]

E. coli with gene knockout in folate metabolism

The second possibility to induce a folate deficiency in nematodes is the use of two different *E. coli* strains:

MH951 is a strain in which the dihydrofolate reductase gene (*folA*) is replaced by a kanamycine resistance. Due to the deletion of *folA*, the enzyme dihydrofolate reductase is no longer expressed. Thus, dihydrofolic acid can not be transformed into tetrahydrofolic acid and leads to the folate deficiency. The kanamycine resistance, which replaced the *folA* gene, can be used as a selection marker.

The strain MH828 is the parental strain of MH951 and was used as their control. Both strains had to grow in LB-media in which thymine (stock concentration 5 mg/ml) was added (final concentration 0.05 mg/ml) over night at 37°C. Furthermore, supplementation of thymine (final concentration 0.05 mg/ml) to NGM-agar plates was necessary to assure viability of these bacteria. As MH951 bacteria contain a kanamycine resistance gene the addition of a final concentration of 0.03 mg/ml kanamycine to the LB-media and agar plates was necessary to use the existing selection marker.

3.2.1.3 Folic acid supplementation studies

The difference between supplemented and non-supplemented nematodes is the addition of folic acid onto the plates with a final concentration of 100 µM. Bacteria are also able to metabolize folic acid, which makes it impossible to guarantee that the total supplement was consumed only by the nematodes if the time span between adding the bacteria and the nematodes on plates is too long. For this reason the plates were supplemented with 1 ml of 2 mM folic acid on top of the 20 ml NGM-agar in a 9 cm plate. Because folic acid is a light sensitive vitamin, the plates were stored at 4°C over night in darkness. On the next morning the bacteria were added and as soon as the plates were dry the nematodes could be transferred to these supplemented plates.

3.2.1.4 Synchronization of worm population

In order to get reliable and repeatable results it is necessary to use an age synchronized worm population. Additionally, the former generation of nematodes has to experience the same treatment like the offspring. After this was done, the egg preparation could be performed. For this, plates with young adult worms were washed off with M9-buffer into 15 ml falcons. The next step was to centrifuge the nematodes at 2500 rpm for two minutes at room temperature before the supernatant was removed. This procedure was repeated until all plates were washed off and the supernatant was absolutely clear. After the last washing cycle, the supernatant could be removed and the worm pellet was replenished onto three milliliters with M9-buffer. Four milliliters of the bleaching solution were added and the falcons had to be shaken for

about five minutes. After that, the microscope was used to make sure, that all nematodes were dead before the worms were centrifuged (2500 rpm, 1.5 min. 20°C) and washed for four times to eliminate all of the bleaching solution. After the last washing step, the supernatant was removed and the worm pellet replenished onto six milliliters M9-buffer and dandled over night at constantly 20°C.

The next day the falcons were again centrifuged and the L1-larvae could be placed onto agar plates which provided the suitable food source and which were prepared the day before their use. Two days later wild type worms had reached the L4 larval stage. At this stage nematodes carry a white crescent in the last third of their body, which makes it possible to recognize them. For metabolic profiling as well as gene expression studies these L4 larvae were washed off the plates with M9-buffer and washed several times until the supernatant was clear.

After the washing steps, the supernatant was removed and the pellet was stored at -20°C in 1.5 ml reaction tubes each tube containing approximately 200 µl worm pellet.

3.2.2 Metabolic profiling via LC-MS in *C. elegans*

In 2009, Hughes et al. published a paper about the metabolomic responses of *Caenorhabditis elegans* to cadmium which gave the impetus to try the analysis of the total metabolic profile of the nematode *C. elegans* via LC-MS [HUGHES et al., 2009]. They used both ^{1}H NMR and UPLC-MS for their measurements and were able to determine the reverse phase chromatography conditions with a gradient of 26 minutes of time. The different conditions of measurement via UPLC and HPLC make it necessary to modify the gradient as well as the sort of mobile phase. For this reason, one aim of this project was to establish the proper conditions for metabolic profiling via LC-MS in *C. elegans*.

3.2.2.1 Sample preparation

The frozen pellet of L4 nematodes needed to be processed in order to be injectable into the LC-MS system. For this, the pellet was defrosted and

centrifuged at 2000 rpm for 1.5 min at room temperature. The supernatant was removed and the pellet resuspended in 200 µl M9-buffer. Before the tubes were vortexed three to four times for 1.5 minutes at full speed, one volume of glass beads was added into each tube. Between each vortex step, the tubes were placed on ice to make sure that the mechanical frictional heat did not affect any metabolites or proteins of interest. A heated needle was used to stab a hole into the lid as well as into the ground of the reaction tube, which was placed onto a 15 ml falcon. After one centrifugation step at 2500 rpm for 1.5 min at room temperature, 200 µl M9-buffer were added in order to wash the glass beads. One more centrifugation step was needed before approximately 400 µl of the supernatant could be transferred into a new reaction tube in which 800 µl of 99.99 % methanol were added (final methanol concentration of 66.66%) in order to remove the proteins. The reaction was incubated for 20 min at -20°C. After the protein precipitation, the tube was again centrifuged at 13000 rpm at 4°C for ten minutes. The next step was to transfer the supernatant into a new reaction tube.

The idea to deep-freeze the metabolites in order to make them storable at that time had to be rejected. As soon as the samples were defrosted not-identifiable substances could be seen macroscopically, which made it impossible to inject the samples into the HPLC-system.

For this reason, they were stored at 4°C over night. The next day they were concentrated (Eppendorf concentrator) at room temperature until they were completely dry before 200 µl of 66.66 % methanol were added. The dried part had to be resuspended. In order to prevent congestion at the HPLC column one more centrifugation step was needed (2500 rpm, 1.5 min, 20°C). Again, the supernatant was transferred into a new reaction tube and 100 µl were ready to be placed into the sample glass vial in order to be injected into to HPLC-system.

3.2.2.2 Reverse Phase High Performance Liquid Chromatography (HPLC)

HPLC is a method which is widely used in analytics. The principle of this process is the separation of a complex sample into its single components. Because of high pressure in the system the sample is transported together with

the mobile phase through the column, in which the stationary phase is placed. The composition of mobile and stationary phase can either be isocratic, which means that there is no change during the process, or it can be with a gradient, which means that the composition of both phases changes over time. The separation is possible on the basis of different interactions between the sample and mobile phase as well as the sample and stationary phase. The higher the interactions are, the longer the component will need to pass the column. To visualize the result, a detector at the end of the system is needed. In case of this thesis a gradient as well as mass spectrometry as a detector were used.

The following gradient was developed to be the best choice for the analysis of the metabolic profiling of the nematode *C. elegans* on HPLC-qTOF (UltiMate 3000, Dionex; micrOTOF-QII, Brucker):
10 µl were injected onto the column. A guard column (Fortis Phenyl, ϕ3µm) was used to protect the separation column. The chromatographically separation was achieved on a Fortis Phenyl, ϕ3µm, 150x 2.1mm at 25°C with a flow of 0.300 ml/min. The ion polarity was positive and the mass range was determined from 100 to 2000 m/z. The chromatography conditions were determined for a 40 min gradient with eluent A: acetonitrile (ACN) plus 0.1 % formic acid and eluent B: dH_2O plus 0.1 % formic acid.
For the first two minutes 0.1 % A and 99.9 % B were held before A was increased linearly up to 25 % over the next ten minutes. Thus, eluent B was consequently reduced down to 75 %. For the following five minutes A was raised up to 35 % and B reduced to 65 %. Between minute 17 and 25 eluent A was again raised, this time up to 45 %, whereas eluent B was reduced down to 55 %. This composition was held for five minutes before A returned to 35 % over the next 5 minutes. The last reduction of A was between minute 35 and 37 in which the eluent was lowered down to 0.1 %. This setting was held until 40 minutes. Eluent B was always increased and decreased to fit eluent A. The software ‘Hystar (version 3.2)’ was used for the analysis.

Table 18: Chromatography conditions (overview)

Retention (min)	**Eluent A: ACN (%)**	**Eluent B: dH2O (%)**
0 min	0.1	99.9
2 min	0.1	99.9
12 min	25.0	75.0
17 min	35.0	65.0
25 min	45.0	55.0
30 min	45.0	55.0
35 min	35.0	65.0
37 min	0.1	99.9
40 min	0.1	99.9

3.2.2.3 Mass Spectroscopy (MS)

Like the high performance liquid chromatography (HPLC), in analytics mass spectrometry (MS) belongs to the methods routinely used. The method is used to identify the mass of unknown molecules in a sample. For this, the sample is ionized via electrospray ionization (ESI). These ions are sped up through an electrical field and fly through a quadrupole in which the separation of the different masses is performed because of their diverse m/z-value. The measurement of the time needed to cross the flight tube (time of flight, TOF) allows the identification of the molecules. The following settings were selected for the analysis of the metabolic profile of the nematode *C. elegans*:

Source	
Nebulizer	0.4 bar
Dry Gas	4.0 l/min
Dry temperature	180°C

Transfer	
Funnel 1 RF	300.0 Vpp
Funnel 2 RF	400.0 Vpp
Hexapole RF	400.0 Vpp

Quadupole	
Ion Energy	5.0 eV
Low Mass	100.00 m/z

Collision Cell	
Collision Energy	10.0 eV
Collision RF	600.0 Vpp

3.2.3 Alterations in mRNA expression of *C. elegans* genes involved in folate metabolism induced by folate depletion and supplementation via realtime RT- PCR

3.2.3.1 Realtime RT-PCR with TaqMan probes

Polymerase chain reaction (PCR) is a technique to amplify small amounts of DNA during a given number of amplification cycles. In each cycle the DNA sequences have to go through three different steps: denaturation, annealing and elongation:

Denaturation: The temperature is raised up to 93-95°C in order to melt the DNA double helix to produce single stranded DNA. This temperature is held for 5-10 minutes.

Annealing: This is the step in which the primer can bind to the single stranded DNA sequences. The temperature stays at 93-95°C and is held for another 15 seconds.

Elongation: In the elongation step the DNA polymerase synthesizes the complementary strand in the direction of 5' to 3'. This step is held for one minute at 60°C.

Realtime RT-PCR is based on the normal PCR principle. In addition to the generation of up to millions of copies of one DNA sequence, realtime PCR is also able to quantify the amount of DNA in each cycle at real time. This is done by measuring the fluorescence signal of each sample after each cycle. The signal raises proportional to the amount of DNA. RT is the short version of reverse transcription and is mentioned to indicate that complementary DNA (cDNA) instead of genomic DNA (gDNA) was used for the analysis. TaqMan probes are one possibility to allow fluorescence. TaqMan probes are tagged with a quencher (3') on one site and with a fluorophore (5') on the other site. These probes are able to anneal within the sequence, which is supposed to be amplified, and are hydrolyzed during the elongation step of the DNA and with that, they are released. This hydrolysis causes the fluorescence because the

quencher is no longer able to suppress the signal of the fluorophore. [HEID et al. 1996]

3.2.3.2 Sample preparation

Before the cDNA could be analyzed via realtime RT-PCR, the frozen worm pellet needed to be processed in two more steps: RNA extraction and reverse transcription. As in the LC-MS project, two strains, RB755 and N2 Bristol wild type, were investigated. RB755 was only fed with OP50 *E. coli.* The wild type worms were either fed with OP50 *E. coli* as control or folate depletion was induced by feeding with MH828 *E. coli* and MH951 *E. coli* or SMX treated OP50 *E. coli.*

RNA Extraction

The Rneasy MiniKit (Qiagen) was used for RNA extraction. Therefore, the worm pellet with the synchronized L4 larvae was defrosted and 1 ml of the given RLT buffer was added. The sample was mixed and 600 µl were transferred into a new reaction tube. One volume of glass beads was added before the tubes were vortexed five times for 1.5 min. In between the samples were cooled down on ice. A heated needle was used to stab a hole into the lid as well as into the ground of the reaction tube, which was placed onto a 15 ml falcon. After one centrifugation step at 2500 rpm for 1.5 min at room temperature (all following steps were performed at room temperature), 100 µl RLT-buffer were added into each reaction tube to wash the glass beads. One more centrifugation step was needed before the reaction tubes could be removed. The falcons were centrifuged at full speed for three minutes and the supernatant could be transferred into a new reaction tube. 700 µl of 70 % ethanol were added into each tube before 700 µl of each sample were placed onto the given column and centrifuged (full speed, 1.5 minutes). The eluate was rejected and the rest of each sample was given onto the column and centrifuged the same way. 700 µl of the given RW1 buffer were added and centrifuged. Again, the eluate was removed. Two times 500 µl of the given RPE buffer were added onto each column. In between the tubes were centrifuged and the eluate removed. In order to dry the membrane, the column tubes had to be centrifuged one more

time at full speed for 1.5 min. After that, the column was transferred into a new reaction tube and 30 µl of the given Rnase free water were added directly onto the membrane. After one minute of incubation, the tubes were centrifuged at full speed for one minute. The RNA was now in the eluate and could be stored at -20°C.

Reverse Transcription

One microgram RNA was used for the reverse transcription into cDNA. For this reason the amount of RNA within one µl of the sample needed to be determined. Both, a NanoDrop (ND1000, PeqLab) or a PicoDrop (PicoDrop, Biozym) are suitable methods for the analysis and were used. The following steps were obeyed for the reverse transcription and the elimination of gDNA:

The given gDNA Wipeout buffer, quantiscript RT buffer, Rnase free water and RT-primer mix as well as the template RNA were defrosted. Each tube, except for the RNA samples, was vortexed in order to mix all substances properly. During the whole process, all tubes were kept on ice.

The total volume of each gDNA elimination reaction was 14 µl: 2 µl gDNA wipeout buffer plus the volume of the template RNA which is equal to 1 µg RNA. If these two substances together were less than 14 µl the samples were replenished with a variable amount of Rnase free water.

Next, the reaction tube was incubated at 42°C (heating block) for two minutes before it was replaced on ice.

The reverse transcription master mix was also prepared and mixed on ice. For this, 1 µl quantiscript reverse transcriptase and 1 µl RT primer mix were added to 4 µl quantiscript RT buffer.

6 µl of the reverse transcription master mix were added to the reaction tube from the gDNA elimination reaction. The final volume was 20 µl and the tubes were again incubated at 42°C (heating block), this time for 15 minutes. For the following 5 minutes the temperature was raised up to 95°C in order to inactivate

the reverse transcriptase. The cDNA was now ready to be analyzed via qPCR or it could be stored at – 20°C.

3.2.3.3 Analysis via realtime RT-PCR

C06A8.1, R03D7.1, Y4C6B.5, C36B1.7 (*dhfr-1*), and C06H2.4 (*folt-1*) (target genes) as well as F40E10.3 (*csq1*), C54G10.3 (*pmp3*), F20H11.3 (*mdh-2*) and Y45F10D.4 (housekeeping genes) were the genes of interest. In order to amplify and quantify the cDNA via realtime RT-PCR a mega master mix was prepared in which the template could be placed for the measurement:

Each reaction was performed in repeated determination. All PCR tubes contained 22.5 µl of the mega master mix and 2.5 µl of the sample cDNA. The mega master mix was prepared on ice for each primer. 12.5 µl TaqMan universal Master Mix, 8.75 µl DEPC H_2O and 1.25 µl gene expression assay mix (including primer plus probes) were used to produce one mega master mix. To ensure that the fluorescence signal is only caused by the amplified DNA, a water sample, consisting of 22.5 µl mega master mix and 2.5 µl DEPC H_2O (control), was additionally measured for each primer.

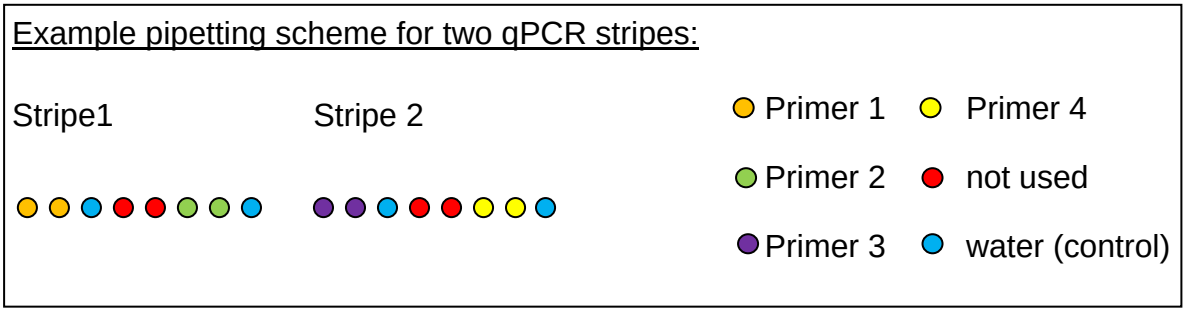


Figure 6: Pipetting scheme for PCR stripes

The realtime RT-PCR temperature program:

Not all of the primers could reach their full potential on the same temperature program. For this reason two different programs were needed. The one for the primers C54G10.3 and F20H11.3 was as follows:

2 min: 50°C
10 min: 95°C
0.15 min: 95°C
1min: 60°C
} 50 cycles

The program for the primers C06H2.4, C06A8.1, C36B1.7, R03D7.1, F40E10.3 and Y45F10D.4 was as follows:

2 min: 50°C
5 min: 93°C
0.15 min: 93°C
1 min: 60°C
} 45 cycles

After the run was successfully completed, the crossing points (CT-values) were transferred into a program called *BestKeeper*, which is a Microsoft® Excel®-based tool to determine stable housekeeping genes via pair-wise correlation analysis [PFAFFL et al. 2004].

BestKeeper uses the method of choice for standardization: relative quantification, which is the standardization with another gene. This so called housekeeping gene (HKG) is supposed to be unaffected by any experimental situation and thus, its expression should stay constant. However, there is also evidence that there is no such gene which is constantly unaffected. Therefore, *BestKeeper* allows the investigation of more than only one potential housekeeping gene (HKG). The program is also able to discover the least stably expressed HKG(s) by calculating and comparing the standard deviations (SD). A SD higher than one indicates an inconstant expression of the gene. Therefore, after identification, the affected gene can be eliminated from the list. Furthermore, the program is able to calculate a *BestKeeper Index* for each treatment out of the remaining HKGs [PFAFFL et al., 2004].

This Index is used in another program called *qgene*, which is also a Microsoft® Excel®-based software tool and which allows the evaluation, the mathematical and statistical analysis as well as the graphical presentation of the quantitative realtime RT-PCR data [MULLER et al. 2002].

4 Results

4.1 Metabolic profiling via LC-MS in *C. elegans*

One aim of this thesis was to establish a protocol for metabolic profiling of the nematode *C. elegans* with HPLC-MS. Hunger is one of the most extreme possibilities to force differences in metabolism and consequently in the metabolic profile. This condition was used to validate the method, because the expected differences in metabolic profiles between normal fed and starved *C. elegans* should be remarkably. In the end, the new developed HPLC-MS method should be sensitive enough to measure predicted differences in the metabolic profile between control worms, and folate depleted and folic acid supplemented worms.

4.1.1 Metabolic profiling of *C. elegans*: Development of a HPLC-MS based method

In our case, there are two important requirements the new developed HPLC-MS method has to fulfill: The sensitivity to show differences in the metabolic profile of differently treated worms and to be reproducible as well as reliable. For developing the protocol the metabolic profiles of non-starved nematodes were compared to the profiles of starved worms. To investigate the reproducibility and reliability, measurements of different biological replicates and different storage conditions were determined. In the end, the method is supposed to be as sensitive as possible to measure differences in the metabolic profile of *C.elegans* induced by different feeding, e.g. folate depletion or folic acid supplementation.

The UPLC gradient developed in the paper of Hughes et al. [HUGHES et al., 2009] served as the starting point for the gradient generated in this thesis. Due to the differences of UPLC and HPLC, different settings for the composition of the solvents as well as flow, gradient and duration of each measurement were tested. After trying different settings, the final one, which was able to perform the best profiles, could be determined. Page 32ff maintains the set up for the

HPLC as well as for mass spectroscopy (MS), which were used for all of the following investigations.

4.1.1.1 Differences of the metabolic profile of starved and non-starved *C. elegans*

As already mentioned above, hunger is a strong possibility to force differences in the metabolic profile. Therefore, wild type nematodes having starved for 17 hours before they were stored at -20°C, were compared with normal fed worms to see whether the requested demands towards the protocoling could actually be met.

The profile of non-starved versus starved wild type nematodes (**Figure 7**) shows differences all the way up to minute 25. Before that, the profile of starving nematodes (pink) deceeds the one of non-starving nematodes (red).

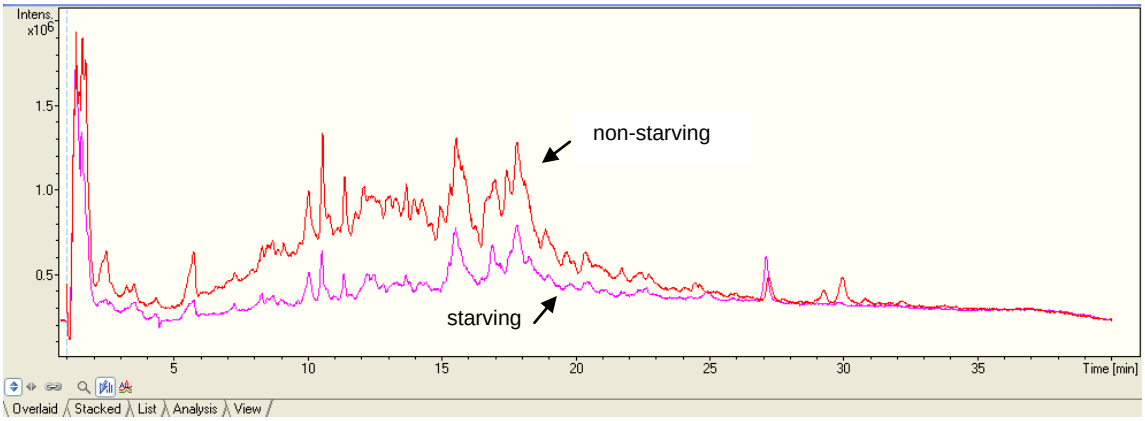


Figure 7 Metabolic profile of non-starved versus starved wild type *C. elegans*. The total ion chromatogramm of a mixed population of starved (pink) and non-starved (red) wild type worms.

For two samples of non-starved and starved wild type nematodes were cultivated, this measurement could be performed two times. The second profile of the same comparison shows a similar result. Again, the profile of non-starved nematodes is higher than the one of starved nematodes (Appendix, Figure 32).

4.1.1.2 Reproducibility and reliability of the method

Due to the massive metabolic differences (non-starved and starved nematodes), alterations within the profiles could be shown with the help of the generated gradient and the MS settings. Nevertheless, it is more important that

the method is reliable and provides repeatable results. This was investigated by the use of different biological replicates, which went under the same treatment.

The two profiles of non-starved wild type nematodes can be seen in **Figure 8**. Mild deviations can be seen between the 6th and 27th minute, but overall, both profiles are alike, which indicates a good reproducibility and reliability.

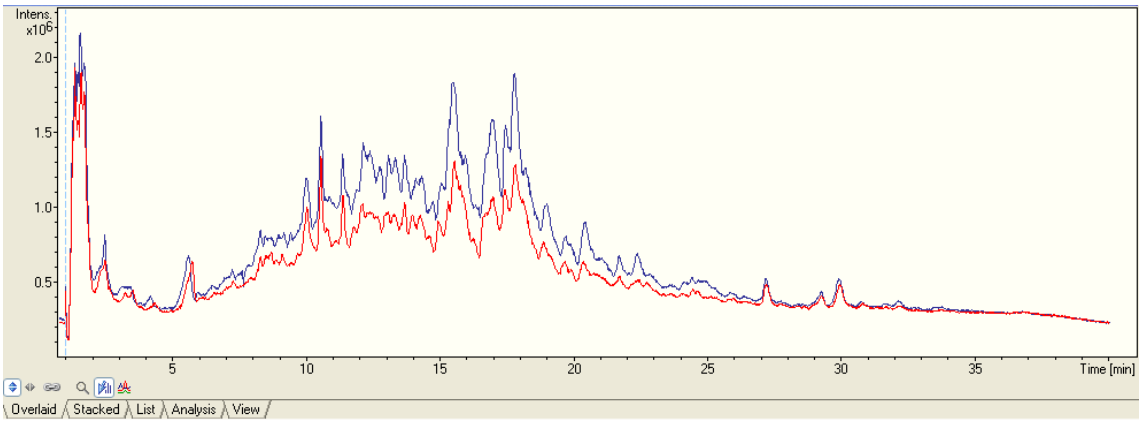


Figure 8: Metabolic profiles of non-starved wild type *C. elegans*. The total ion chromatogramms of a mixed population of non-starved N2 wild type nematodes.

Same comparison as above was also made for starved nematodes. Again, the profils showed mild derivations, but in all, the reproducibility and reliability could be proven (Appendix, Figure 30).

4.1.1.3 Influence of storage on reproducibility

Since alterations under different treatments (non-starved and starved) as well as the reproducibility of the method could be shown, the investigation of the influence of storage was the last part of interest.

To see, whether storage would cause any alterations in the metabolic profiles, all samples have been stored in their reaction tubes in darkness at 4°C for three weeks. After that time, they have again been injected into the HPLC system and the identical chromatographically conditions from above were used for the separation.

Figure 9 shows the metabolic profile of the non-stored and stored samples of the non-starved wild type population.

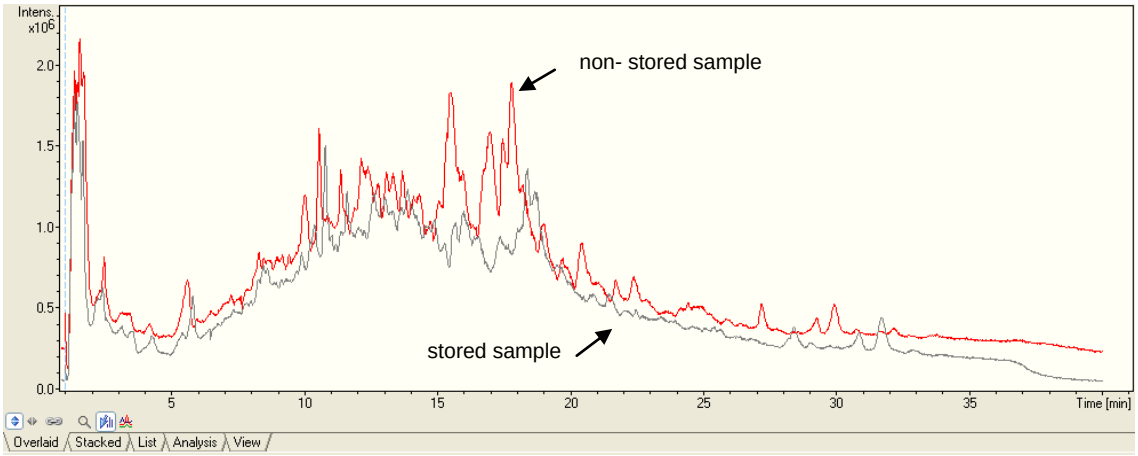


Figure 9: Metabolic profile of non-stored and stored wild type *C. elegans*. Total ion chromatogramm of a non-starving, mixed population of wild type nematodes. The red graph shows the profile of the sample without storage. The grey graph shows the stored sample.

As it can be seen, storage causes a reduced intensity of the peaks and especially between minute 15 and 20 totally different peaks. Therefore, storage leads to remarkable, so far unexplainable distinctions.

Again, the same measurement was also made for non-stored and stored samples of starved nematodes with the same results (Appendix, Figure 31).

Further measurements of non-stored and stored samples were made. Non-supplemented and supplemented wild type nematodes fed with either normal OP50, MH828, MH951, 0 µM or 10 µM treated SMX OP50 bacteria as well as RB755 nematodes fed with OP50 bacteria showed that all profiles of stored samples differ from their non-stored partner and therefore, storage seems to have an impact on the outcome. Thus, the remaining graphs are presented in the appendix (Appendix, Figure 32 until Figure 45; page 101ff).

In summary, a protocol, which meets the following requests was developed and validated: It shows differences in the metabolic profile of control worms versus starving nematodes. Additionally, the method seems to provide reproducible results as long as the regeneration and storage is comparable. Furthermore, it could be shown that storage leads to a change within the measured data. Therefore, a protocol for HPLC-MS was developed, which is suitable to measure the metabolic profile in the nematode *C. elegans*.

4.1.2 Metabolic profiling of supplemented versus non-supplemented *C. elegans* with the new developed HPLC-MS method

On this occasion the total ion chromatograms of nematodes supplemented with 0.1 mM folic acid were compared to non-supplemented nematodes. To minimize age dependent differences in the profiles, synchronized L4 larvae were used for the measurements.

The profiles of supplemented and non-supplemented N2 wild type nematodes fed with OP50 bacteria can be seen in **Figure 10**.

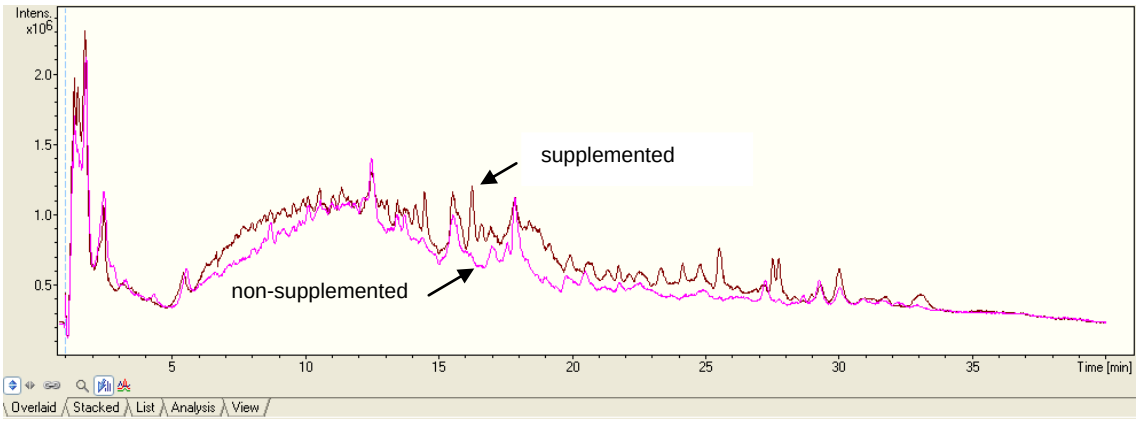


Figure 10: Metabolic profile of wild type *C. elegans* grown on OP50 *E.coli* with and without folic acid supplementation. Total ion chromatogramm of supplemented (brown) and non-supplemented (pink) L4 wild type worms

As the supplementation is the only difference between these two samples, it seems to be responsible for the distinctions within the profiles. In the beginning (unil minute six) the chromatogram is similar, but than the graph of supplemented nematodes (brown) exceeds the one of non-supplemented nematodes (pink) with different peaks.

Like wild type nematodes fed with OP50 bacteria, supplemented and non-supplemented RB755 nematodes show alterations even though to different extent (**Figure 11**). RB755 nematodes have a gene deletion in the methionine synthase gene *metr-1*.

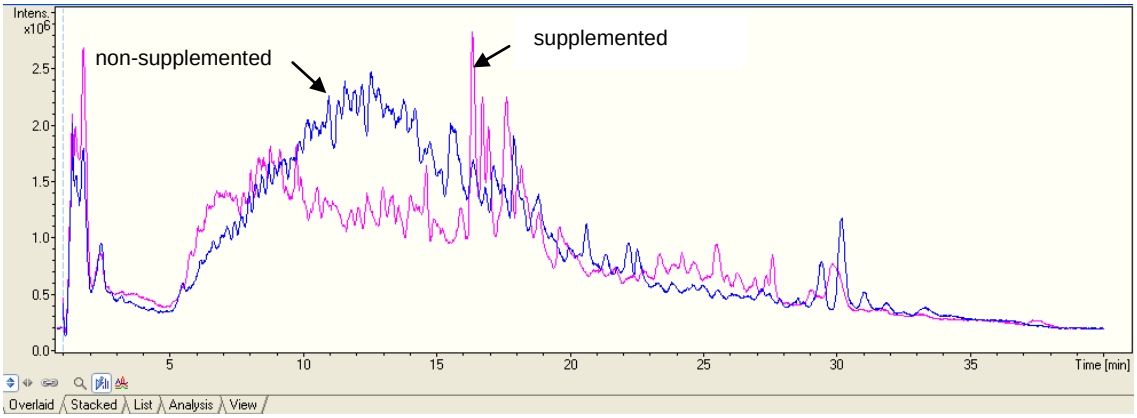


Figure 11: Metabolic profile of supplemented and non-supplemented RB755 *C. elegans*. Total ion chromatogramm of supplemented (pink) and non-supplemented (blue) L4 RB755 nematodes fed with OP50 bacteria

From minute 3 up to minute 10, the profile of supplemented (pink) nematodes exceeds the one of non-supplemented (blue) nematodes which changes from minute 10 on until minute 16. Within this part of the profile, the blue graph exceeds the pink one. With a short break around minute 17, the pink graph is again higher than the blue one between minute 16 and 18. Minute 18 until minute 23 show that the blue graph is higher than the pink one. Up to minute 29 the profile of supplemented nematodes exceeds the one of non-supplemented ones.

Figure 12 shows the profiles of wild type nematodes fed with MH828 *E. coli* with and without folic acid supplementation. MH828 are a special *E. coli* strain with a *thyA* deficiency and are used as a control for MH951 bacteria, which have a knockout in the *folA* (dihydrofolate reductase) gene.

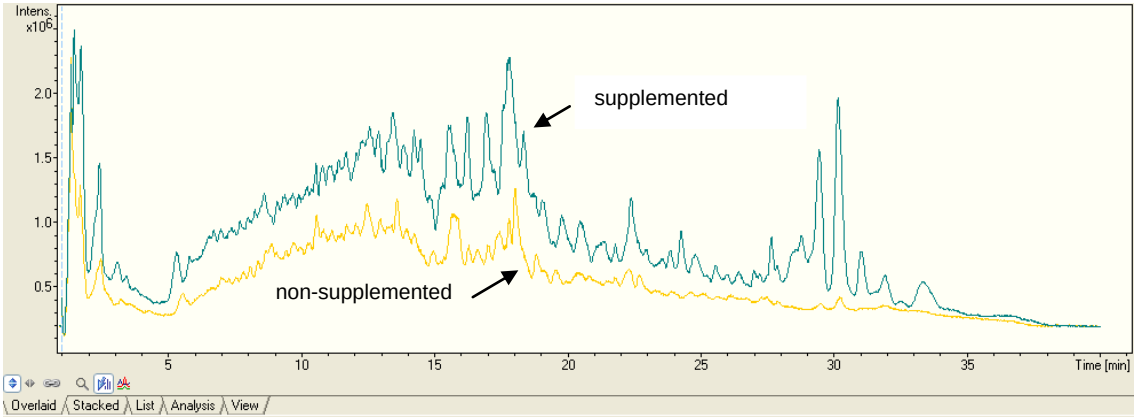


Figure 12: Metabolic profile of supplemented and non-supplemented wild type nematodes (MH828). Total ion chromatogramm of supplemented (blue) and non-supplemented (yellow) L4 wild type nematodes fed with MH828 bacteria.

The profile of supplemented nematodes (blue line) is over all time points higher and with more peaks than the one of non-supplemented nematodes (yellow line). Hence, supplementation seems to alter the metabolic profil of these nematodes drastically.

The differences in the profiles of supplemented and non-supplemented nematodes fed with MH951 bacteria are not as visible as those of nematodes fed with MH828. Nematodes fed with MH951 *E. coli* are supposed to have a folate deficiency. Most of the time, the blue graph (supplemented) exceeds the brown one (non-supplemented) (**Figure 13**).

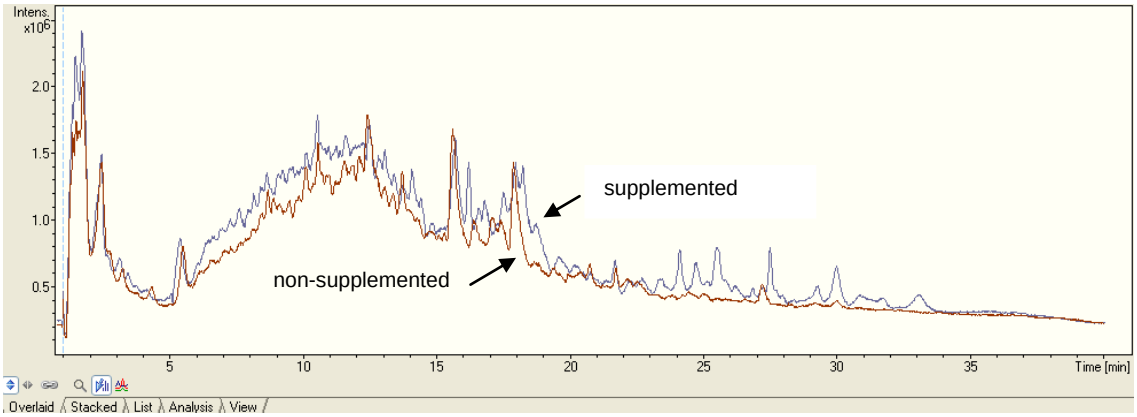


Figure 13: Metabolic profile of supplemented and non-supplemented wild type *C. elegans* (MH951). Total ion chromatogramm of supplemented (blue) and non-supplemented (brown) L4 wild type nematodes fed with MH951 bacteria.

From minute 23 on, the chromatogram of supplemented nematodes shows still peaks, whereas almost none can be seen in non-supplemented worms. Therefore, in this case the alterations seem to be located at the last part of the gradient.

Like above, the profiles of nematodes fed with 0 µM Sufamethoxazole (SMX) treated OP50 bacteria with and without additional folic acid show differences (**Figure 14**). Feeding bacteria grown in minimal media with 0 µM SMX to the nematodes are used as control for feeding *E. coli* with inhibited folic acid biosynthesis, because of adding 10 µM SMX into the growth media.

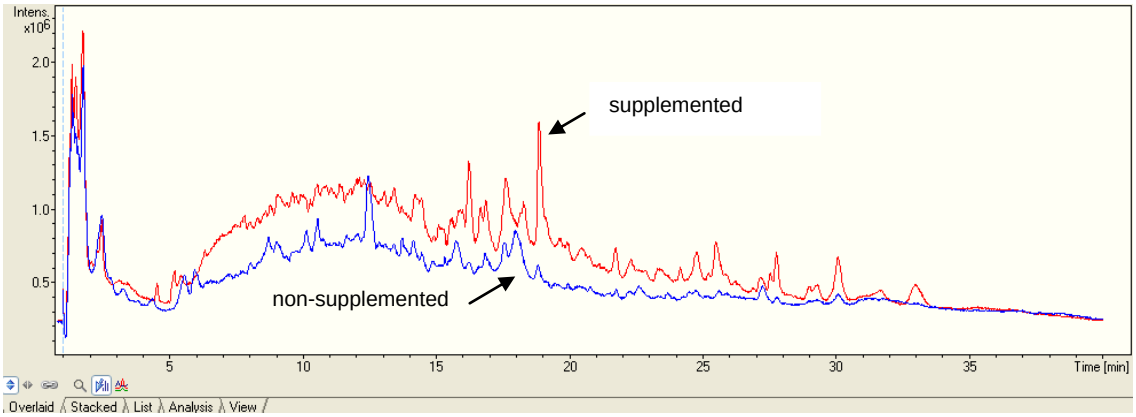


Figure 14: Metabolic profile of non-supplemented and supplemented wildtype *C. elegans* (OP50 + 0µM SMX). Total ion chromatogramm of supplemented (red) and non-supplemented (blue) L4 wild type nematodes fed with 0µM SMX treated OP50 bacteria.

The peaks of supplemented nematodes (red) are all the time higher and different than the one of non-supplemented worms (blue).

Figure 15 presents the alterations in the profile of non-supplemented and supplemented nematodes fed with 10 µM SMX treated OP50 bacteria. The addition of SMX to the OP50 bacteria causes a folic acid deficiency in the nematodes which was thought to be removable by supplementation.

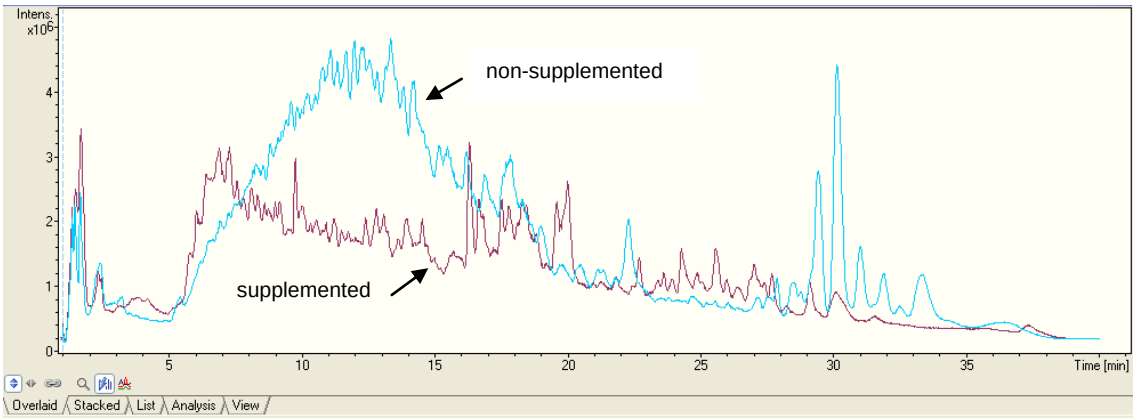


Figure 15: Metabolic profile of supplemented and non-supplemented wild type *C. elegans* (OP50 + 10 µM SMX). Total ion chromatogramm of supplemented (purple) and non-supplemented (blue) L4 wild type nematodes fed with 10 µM SMX treated OP50 bacteria.

This time the profile is noticable between the 7th and the 17th minute. Within this part, the profile of non-supplemented nematodes exceeds the profile of the supplemented population.

Due to these results it can be said that the implemented method is able to show differences in profiles of nematodes which were exposed to a less massive difference than hunger. Furthermore, the outcome of each measurement

indicates that a supplementation with folic acid has a visible impact on the profiles.

4.2 Alterations in mRNA expression of *C. elegans* genes involved in folate metabolism induced by folate depletion and supplementation

Recent studies in our group have investigated two different possibilities to induce a folate deficiency in the nematode *C. elegans*. The aim of the second part of the present thesis was to investigate the different mRNA expression of five genes out of the folic acid metabolism in *C. elegans* to see if the expression of the target genes C06H2.4 (*folt-1)*, C06A8.1 *(mthfr-1)*, R03D7.1 *(metr-1),* C36B1.7 *(dhf1*) and Y4C6B.5 (predicted homologue to mammalian proton-coupled folate transporter) is influenced by folate depletion. In a next step alterations in the gene expression were measured after supplementation with 0.1 mM folic acid.

4.2.1 Selection of stable expressed housekeeping genes (HKG) for investigations in the folate metabolism in *C. elegans*

The expression of reference genes should stay constant in all samples under investigation and under all experimental conditions. In 2008, Hoogewijs et al. published an article about reliable reference genes for quantitative gene expression analysis in *C. elegans*. The aim of the study was to identify reference genes within the nematode which can reliably be used for an investigation of gene expression. Among others, Hoogewijs et al. tested four genes which were also selected for validation of their stability in this thesis: Y45F10D.4, F40E10.3 (*csq-1),* F20H11.3 (*mdh-2*) and C54G10.3 *(pmp-3)* [HOOGEWIJS et al., 2008].

a) Y45F10D.4

This gene encodes for an iron-sulfur cluster assembly enzyme, which is needed to allow the survival of embryos and larvae. This enzyme is orthologous to the human NIFUN (ISCU = Iron-sulfur cluster assembly enzyme, mitochondrial)

[PHOSPHOSITEPLUS®] as well as ISU1 and ISU2 in *Saccharomyces cerevisiae* [WORMBASE].

b) F4E10.3 (*csq-1*)

The protein **C**al**S**e**Q**uestrin (*csq1*) is required for calcium ion homeostasis. *In vitro*, the encoded protein binds Ca(++) and buffers the sarcoplasmatical stores. The expression of CSQ-1 can be seen in body wall muscle cells and vulval muscles as well as in the pharynx [WORMBASE]. F40E10.3 is orthologous to the CASQ1 and CASQ2 in humans where CASQ1 is a Ca++ binding protein in muscles cells. The isoform within the heart is called CASQ2. [WEI et al., 2009]

c) F20H11.3 (*mdh-2*)

The encoded protein MDH-2 is homologue to mammalian malate dehydrogenase. In larvae and adults, the gene is expressed in the pharynx, intestine and body wall muscles. The biological function contains the determination of the adult life span, the embryonic development ending in birth or egg hatching, growth, nematode larval development, positive regulation of growth rate as well as receptor-mediated endocytosis. The protein is also thought to be part of malate metabolic processes, cellular carbohydrate metabolic processes, oxidation and reduction [WORMBASE].

d) C54G10.3 (*pmp-3*)

Pmp-3 (**p**eroxisomal **m**embrane **p**rotein) encodes for an ABC transporter which is thought to be orthologous to the human ABCD4 transporter, a protein involved in the peroxisomal import of fatty acids into organelles. The transporter is integrated into the membrane and its molecular functions contain ATP binding, ATPase activity, NTPase activity as well as nucleotide binding [WORMBASE].

The intention of this choice of target genes was to find at least two genes which can be used for investigations of a gene expression study within the folic acid metabolism of the nematode *C. elegans*. For this, the expression of the four genes within wild type nematodes fed with either normal OP50 bacteria (control)

or with MH828, MH951 or SMX treated OP50 bacteria (0 µM or 10 µM) was evaluated. Realtime RT-PCR was used to determine the CT-values. CT stands for **c**ycle **t**hreshold and indicates the point at which the fluorescence within the PCR crosses the threshold limit. The threshold limit is normally ten times the standard deviation of the base line [HEID et al., 1996].

The program *BestKeeper* was then applied to identify the genes with the most stable expression under the five different treatments. The two best performing genes were selected to act as housekeeping genes within this study.

Sample:	**C54G10.3 HKG 1**	**F20H11.3 HKG 2**	**F40E10.3 HKG 3**	**Y45F10D.4 HKG 4**
N2 (OP50)	36,00	29,84	28,92	31,00
N2 (OP50)	35,90	30,00	28,63	31,67
N2 (MH828)	34,80	31,00	34,30	33,32
N2 (MH828)	35,29	30,70	34,30	33,33
N2 (MH951)	33,00	26,86	31,73	33,00
N2 (MH951)	32,48	26,98	31,00	32,50
N2 (OP50)+ 10 µM SMX	34,58	28,91	31,07	32,14
N2 (OP50)+ 10 µM SMX	34,22	29,00	30,84	32,15
N2 (OP50)+ 0 µM SMX	34,00	29,74	30,62	32,97
N2 (OP50)+ 0 µM SMX	34,24	29,78	30,36	32,64
	HKG 1	**HKG 2**	**HKG 3**	**HKG 4**
n	10	10	10	10
geo Mean [CP]	**34,43**	**29,25**	**31,12**	**32,46**
ar Mean [CP]	**34,45**	**29,28**	**31,17**	**32,47**
min [CP]	32,48	26,86	28,63	31,00
max [CP]	36,00	31,00	34,30	33,33
std dev [± CP]	**0,86**	**1,07**	**1,36**	**0,59**
CV [% CP]	**2,50**	**3,67**	**4,36**	**1,80**

Table 19: Data of the program *BestKeeper.* CT-values of all HKG being tested (pink). Description of the tested strains and food sources can be found in the green box. F20H11.3 and F40E10.3 were rejected (red). C54G10.3 and Y45F10D.4 were accepted because they could achieve a standard deviation lower than one (blue).

The data shown in **Table 19** strongly suggests the selection of C54G10.3 (*pmp-3*) and Y45F10D.4 as stable expressed housekeeping genes for investigation of folate metabolism in *C.elegans*. CT-values of F20H11.3 as well as F40E10.3 showed a standard deviation greater than one [1.07 (F20H11.3) and 1.37 (F40E10.3)] which indicates an altered expression under the different

treatments. The two chosen housekeeping genes (C54G10.3 and Y45F10D.4) were used for all further expression analysis.

4.2.2 Target genes (TG)

In order to get a complete overview of changes within the folate metabolism (**Figure 16**) in *C. elegans,* induced by folate deficiency or folic acid supplementation, five target genes were chosen. Beneath them are two genes which code for predicted folate transporters (Y4C6B.5 and *folt-1*), two genes which code for predicted key enzymes in the folate metabolism (*dhfr-1* and *mthf-1*) and one gene which is involved in a side chain of the folate metabolism, namely methionine synthase (*metr-1*).

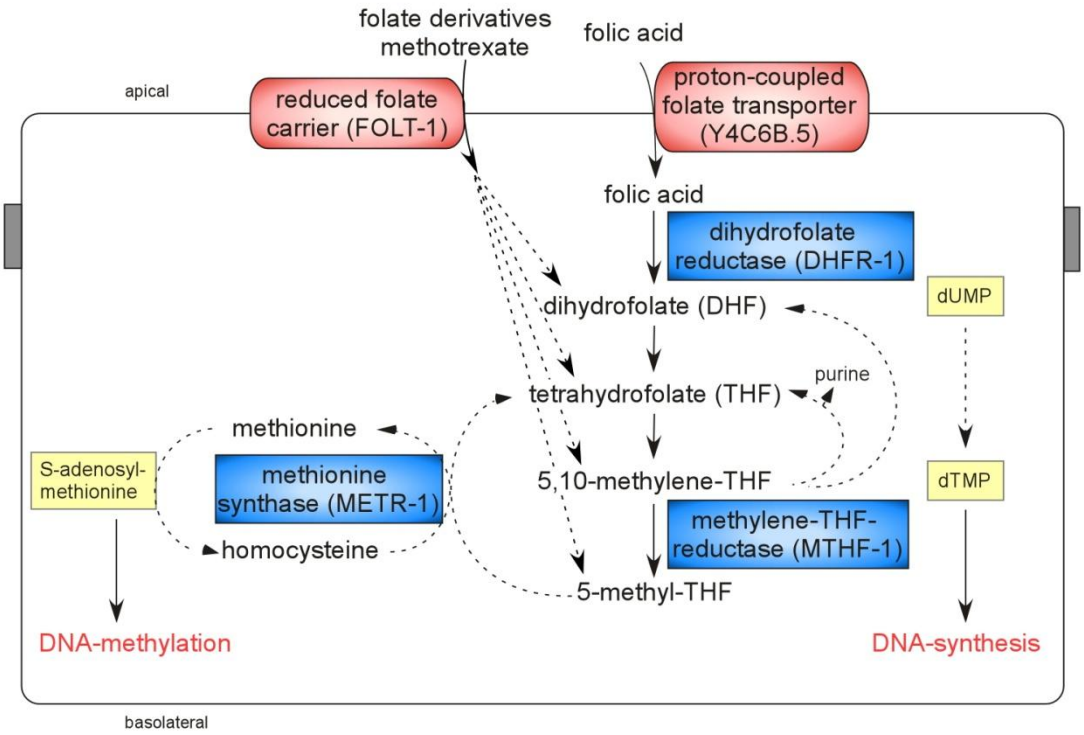


Figure 16: Overview of the folic acid metabolism within a cell. Folic acid and folates enters through FOLT-1 (Reduced Folate Carrier) or Y4C6B.5 located on the apical side of the cell. With the help of dihydrofolate reductase (DHFR-1) folic acid is transferred into dihydrofolate (DHF) which is processed into tetrahydrofolate (THF) before it becomes 5,10-methylene-THF. Methylene-THF-reductase (MTHF-1) is then needed to reduce 5,10-methylene-THF to 5-methyl-THF which can together with homoccystein and the help of the methionine synthase (METR-1) be transferred into THF and methionine (needed for DNA-methylation) [PROVIDED BY Dr. J. BENNER].

a) C06H2.4 (*folt-1*)

FOLT-1 belongs to the folate transporter family and encodes a folate transporter, which is required for the absorption of folate. The activity of FOLT-1 is proton-dependent, but sodium-independent and is inhibited by a folate derivate, sulfasalazine as well as anion transport inhibitors (e.g. 4,4-diisothio-cyanatostilbene-2,2-disulphonic acid (DIDS)). In larvae, an expression can be found in the pharynx, intestine, body wall muscle and unidentified cells in the head and tail. In adults, an additional expression is seen in the reproductive system, vulval muscle and gonad sheath cells [WORMBASE]. C06H2.4 is orthologous to the human SLC19A1 (**S**o**l**ute **c**arrier family 19), also called reduced folate carrier (RFC) [WORMBASE]. RFC is a transmembran anion exchanger and is unable to bind folic acid. RFC shows a high affinity towards reduced folates (K_t= 2-4 µM) whereas it has a low affinity for folic acid (K_i= 200 µM) and is neither sodium nor proton dependent. In almost all peripheral tissues of mammals, RFC is the main transporter because its optimal activity is reached at physiological (7.4) pH- value [ZHAO et al. 2009].

b) Y4C6B.5

The encoded protein belongs to the major facilitator superfamily (MFS), a large group of transporters containing uniporters, symporters and antiporters. The gene is predicted to code for the transporter ADD1 (= adducing 1 alpha), a transmembrane transport protein. The expression of Y4C6B.5 can be observed temporarily in the intestine of all post-embryonic stages. On occasion the protein is also expressed in the nerve cells of the pharynx [WORMBASE]. BLASTP results suggest that this transporter is also homologous to the mammalian proton-coupled folate transporter (PCFT, SLC46A1) with a similarity of 40% [personal BLASTP result]. The transporter has similar affinities for reduced (5-methyl-THF) as well as oxidized (folic acid) folates, is independent of sodium but proton dependent. The transporter is expressed within the small intestine, there mainly in the duodenum and jejunum, but also in the kidney, liver, placenta, retina and the brain. Genetically, there is no relationship between RFC and PCFT. The transporter is specialized for low pH-values, a

quality which makes it so important for the absorption within the small intestine [ZHAO et al., 2009].

c) C36B1.7 (*dhfr-1*)

C36B1.7 belongs to the dihydrofolate reductase family. The molecular functions are dihydrofolate reductase activity, oxidoreductase activity and NADP(H) binding [WORMBASE]. In mammals, the dihydrofolate reductase is responsible to reduce folic acid to DHF (dihydrofolate) and THF (tetrahydrofolate) [ZHAO et al., 2009].

d) C06A8.1 (*mthf-1*)

The target gene C06A8.1 is orthologous to the human methylene-tetrahydrofolate reductase. In larvae, the protein MTHF-1 can be found in the pharynx, intestine, hypodermis, nervous system, nerve ring, head neurons, pharyngeal neurons as well as in unidentified cells in the tail. Adult nematodes show an additional expression within the reproductive system and vulval muscle but none in the pharyngeal neurons [WORMBASE]. The MTHFR in mammals is needed to reduce the 5,10-methylenetetrahydrofolate into 5-methylentetrahydrofolate which is needed for the elimination reaction of homocysteine (HC) out of the system [ZHAO et al., 2009].

e) R03D7.1 (*metr-1*)

R03D7.1 is orthologous to the methionine synthase within the human genome. In larvae and adults, the gene can be discovered within the intestine, hypodermis, seam cells and pharynx [WORMBASE]. The mammalian methionine synthase is responsible to regenerate methionine from homocysteine whereas in the same step, 5-methyltetrahydrofolate becomes tetrahydrofolate (THF) [ZHAO et al., 2009].

4.2.3 Comparison of the differently treated worm samples

Two different strains (N2 var. Bristol wild type and RB755) of the nematode *C. elegans* were used, whereas wild type nematodes were fed with five different diets: OP50 bacteria, MH828 bacteria, MH951 bacteria as well as SMX treated OP50 bacteria (0µM and 10µM). Normal OP50 bacteria served as the food source of the strain RB755.

For a better understanding, **Figure 17** describes the connection of strains and different feeding types. The difference of wild type and RB755 nematodes in the genetic background ('strain-level'), whereas wild type nematodes fed with MH828 and MH951 bacteria as well as SMX treated OP50 bacteria have the differences on the 'food-level'.

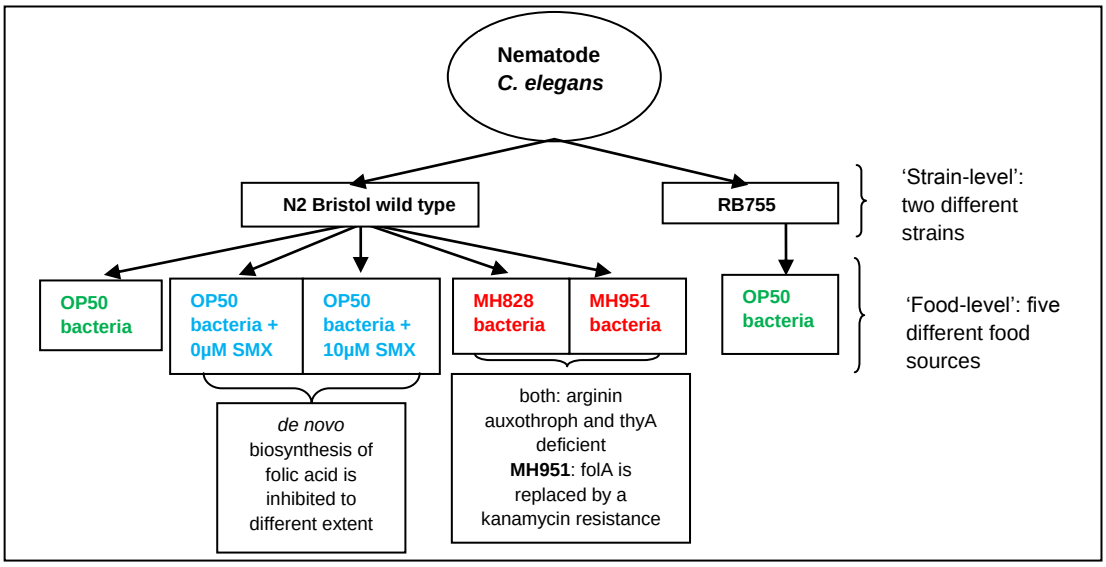


Figure 17 Visualization of the comparisons. N2 wild type nematodes were compared to RB755, both fed with normal OP50 bacteria (green). Wild type nematodes fed with MH828 bacteria (control) were likened to nematodes fed with MH951 bacteria (red) and those being fed with 0µM (control) and 10µM SMX treated OP50 bacteria were compared to each other (blue).

The difference in the genetic background of N2 and RB755 nematodes concerns a side pathway of the folic acid metabolism. RB755 nematodes possess a deletion of about 2500 bp within the methionine synthase (*metr-1*) gene. This extinction results in the inability to synthesise the enzyme. Therefore, normal fed N2 nematodes and genetically modified RB755

nematodes were compared to each other whereas the wild type served as the control (**Figure 17**, green).

Folate deficiency was induced in *C. elegans* by the two different ways explained earlier in this thesis (page 29ff). The deletion of the *folA* gene within the MH951 bacteria is thought to influence the metabolism. MH828 and MH951 are both arginine auxotroph and *thy A* deficient *E. coli* bacteria strains, whereas MH951 has a deletion in the *folA* gene and hence reduced folate concentration, which when fed to the worms induces a folate deficiency. Therefore, wild type nematodes fed with MH951 bacteria were compared to their control: nematodes fed with MH828 bacteria (**Figure 17**, red).

The greatest interest concerning this part was to see whether a supplementation of MH951 fed nematodes with 100 µM folic acid is able to bring the expression levels of all target genes back to the level of non-supplemented nematodes fed with MH828 bacteria. This would show that an external supplementation of the nematodes with folic acid could be used to compensate the deficiency caused by the genetically modified bacteria.

The addition of 10 µM SMX to OP50 bacteria inhibits the *de novo* biosynthesis of folic acid within the bacteria in a greater intensity. To see the influence of SMX, nematodes fed with 10 µM SMX treated OP50 bacteria were compared to their control: nematodes fed with 0 µM treated OP50 bacteria (**Figure 17**, blue). Here, too, the interest was to find out whether the supplementation with 100 µM folic acid is capable to compensate the folic acid deficiency induced by SMX and bring the expression levels of the target genes of nematodes fed with 10 µM treated OP50 bacteria back to control level.

4.2.4 Different gene expression of *folt-1* (C06H2.4)

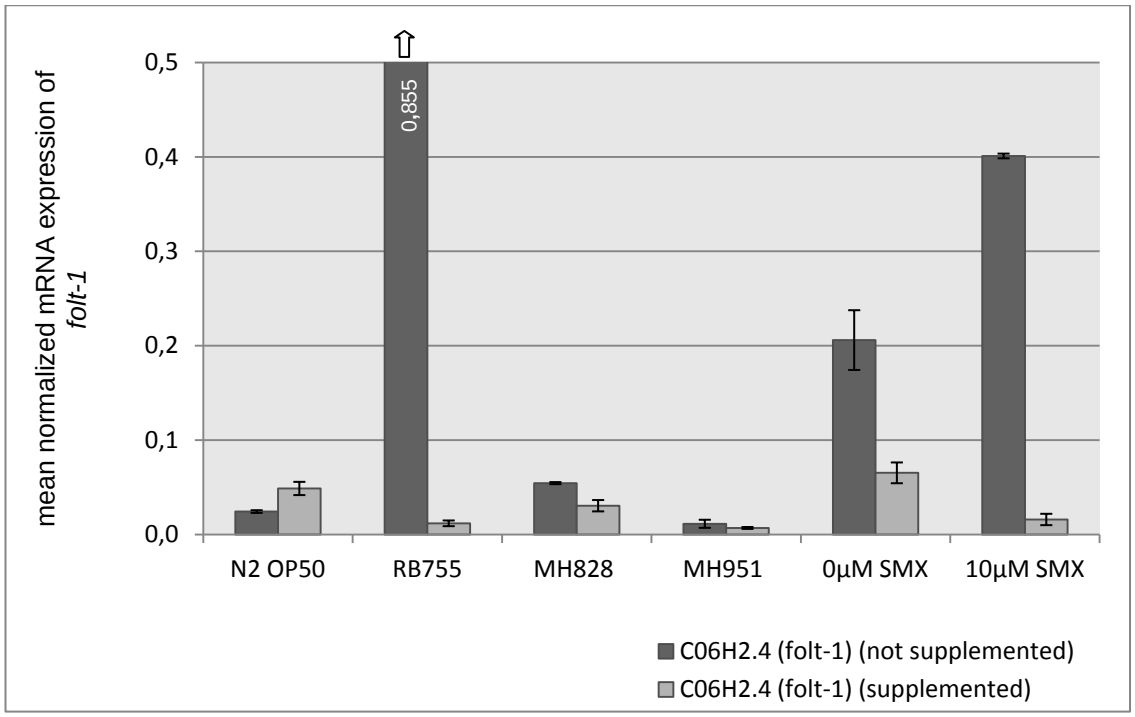


Figure 18: Different expression of the target gene C06H2.4 (*folt-1*) of non-supplemented (dark) and supplemented (light) nematodes. The data is given in absolute values for both strains (N2 and RB755) as well as the different ways of feeding within the wild type (MH828 *E. coli* bacteria, MH951 *E. coli* bacteria and OP50 bacteria either grown in 0 µM or 10 µM Sulfamethoxazole).

N2 var. versus RB755:

The expression of *folt-1* in non-supplemented RB755 (0.855± 0.043) exceeds the one of non-supplemented wild type nematodes (0.024± 0.001). In contrast to that, supplemented wild type nematodes show a higher expression of *folt-1* in the wild type (0.049± 0.007) compared to RB755 (0.012± 0.003).

Figure 18 shows that a supplementation of the wild type leads to an up regulation of *folt-1* whereas in RB755, the gene is down regulated because of a supplementation.

MH828 versus MH951 bacteria:

Folt-1 is higher expressed in non-supplemented nematodes fed with MH828 (0.054± 0.001) than in non-supplemented ones fed with MH951 bacteria (0.011± 0.004). A similar behavior can be observed under supplementation. The expression in supplemented nematodes fed with MH828 bacteria (0.031± 0.006) exceeds the one of supplemented nematodes fed with MH951 bacteria (0.007± 0.001). Therefore, a supplementation results in a down regulation of *folt-1* in both, nematodes fed with either MH828 or MH951 bacteria. As it can be seen, a supplementation of the nematodes fed with MH951 bacteria (**Figure 18**) is not able to bring the expression level back to the one of non-supplemented MH828 fed nematodes.

0 µM versus 10 µM SMX treated OP50 bacteria:

In non-supplemented nematodes fed with 10 µM SMX treated OP50 bacteria (0.401± 0.003), the expression of *folt-1* is higher than in control worms (non-supplemented) (0.206± 0.032). In contrast to that, supplemented nematodes fed with 0 µM SMX treated OP50 bacteria show a higher expression (0.065± 0.011) of *folt-1* than supplemented nematodes fed with 10 µM SMX treated bacteria (0.016± 0.006). In both feeding types, a supplementation leads to a down regulation of the gene. Again, a supplementation of nematodes fed with 10 µM SMX treated OP50 bacteria (**Figure 18**) is unable to bring the expression level back to where it is in non-supplemented nematodes fed with 0 µM SMX treated OP50 bacteria.

4.2.5 Different gene expression of Y4C6B.5

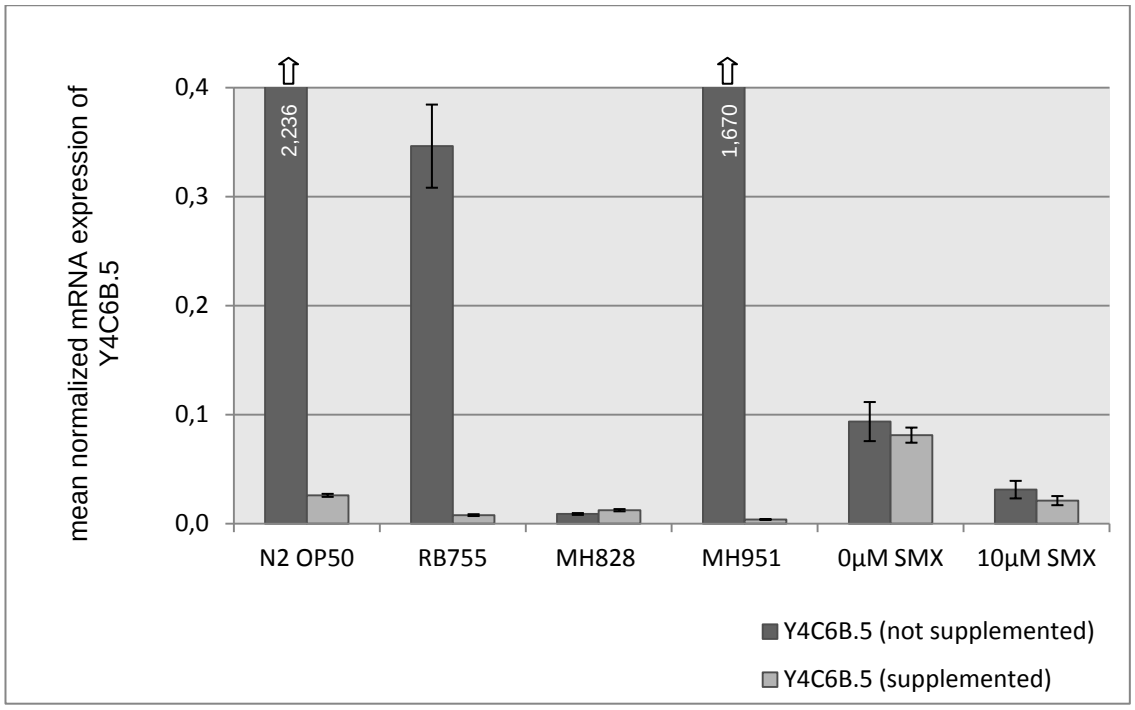


Figure 19 Different expression levels of the target gene Y4C6B.5 of non-supplemented (dark) and supplemented (light) nematodes. The data is given in absolute values and both strains (N2 and RB755) as well as the different ways of feeding within the wild type (MH828 bacteria, MH951 bacteria and OP50 bacteria either grown in 0µM or 10µM Sulfamethoxazole).

N2 versus RB755:

The expression of Y4C6B.5 in non-supplemented wild type nematodes (2.236± 0.184) is higher than in non-supplemented RB755 (0.346± 0.038). In both strains, a supplementation results in a down regulation of the gene. Furthermore, the expression under supplementation (**Figure 19**) is higher in the wild type nematodes (0.026± 0.001) than in RB755 (0.008± 0.001).

MH828 versus MH951 bacteria:

In non-supplemented nematodes fed with MH951 bacteria (1.670± 0.259) the expression of Y4C6B.5 exceeds the one of non-supplemented nematodes fed with MH828 bacteria (0.009± 0.001). Supplemented nematodes fed with MH828 bacteria (0.012± 0.001) show a higher expression than supplemented nematodes fed with MH951 bacteria (0.004± 0.000). In nematodes fed with MH828 a supplementation leads to a slight up regulation, whereas in those being fed with MH951 bacteria the supplementation leads to clearly visible

down regulation (**Figure 19**). Although the expression level of supplemented nematodes fed with MH951 bacteria is not exactly the equivalent of non-supplemented MH828 fed nematodes, the approach to the same value is not to ignore.

0 µM versus 10 µM SMX treated OP50 bacteria:

In non-supplemented nematodes, the expression of Y4C6B.5 is higher in those nematodes being fed with 0 µM SMX treated OP50 bacteria (0.094± 0.018) than in 10 µM SMX treated bacteria (0.031± 0.008). Same can be seen in supplemented nematodes (0.081± 0.007 and 0.021± 0.004). In both, 10 µM and 0 µM SMX treated OP50 bacteria, a supplementation of the nematodes results in a down regulation of the gene. A supplementation of nematodes being fed with 10 µM SMX treated OP50 bacteria is unable to bring the expression level of Y4C6B.5 back to where it is in non-supplemented nematodes being fed with 0 µM SMX treated OP50 bacteria (**Figure 19**).

4.2.6 Different gene expression of *dhfr-1* (C36B1.7)

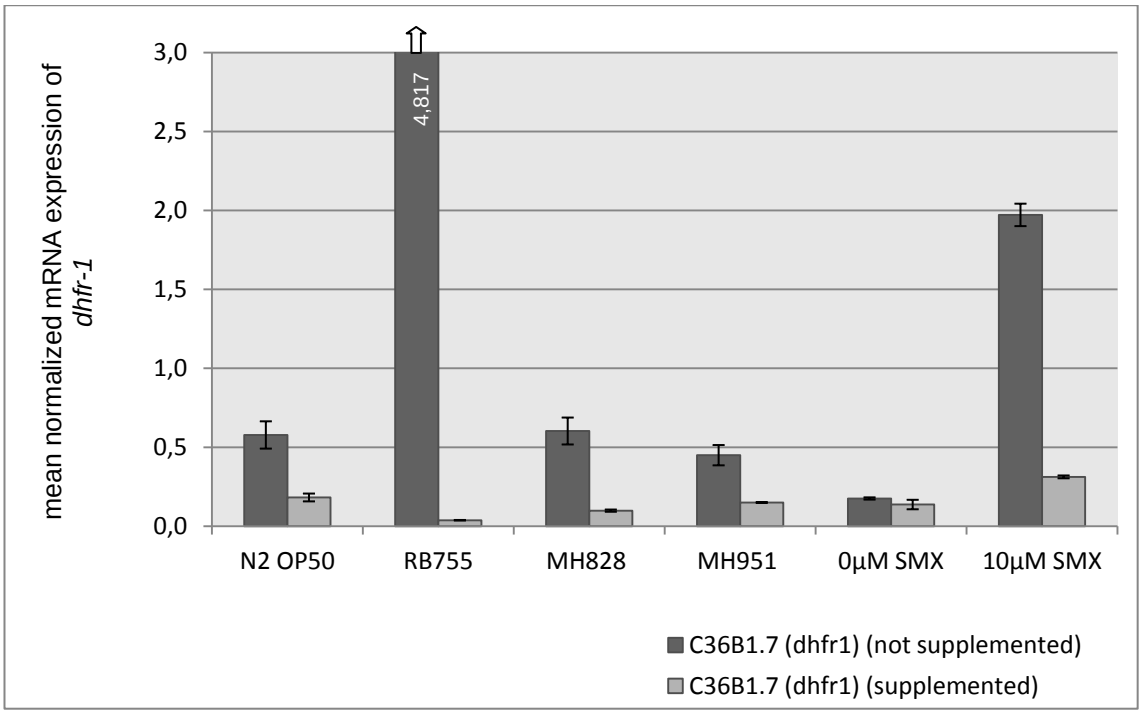


Figure 20 Different expression levels of the target gene C36B1.7 (*dhfr-1*) of non-supplemented (dark) and supplemented (light) nematodes. The data is given in absolute values for both strains (N2 and RB755) as well as the different ways of feeding within the wild type (MH828 *E. coli* bacteria, MH951 *E. coli* bacteria and OP50 bacteria either grown in 0 µM or 10 µM Sulfamethoxazole).

N2 versus RB755:

Non-supplemented wild type nematodes show a lower expression (0.578± 0.086) of C36B1.7 than non-supplemented RB755 (4.817± 0.639). In contrast to that, in supplemented nematodes, the expression is higher in wild type nematodes (0.182± 0.025) than in RB755 (0.038± 0.002) (**Figure 20**). In both strains, a supplementation leads to a down regulation of the gene.

MH828 versus MH951 bacteria:

The expression of C36B1.7 in non-supplemented nematodes fed with MH828 bacteria (0.603± 0.085) exceeds the one of those being non-supplemented and fed with MH951 bacteria (0.405± 0.064). In supplemented nematodes, the expression is higher in those being fed with MH951 bacteria (0.150± 0.000) compared to those being fed with MH828 (0.098± 0.007). Again, in both strain, a supplementation leads to a down regulation of the gene. Furthermore, a

supplementation of MH951 fed nematodes (**Figure 20**) can not reuptake the expression level to that of non-supplemented nematodes fed with MH828 bacteria.

0 µM versus 10 µM SMX treated OP50 bacteria:

Nematodes fed with 0 µM SMX treated OP50 bacteria show a lower expression of C36B1.7 (0.175± 0.007) than those being non-supplemented and fed with 10 µM SMX treated OP50 bacteria (1.972± 0.071). Same behavior can be observed under supplementation. Again, the expression in higher in those being fed with 10 µM SMX treated OP50 bacteria (0.312± 0.009) than in those being fed with 0 µM SMX treated bacteria (0.137± 0.030). As above, the value of supplemented nematodes fed with 10 µM SMX treated bacteria (**Figure 20**) is no match to non-supplemented nematodes being fed with 0 µM SMX treated bacteria.

4.2.7 Different gene expression of *mthf-1* (C06A8.1)

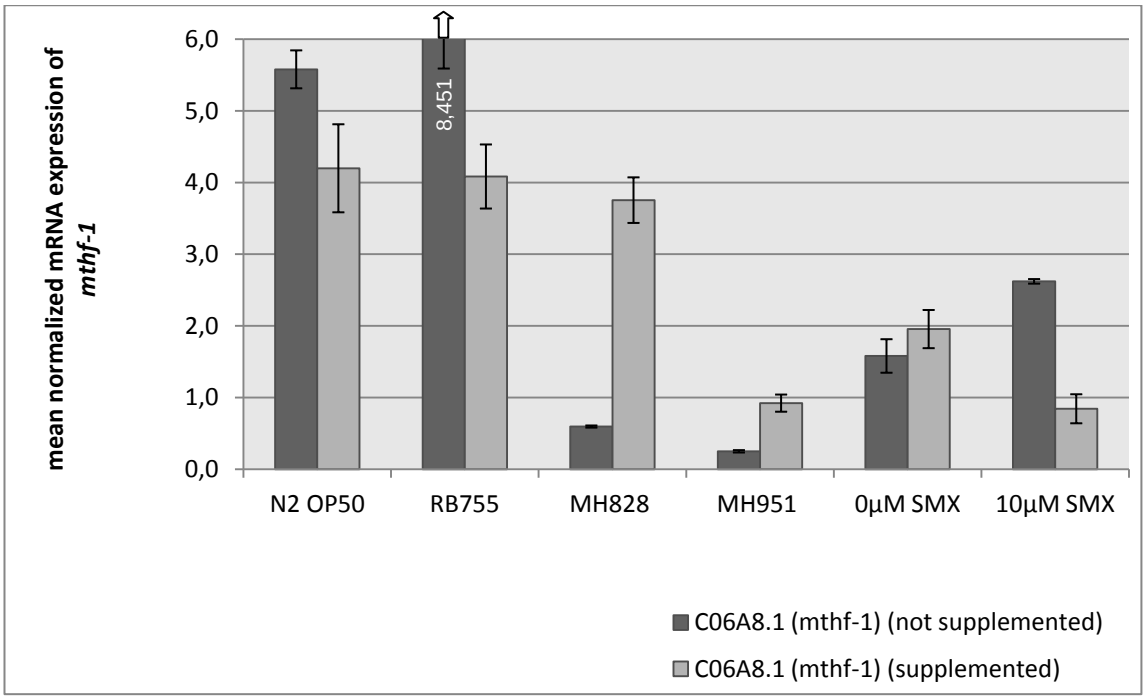


Figure 21 Different expression levels of the target gene C06A8.1 (*mthf-1*) of non-supplemented (dark) and supplemented (light) nematodes. The data is given in absolute values and both strains (N2 and RB755) as well as the different ways of feeding within the wild type (MH828 bacteria, MH951 bacteria and OP50 bacteria either grown in 0 µM or 10 µM Sulfamethoxazole).

N2 versus RB755:

Both, wild type and RB755 nematodes, show a higher expression of C06A8.1 in non-supplemented nematodes (5.578± 0.265 (N2) and 8.451± 2.861(RB755)) than in those who were supplemented (4.198± 0.614 (N2) and 4.084± 0.447 (RB755)). Therefore, it can be said that a supplementation with folic acid leads to a down regulation of the gene *mthf-1* in *C. elegans* (**Figure 21**).

MH828 versus MH951 bacteria:

In wild type nematodes fed with MH828 the expression of mthf-1 is lower in non-supplemented nematodes (0.596± 0.015) compared to those who were supplemented (3.754± 0.318). A similar behavior can be observed in nematodes fed with MH951 bacteria. When compared to the non-supplemented ones (0.251± 0.017), a higher expression of the gene can be seen in the supplemented nematodes (0.923± 0.120). In this case, a supplementation

leads to an up regulation of the expression. As it could be observed so far in the other genes, a supplementation of nematodes fed with MH951 bacteria is not able to result in the same expression level as in non-supplemented nematodes fed with MH828 bacteria (**Figure 21**).

0 µM versus 10 µM SMX treated OP50 bacteria:

Nematodes fed with 0 µM SMX treated OP50 bacteria show a higher expression when supplemented (1.956± 0.266) when compared to non-supplemented ones (1.581± 0.234). In contrast to that, a supplementation of nematodes fed with 10 µM SMX treated OP50 bacteria leads to lower expression of *mthf-1* (0.884± 0.203) in *C. elegans* compared to the non-supplemented nematodes (2.621± 0.032). Therefore, supplementation in the first group, a supplementation leads to an up regulation of the gene expression, whereas in the second group a down regulation can be observed. Supplemented nematodes supplied with 10 µM SMX treated OP50 bacteria (**Figure 21**) do not show the same expression level as non-supplemented nematodes fed with 0 µM SMX treated OP50 bacteria.

4.2.8 Different gene expression of *metr-1* (R03D7.1)

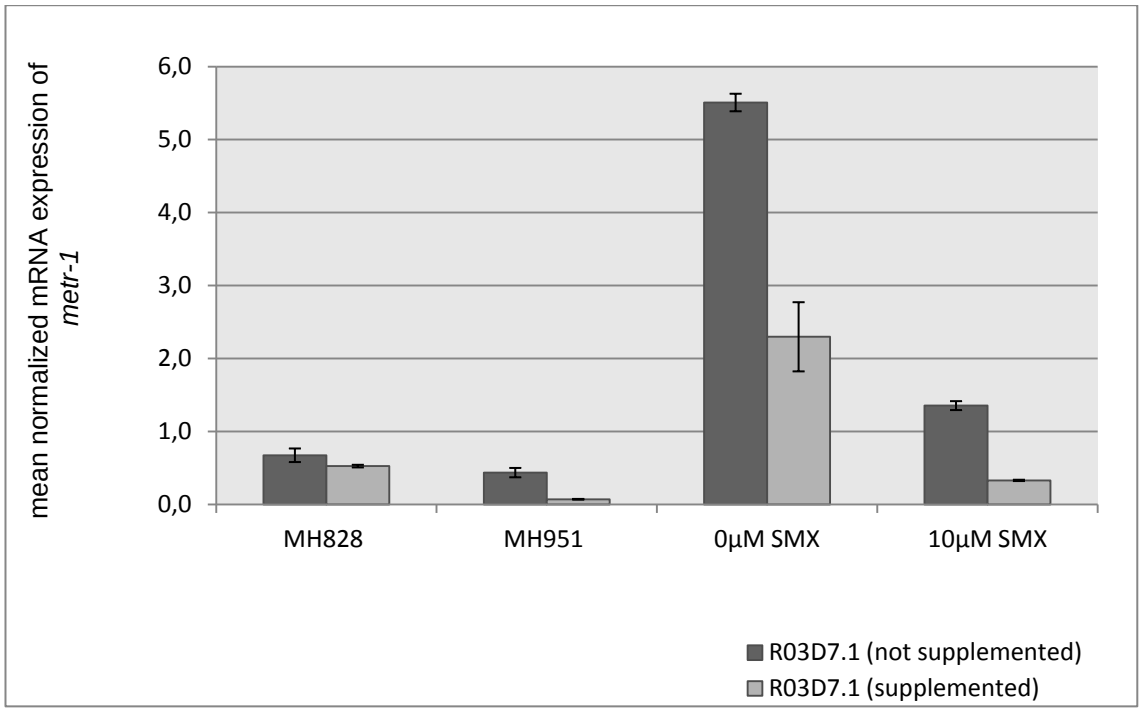


Figure 22 Different expression levels of the target gene R03D7.1 (*metr-1*) of non-supplemented (dark) and supplemented (light) nematodes. The data is given in absolute values and both strains (N2 and RB755) as well as the different ways of feeding within the wild type (MH828 bacteria, MH951 bacteria and OP50 bacteria either grown in 0 µM or 10 µM Sulfamethoxazole).

MH828 versus MH951 bacteria:

In both, wild type nematodes fed with either MH828 or MH951, a supplementation leads to a slight down regulation of the gene. The expression level of supplemented nematodes supplied with MH951 bacteria (0.070± 0.005) can not reach the level of non-supplemented nematodes fed with MH828 (0.674± 0.093). Non-supplemented MH951 fed nematodes express the *metr-1* gene slightly lower (0.436± 0.064) than supplemented nematodes fed with MH828 (0.525± 0.018) (**Figure 22**). As it can be seen, a supplementation of MH951 fed nematodes can not undo the effect caused by the deletion of the *folA* gene in the bacteria.

0 µM versus 10 µM SMX treated OP50 bacteria:

A supplementation of wild type nematodes, fed with 0 µM SMX treated OP50 bacteria, leads to a down regulation of the gene *metr-1* (2.298± 0.474) (non-supplemented: 5.508± 0.120). Non-supplemented nematodes fed with 10 µM SMX treated OP50 bacteria show a lower expression (1.354± 0.061) than nematodes fed with 0 µM SMX treated bacteria but a higher expression than supplemented nematodes supplied with 10 µM SMX treated bacteria (0.329± 0.009). In both cases, a supplementation leads to a down regulation of *metr-1*. As it can be seen, the effect caused by the addition of 10 µM SMX in the growth media of the bacteria can not be undone by a supplementation with 0.1 mM folic acid (**Figure 22**).

5 Discussion

5.1 Folate supplementation changes metabolic profiles in the nematode *C. elegans*

Mild deviations in the profiles of non-starving and starving nematodes could be seen in the results above. Since the amount of nematodes within the analyzed sample was determined at 200 µl worm pellet and therefore, the exact number of worms can not be guaranteed to be constantly the same, the mentioned deviations might rather occur due to this inaccuracy than an insufficient gradient.

There is one major bottom line, which can be drawn out of the experiment concerning the changes in the metabolic profile:
In wild type nematodes fed with either normal OP50, MH828, MH951, 0 µM or 10 µM SMX treated OP50 bacteria as well as RB755 nematodes fed with OP50 bacteria, folic acid supplementation led to changes in the metabolic profiles to different extent. To see, which metabolites are actually affected, a principal component analysis (PCA) might help to reveal the answer. The PCA can show whether the profiles are able to cluster as well as whether there really are differences in the main components. Searching databases for the different masses found in the profiles might be another attempt which could help to find out which pathways might be affected and to which extend this occurs.

In their paper about limitations and recommendations in mass-spectrometry-based metabolomics in nutrition, Scalbert et al. addressed critical and difficult steps in metabolomic research. Unsolved problems such as non-optimized study protocols, unwanted sources of variations associated to sample collection and storage, limited quality of MS analysis, insufficient exploitation of the data and data overfitting as well as the want of well determined and standardized methods and practice are some of the reasons why the progress in this research field is after all cumbered. Furthermore, since the identification of the metabolites is one of the essential but also difficult steps in metabolomics, Scalbert et al. recommended the use of a standard set, which is externally

spiked in retention markers and consists out of five to seven compounds [SCALBERT et al., 2009].

As already claimed from Scalbert et al., for future investigations dealing with metabolic profiling of *C. elegans* it might be interesting to use spiked samples in order to see how much of the originally used amount of nematodes get lost because of reprocessing steps.

Furthermore, the creation of reference levels for worm samples might be helpful to refer the LC-MS data to. Since in this study, a 200 µl worm pellet was used for each analysis, the actual reason for the mild deviation mentioned at the beginning of this part can only be guessed rather than explained. Therefore, the determination of the DNA concentration, the examination of the amount of protein within a sample measured after the shredding step but before the protein precipitation as well as the weighing of each used sample or even the count of the number of nematodes might be good starting points in order to achieve more precise results and interpretations of the produced data.

5.2 Folate depletion and supplementation influence gene expression of folate metabolism in *C. elegans*

Folate depletion was induced in two different ways. First, two different *E. coli* bacteria strains were used: MH828 and MH951. The parental strain (MH828) was used as control for MH951 which contain a kanamycine resistance instead of the *folA.* Due to the *folA* deletion, the enzyme dihydrofolate reductase is no longer expressed. Therefore, dihydrofolic acid (DHF) can not be transformed into tetrahydrofolic acid (THF) acid which leads to the folate deficiency.

The second possibility to induce the folate deficiency in *C. elegans* is to feed OP50 bacteria which were grown in Sulfamethoxazole (SMX). SMX, a sulfonamid, competes against pABA (p-amino-benzoic acid) and therefore lowers concentration dependent the biosynthesis rate of folic acid and folates. Previous studies in our group have shown that 10 µM SMX are sufficient to induce a folate deficiency in worms in the end. Furthermore, bacteria grown in 0 µM SMX were used as control for those grown in 10 µM SMX. After the

induction of the folate depletion, folic acid was supplemented and the mRNA expression of all target genes was measured in nematodes fed with 0 and 10 µM SMX as well as MH828 and MH951. Figure 16 (p.52) as well as Figure 18 (p.57), Figure 19 (p.59), Figure 20 (p.61), Figure 21 (p.63) and Figure 22 (p.65) show the data for the respective comparison and might help to understand the following.

5.2.1 Differences in gene expression of supplemented and non-supplemented N2 and RB755 nematodes

The following presents the explanation and interpretation of the results obtained in the second project of this master thesis about the different expression of selected housekeeping genes due to folic acid supplementation.

5.2.1.1 The expression of the predicted folic acid transporter Y4C6B.5 is altered by folic acid supplementation

When wild type and RB755 nematodes are compared, the expression of *folt-1* in normal OP50 fed nematodes is markedly higher in RB755, whereas the second putative transporter Y4C6B.5 is lower expressed. Therefore, the main transporter in RB755 nematodes seems to be FOLT-1, which is responsible to transport folate derivatives into the cell. In contrast to that, Y4C6B.5, the transporter responsible for folic acid intake, seems to be preferred in N2 wild type nematodes. Hence, the deletion of the methionine synthase gene (*metr-1*) in RB755 worms, which is needed for the remethylation of homocysteine to methionine, seems to have an impact on which transporter is favored.

In wild type nematodes, more folic acid is able to enter the cell, whereas RB755 nematodes prefer the absorption of folate derivates. This behavior might occur due to the fact that RB755 nematodes are unable to form THF out of 5-methyl THF. Therefore, it might be possible that RB755 nematodes raise the intake of folate derivatives in order to maintain the intracellular pool of THF.

In both strains under folic acid supplementation, the Y4C6B.5 expression is strongly decreased compared to non-supplemented wild type and RB755

nematodes. This could be due to an increased folic acid concentration outside the cell that is the only substrate for Y4C6B.5. Hence, fewer membrane transporters are needed to reach the same final intracellular folic acid concentration as in case of a folic acid depleted diet. Also, McCormick posted that a high folate status can be harmful. High folate levels increase the risk of cancer and might be able to induce or worsen neurological damage caused by a vitamin B_{12} deficiency. Furthermore, high folate concentrations might be responsible for a specific and significant decrease in folate uptake in intestinal cells [MCCORMICK, 2010].

The same effect was described by Balamurugan et al. for the folate transporter FOLT-1 [BALAMURUGAN et al., 2007]. Supplementation of folic acid to wild type nematodes decreased the mRNA expression and function of FOLT-1. The same effect was seen for RB755 in this study. Unfortunately, in our case we observed the oppositional effect for wild type worms. They show a higher expression of *folt-1* in supplemented nematodes when compared to the non-supplemented ones. Nevertheless, since this study focused on a supplementation with folic acid instead of folates, physiologically there should be no relevance for this occurrence. Therefore, the decisive relation between folic acid supplementation and transporters concerns Y4C6B.5 instead of FOLT-1.

For Y4C6B.5 in the wild type and RB755 strain was downregulated through supplementation, the genetical difference between both strains can not have an influence on their reaction of the expression towards a supplementation. Since Y4C6B.5 is also responsible for the folate transport, the lower expression under supplementation agrees with the findings of Balamurugan et al. [BALAMURUGAN et al., 2007].

5.2.1.2 Knockout of the methionine synthase leads to enhanced *dhfr-1* expression

When compared to wild type nematodes, the gene loading for the enzyme dihydrofolate reductase is higher expressed in RB755 nematodes. As shown above, RB755 worms seem to take up more folate derivatives instead of folic

acid compared to wild type, because of higher *folt-1* expression. This might induce *dhfr-1* expression in some way. Also, the lack of methionine synthase might have and influence on the expression of *dhfr-1*.

Since METR-1 is missing, 5-MTHF can no longer be transformed into THF. Therefore, an increase in the DHFR-1 expression might occur in order to raise the conversion of the previous substances into the biologically active THF.

In both strains, the supplementation led to an unexplainable decreased expression of *dhfr-1*. This is in contrast to the findings of Gao et al., who were able to show that a supplementation with folic acid in patients suffering hyperhomocysteinemia as well as in mice increases the expression of dihydrofolate reductase (DHFR) [GAO et al., 2009]. In the nematode *C. elegans* the opposite effect could be discovered.

5.2.1.3 The expression of *mthf-1* is influenced by the deletion of the methionine synthase

The expression of *mthf-1* is higher in RB755 than in N2 wild type nematodes, which could be the result of the *metr-1* deletion. As the expression of the upstream enzyme (*dhfr-1*) in RB755 was increased when compared to the wild type, there could be more decomposition products which need to be further processed and therefore the expression of *mthf-1* is increased as well.

In both strains, due to the supplementation, the expression of *mthf-1* is decreased which might indicate that a high availability of folic acid leads to a lower expression of enzymes to maintain the supply. Thus, supportive investigations might be interesting for further analysis. There also might be an association between a folic acid supplementation and the lower expression of *mthf-1* in supplemented nematodes. In 2008, Smith et al. published a paper in which they stated that a high concentration of folic acid could be responsible for the inhibition of the formation of 5-methyl-THF because dihydrofolate is a possible inhibitor of MTHF [SMITH et al., 2008].

5.2.1.4 *Metr-1*

Main parts of the gene encoding for this enzyme are deleted in RB755 nematodes and therefore no expression should be detected. Hence, there is no need for a comparison and thus for a discussion of the mRNA expression within the two strains in this thesis.

5.2.1.5 Conclusion of N2 versus RB755 *C. elegans*

With the exception of Y4C6B.5, which is lower expressed when compared to N2 wild type, the expression of all investigated genes (*folt-1, mthf-1 and dhfr-1*) is enhanced in methionine synthase knockout worms. The deletion of the *metr-1* gene seems to have an impact on the choice of the preferred transporter and therefore the folic acid related substrates available in the cell. Furthermore, with the exception of *folt-1* in N2 wild type nematodes, a supplementation leads to a decreased expression of the genes, which can not be explained so far.

5.2.2 Differences in supplemented and non-supplemented N2 wild type nematodes fed with either MH828 or MH951 bacteria

The following samples differ in their food source. On the one hand the nematodes were fed with MH828 and on the other one with MH951 bacteria. The difference of these two bacteria strains is the deletion in the gene *folA* encoding for a dihydrofolate reductase within MH951. Normally, this gene would be needed to degrade DHF to THF. With its deletion this step is no longer possible and a folate deficiency is predicted.

5.2.2.1 Folate deficiency induced the expression of Y4C6B.5

In nematodes fed with MH828 bacteria, the transport of folic acid and its derivates seems to occur with the help of FOLT-1 which indicates that more reduced folate derivatives enter the cell. The second possible transporter of folic acid, Y4C6B.5, homologous to the mammalian proton-coupled folate transporter is higher expressed in nematodes, which were exposed to the folate deficiency caused by MH951 bacteria. In their review, Iferagan and Assafara present that in mammals one possible mechanism to adapt to a folate deficiency is the overexpression of PCFT [IFERGAN and ASSARAF, 2008] also seen in these

worms. Hence, nematodes fed with MH951 bacteria seem to prefer the transport through Y4C6B.5 which indicates that compared to folate derivatives more folic acid is taken up. The deficiency appears to have an effect on which transporter is favored and therefore higher expressed in wild type nematodes. Furthermore, the observation that at least one of the two transporters is remarkably higher expressed due to the deficiency meets the outcome of the study of Said et al. They were able to find out that a dietary folate deficiency in rats is responsible for a significant increase in the folate uptake [SAID et al., 2000].

The assumption that a deficiency causes a higher expression of the enzymes needed for the depletion might also be conceivable. This might indicate that cells suffering a deficiency desperately try to receive the wanted substrate. Therefore, they raise the expression of the affected enzymes in order to maximize the chance to obtain a minimal supply [SAID et al. 2000]. In case of Y4C6B.5, this thesis might be appropriate.

Under folic acid supplementation the expression of *folt-1* is significantly reduced in both groups, whereas the expression of Y4C6B.5 is only drastically decreased in worms fed with MH951 bacteria. Therefore, as mentioned earlier in this thesis, the assumption that as soon as the supply with folic acid is sufficient, fewer transporters are needed to ensure an adequate intake is applicable. This regulatory effect of high substrate levels on both folate transporters was also seen in rats [DEV et al., 2011] and in pancreatic cells [SAID et al., 2010]. Furthermore, the argument of the selfregulation of each cell in order to protect itself against a high folate and folic acid intake and therefore unwanted effects might also be suitable [MCCORMICK, 2010].

5.2.2.2 There are no appreciable differences in the expression of *dhfr-1* due to a folate deficiency

This enzyme is slightly higher expressed in nematodes fed with control MH828 bacteria than in those being fed with MH951. For the expression of Y4C6B.5 in MH951 fed nematodes is extremely high, a sufficient amount of folic acid

appears to be available and therefore, a normal *dhfr-1* level seems to be satisfying.

In yeast, it could be shown that there is evidence that due to a folic acid deficiency the activity of DHFR-1 is markedly higher. In this case, the reason for the effect could not be found on mRNA level but on protein level. Therefore, the higher activity due to the deficiency seems to be caused by posttranscriptional mechanisms [ZHU et al., 2002]. To find out whether this occurrence can also be found in *C. elegans* might be interesting and worth of further investigations.

Comparable with wild type and RB755, folic acid supplementation induced a distinct decrease in expression of *dhfr-1* in both MH828 and MH951 fed *C. elegans*, which cannot be explained so far.

5.2.2.3 Supplementation leads to a higher expression of *mthf-1*

Like *dhfr-1*, *mthf-1* is higher expressed in those nematodes that do not suffer a folate deficiency. This observation supports the previous statement, which said that due to the missing stimulation through folic acid and its derivatives the expression level of *mthf-1* is kept on a lower level. Therefore, the higher expression in nematodes being fed with MH828 compared to those who received MH951 bacteria, results from the permanent replenishment of the vitamin through the food source.

A conspicuous result can be seen after supplementation. Nematodes fed with MH828 as well as with MH951 bacteria both show a higher expression of *mthf-1*. This result might be an indication that under supplementation a higher production of 5-methyl-THF is needed than without a supplementation. This might appear due to high homocysteine levels during deficiency which should be measured in further studies. 5-methyl-THF is needed for the remethylation of methionine, but when it is unavailable, because of folate deficiency in the diet, levels of homocysteine could elevate. High levels of homocysteine are known to be harmful for cells [WOO et al., 1997]. Thus, they try to eliminate homocysteine as soon as enough folic acid is available, e.g. because of a supplementation. Since the remethylation step of methionine also leads to the

formation of THF which enhances the generation of 5-methyl- THF (+ *mthf-1!!!*), all the other investigated genes might but do not necessarily have to be higher expressed to support the thesis that cells raise the expression of *mthf-1* in order to quickly eliminate the accumulation of homocysteine.

5.2.2.4 Control nematodes show a higher expression of *metr-1* than those fed with MH951 bacteria

Like all other enzymes being investigated and involved in folate degradation pathway, *metr-1* is higher expressed in control nematodes fed with MH828 bacteria when compared to those being fed with MH951 and folate deficiency. As visible in **Figure 16** the enzyme *metr-1* is involved in a side pathway of the folic acid metabolism, which is responsible for the remethylation of homocysteine back to methionine. When the overall availability of folic acid is insufficient, the remethylation of homocysteine is also influenced.

As the expression behavior of non-supplemented nematodes followed the one observed in the previous enzymes, *folt-1,* Y4C6B.5, *dhfr-1* and *mthf-1*, supplementation did not result in any alteration concerning *metr-1*.

5.2.2.5 Non-supplemented nematodes fed with MH828 compared to those being supplemented and fed with MH951 bacteria

Besides the interest in how the expression of the presented genes might change due to an enforced folate deficiency and/or a folic acid supplementation, one of the biggest demands towards this thesis was to find out whether the administration of the folic acid could rescue the effect on gene expression caused by folate deficiency to the level control worms. None of the investigated genes in supplemented nematodes fed with MH951 bacteria was expressed on comparable level of those measured in control worms without supplementation.

5.2.2.6 Summary

According to the presented results, the intake of folic acid is preferred in nematodes, which suffer from a folate deficiency induced by feeding MH951 *E. coli*. This is indicated by the extremely high expression of Y4C6B.5 compared with control worms with higher *folt-1* expression. Therefore, due to the folat

deficiency a switch in which transporter is favored occurs. *Dhfr-1*, *mthf-1* and *metr-1* show a higher expression in control nematodes which could be due to stimulation of sufficient intracellular levels of folic acid and folate. For most of the investigated enzymes folic acid supplementation leads to decreased expression.

5.2.3 Differences in supplemented and non-supplemented wild type nematodes fed with SMX (0 µM or 10µM) treated OP50 bacteria

In contrast to above, the deficiency described in this chapter was not induced by the use of genetically modified bacteria (MH951) but by normal OP50 bacteria which were grown in a minimal media in which 10 µM sulfamethoxazole (SMX) were added. SMX competitively inhibits the folic acid *de novo* biosynthesis in bacteria.

5.2.3.1 Two different ways to induce a folate deficiency show different preference in the main transporter

In contrast to the expression results of nematodes fed with either MH828 or MH951 bacteria, nematodes suffering from folate deficiency, induced by the use of 10 µM SMX, seem to prefer the expression of *folt-1* whereas Y4C6B.5 is more important for control worms fed with 0 µM SMX treated OP50 bacteria. Therefore, two different ways to induce the folate deficiency generate two opposite results concerning the preference for one of the possible transporters.

The sulfonamid SMX inhibits the *de novo* synthesis of folic acid. Thus and because of the bacteria were grown in minimal media, no folic acid is available for the nematodes. Therefore, the lower expression of Y4C6B.5 might be explained by the missing availability of folic acid. In contrast to that, MH951 and MH828 bacteria were grown in full LB media, which allowed the intake of external folic acid. Furthermore, the *de novo* biosynthesis of folic acid within the bacteria was unaffected. Due to the *dhfr-1* deficiency within MH951 bacteria, the concentration of folates could be reduced which might have induced the lower expression of *folt-1*.

In both feeding types, a folic acid supplementation led to lower expression of *folt-1* as well as Y4C6B.5. As mentioned in the text above, current literature supports these findings [SAID et al., 2010; MCCORMICK, 2010; DEV et al., 2011]. Again, this indicates that fewer transporters are needed for a sufficient supply as soon as the folic acid maintenance is ensured, e.g. through supplementation.

5.2.3.2 Folate deficiency induces a higher expression of *dhfr-1*

The enzyme *dhfr-1* is higher expressed in nematodes with folate deficiency than in control. In the comparison of N2 wild type and RB755 nematodes, *dhfr-1* was higher expressed in those who used FOLT-1 as their main transporter. Here, same observation could be made. Nematodes fed with 10 µM SMX treated bacteria showed a higher *folt-1* expression. And like RB755, they also show a higher expression of *dhfr-1*. This could be seen as a support of the explanation made earlier: The folates transported by FOLT-1, or in this case the expression of the transporter itself, might be responsible for a subsequent raise in the expression of *dhfr-1* and *mthfr-1* respectively.

In the cases in which OP50 were selected as food source, grown in either normal or minimal medium, the expression of transporters and enzymes involved in folate metabolism seems to tend into the same direction.

As for Y4C6B.5 and *folt-1*, folic acid supplementation led to a lower expression of *dhfr-1*. The reasons or attempts of explanations can be found above.

5.2.3.3 Higher expression of *mthf-1* due to folate deficiency

Like the enzyme *dhfr-1*, the expression of *mthf-1* is clearly increased in the nematodes suffering a folate deficiency. As soon as there is an alteration in the expression behavior due to an intervention, there might be two possibilities that are able to serve as an explanation.

First, previous steps within the affected pathway induce following reactions to adapt and therefore change their original behavior. Therefore, in this case, the higher expression of *mthf-1* in nematodes fed with 10 µM SMX treated bacteria compared with control is caused through the folate deficiency which forces cells

to express genes coding for enzymes of the folic acid degradation pathway in order to raise the possibility of an adequate supply.

The second possibility would be that reactions downstream of the affected pathway influence the expression of upstream enzymes through a so called feedback mechanism. Therefore, the cell might try to rise the generation of 5-methyl-THF in order to keep the elimination of homocysteine up as good as possible. For this generation, MTHF-1 is needed and therefore higher expressed.

Mthf-1 expression in supplemented nematodes fed with 10 µM SMX followed the tendency seen in all other enzymes under supplementation. As above, the interpretation can be found there.

Only in case of nematodes fed with 0 µM SMX, a so far non explainable divergence could be seen. Here, supplementation with folic acid led to a higher expression of *mthf-1* than in their non-supplemented control.

5.2.3.4 Folate deficiency leads to a lower expression of *metr-1*

As seen in nematodes that were fed with MH951 bacteria and therefore also suffered a folate deficiency, the expression of *metr-1* is decreased in those worms that were fed with 10 µM SMX treated bacteria. This might be explained by the assumption that through the availability of folic acid and its derivatives in nematodes fed with 0 µM SMX treated bacteria, the requirement of METR-1 is higher.

For this enzyme, no variation within the behavior of supplementation compared to above could be found.

5.2.3.5 Comparison of non-supplemented nematodes fed with 0 µM SMX treated OP50 and supplemented ones fed with 10 µM SMX treated bacteria

Again, additionally to the interest in which way a folic acid supplementation influences the expression of the selected genes, it was also attractive to find out whether the deliberately generated folate deficiency could be undone by an external folic acid supplementation. Against the expectations, the present

results show that neither the expression of *folt-1*, Y4C6B.5 and *dhfr-1* nor *mthfr-1* and *metr-1* in supplemented nematodes fed with 10 µM SMX treated bacteria could be brought back on to the level of control worms. With the exception of *dhfr-1*, the mean normalized mRNA expression of the genes in nematodes fed with 10 µM SMX treated bacteria stayed under the amount of the ones who were fed with 0 µM SMX treated OP50. Due to this outcome, it can be said that an external supplementation of nematodes who suffered a folate deficiency might not be sufficient to revise the effect caused by the previous deficiency.

5.2.3.6 Taken together

As seen in the previous comparison, nematodes suffering a folate deficiency seem to prefer the absorption of folate derivatives. The expression level of *dhfr-1* as well as *mthf-1* is increased in those nematodes who received the 10 µM SMX treated OP50 bacteria. Those nematodes, who suffered the folate deficiency showed a lower expression of *metr-1* which was explained by the assumption that the availability of folic acid and its derivatives in nematodes fed with 0 µM SMX treated bacteria, results in a higher requirement of *metr-1.* In this case and comparable to the relation seen in nematodes fed with either MH828 or MH951 bacteria, a folate deficiency seems to cause a decrease in the expression of affected enzymes. With the exception of *mthf-1* in nematodes fed with 0 µM SMX treated OP50 bacteria, all genes under investigations were lower expressed than their non-supplemented partners.

6 Summary

6.1 Abstract

The presented master thesis contains two different projects, which both deal with the nematode *C. elegans*. The aim of the first part of this work was to develop a HPLC-MS method, which was able to show reliably and reproducibly the differences in the metabolic profiles of nematodes. Therefore, a mixed population of starved and non-starved nematodes was analyzed under the same conditions. With that, it could be shown that the developed HPLC gradient is able to match the requested demand. Additionally to the reproducibility of the measurements, the gradient was used to reveal that storage leads to alterations within the metabolic profiles. Furthermore, when compared to the control population it could be shown that different treatments of the worm population- in this case a supplementation with folic acid - is the reason for alterations in the metabolic profiles. In the end, research approaches for further investigation were shown, which might be able to explain and support the statements made in this thesis.

The second project within this work investigated the impact of a folic acid deficiency onto the expression of five genes, which were selected out of the folic acid metabolism: *folt-1,* Y4C6B.5, *dhfr-1, mthf-1* und *metr-1*. Beside the investigation of differently fed wild type worms, RB755 nematodes, a second, methionine synthase deficient strain, was analyzed. Within this project it was also investigated whether the effect of the induced deficiency could be rescued by a folic acid supplementation. It could be shown that not only the selection of the strain as well as the choice of the food source have an impact on gene expression, but also the supplementation with folic acid leads to different data when compared to control worms. Within the discussion, it was possible to draw similarities of the current literature and the results of this work. Furthermore, new insights into this quite uninvestigated research area could be given.

6.2 Zusammenfassung

Die vorliegende Masterarbeit besteht aus zwei voneinander unabhängigen Projekten, die sich beide mit dem Nematoden *C. elegans* beschäftigen. Der erste Teil dieser Arbeit hatte zunächst zur Aufgabe eine HPLC-MS Methode zu entwickeln mit der man Unterschiede in den metabolischen Profilen der Würmer zuverlässig und vor allem auch reproduzierbar messen kann. Dafür wurden gemischte, hungernde und nicht hungernde Wurmpopulationen verwendet die anschließend unter den gleichen Bedingungen analysiert wurden. Dadurch konnte gezeigt werden, dass der entwickelte HPLC Gradient durchaus in der Lage ist Unterschiede in den metabolischen Profilen aufzuzeigen. Zusätzlich zu der Reproduzierbarkeit der Messungen wurde der Einfluss der Lagerung auf das Ergebnis untersucht. Desweiteren konnte mit Hilfe der entwickelten Methode gezeigt werden, dass unterschiedliche Behandlungsarten, in diesem Fall eine Folsäuresupplementierung der Wurmpopulationen, zu anderen metabolischen Profilen führen als sie die jeweiligen Kontrollpopulationen zeigen. Abschließend wurden noch für die Zukunft interessante Forschungsansätze aufgezeigt mit deren Hilfe genauere Aussagen über die Veränderungen innerhalb der einzelnen metabolischen Profile möglich sind.

Das zweite Projekt innerhalb dieser Arbeit untersuchte die Auswirkungen einer Folsäuredefizienz auf die Genexpression von fünf aus dem Folsäurestoffwechsel ausgesuchten Genen: *folt-1,* Y4C6B.5, *dhfr-1, mthf-1* und *metr-1*. Die Expressionsstudien wurden neben unterschiedlich gefütterten Wildtyp Nematoden zusätzlich noch an einem zweiten, Methioninsynthase-defizienten Stamm durchgeführt. Innerhalb von diesem Projekt wurde ebenfalls untersucht, ob die durch das Defizit ausgelösten Veränderungen durch die Gabe einer Folsäuresupplementierung wieder ausgeglichen werden können. Dabei konnte zum einen gezeigt werden, dass nicht nur der Nematodenstamm sowie die Futterwahl, sondern auch die Folsäuresupplementierung zu einer veränderten Expression der analysierten Gene führt. Im Diskussionsteil dieser Arbeit konnten schließlich sowohl Parallelen zu bereits bekannten Ergebnissen in der Literatur aufgezeigt werden als auch neue Erkenntnisse über dieses, bisher doch noch recht schlecht untersuchte Thema dargestellt werden.

Erklärung

Hiermit erkläre ich, Helen Fichtner, die vorliegende Arbeit selbstständig verfasst und keine anderen als die angegeben Quellen benutzt zu haben. Desweiteren versichere ich, die Stellen im Text gekennzeichnet zu haben, die dem Wortlaut oder Sinn nach aus anderen Arbeiten entnommen wurden.

Datum/Unterschrift

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8 Appendix

1. Remaining graphs of the gene expression study

A)

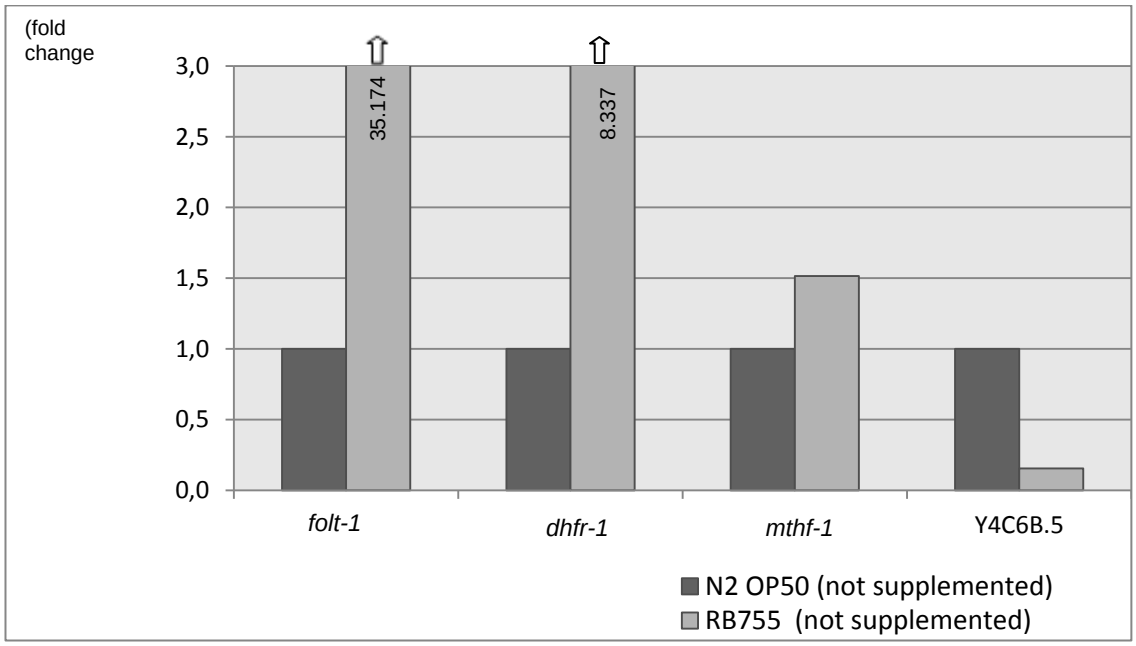

Figure 23: Fold change of non-supplemented N2 wild type nematodes (dark) compared to non-supplemented RB755 (light). With the exception of Y4C6B.5, the expression of non-supplemented RB755 exceeds the one of non-supplemented N2 wild type nematodes.

B)

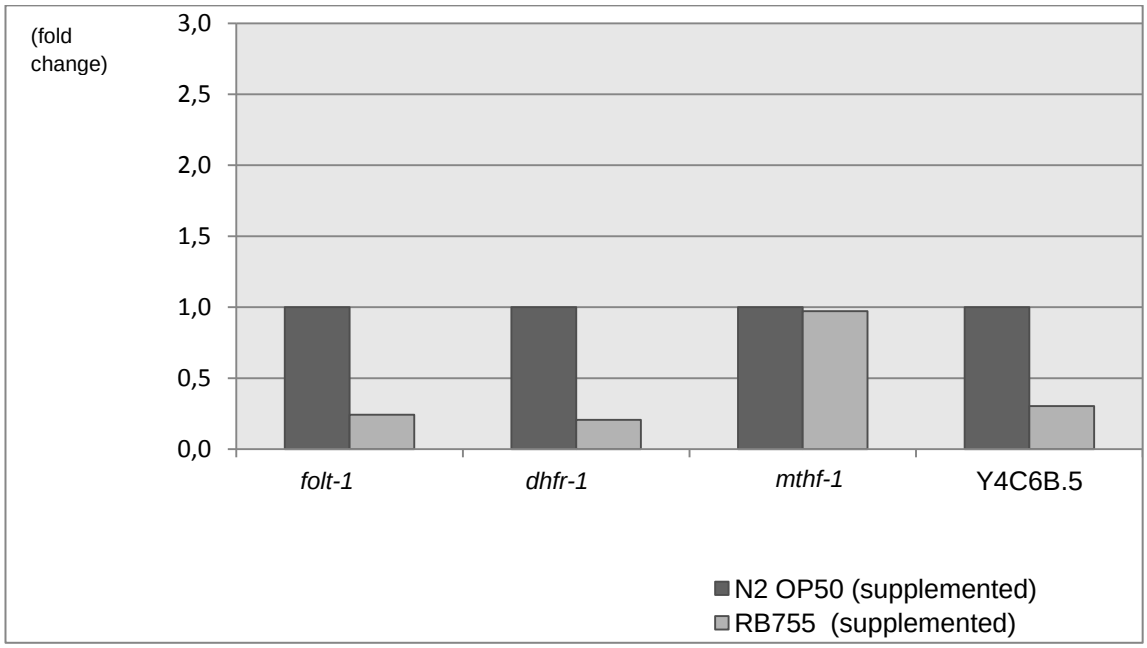

Figure 24: Fold change of supplemented N2 wild type nematodes (dark) compared to supplemented RB755 (light). In all cases, the expression of supplemented RB755 is lower when compared to supplemented N2 wild type nematodes.

C)

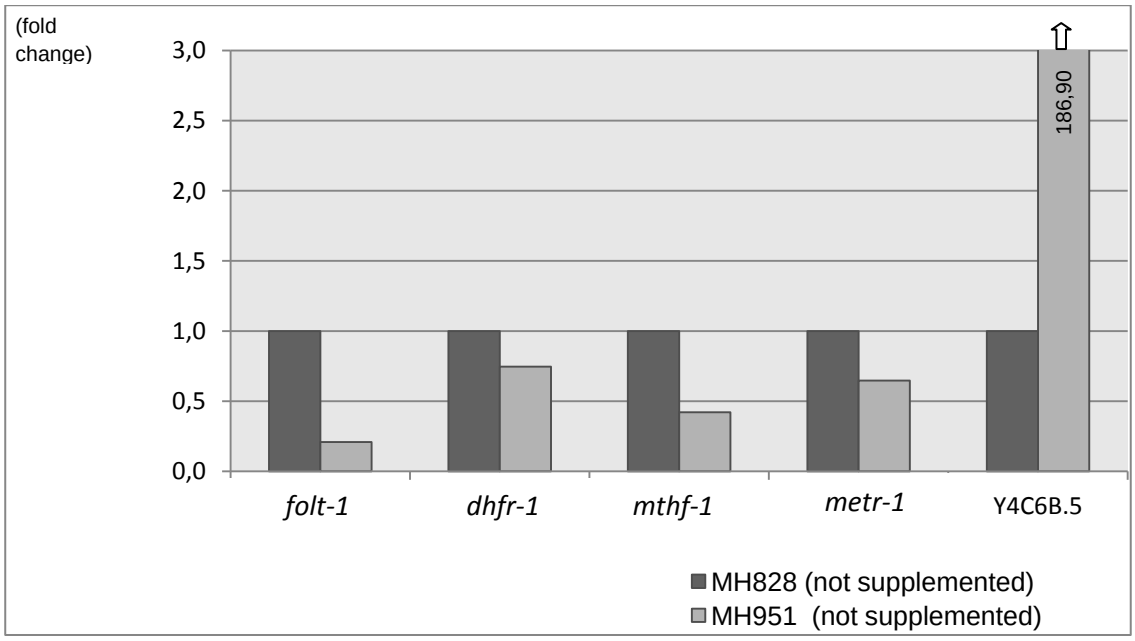

Figure 25: Fold change of non-supplemented N2 (MH828) (dark) compared to non-supplemented N2 (MH951) (light). With the exception of Y4C6B.5, the expression of non-supplemented N2 (MH828) exceeds the one of non-supplemented N2 (MH951) nematodes.

D)

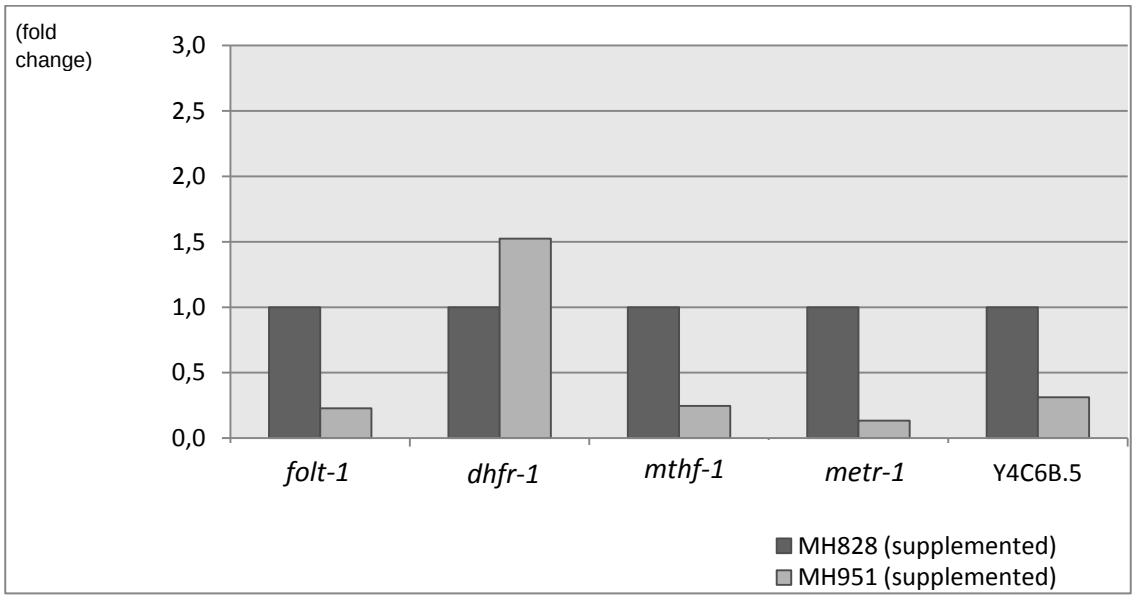

Figure 26: Fold change of supplemented N2 (MH828) (dark) compared to supplemented N2 (MH951) (light). With the exception of *dhfr-1*, the expression of supplemented N2 (MH828) exceeds the one of supplemented N2 (MH951) nematodes.

E)

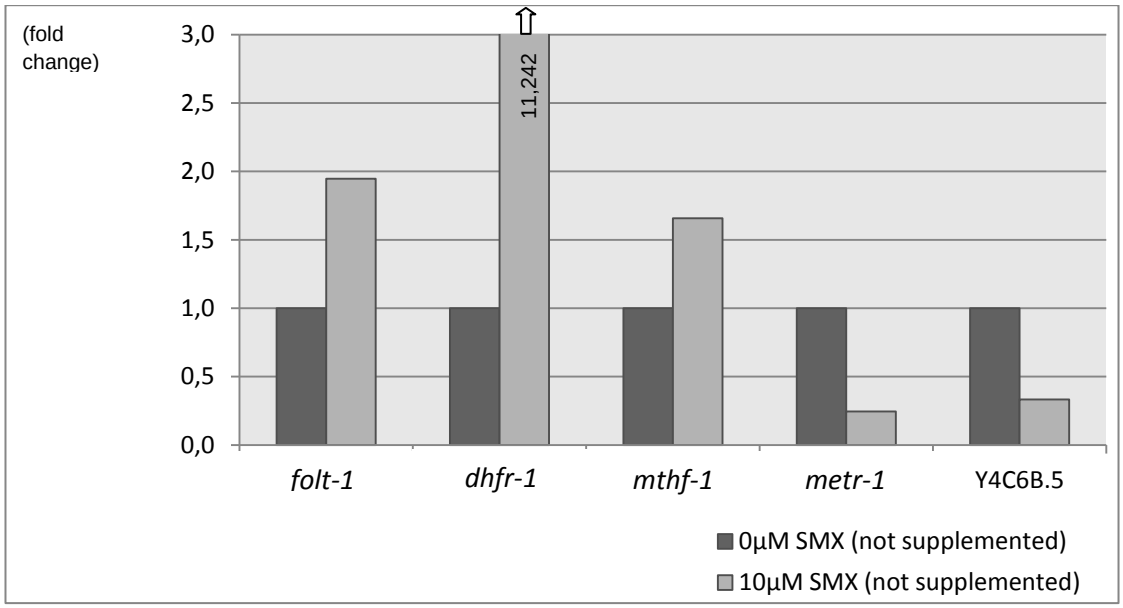

Figure 27: Fold change of non-supplemented N2 fed with 0 µM treated OP50 bacteria (dark) compared to non-supplemented N2 fed with 10 µM treated OP50 bacteria (light). *Metr-1* and Y4C6B.5 are lower expressed in nematodes being fed with 10 µM treated OP50 bacteria. Folt-1, dhfr-1 and *mthf-1* are all higher expressed when the nematodes were fed with the 10 µM treated OP50 bacteria.

F)

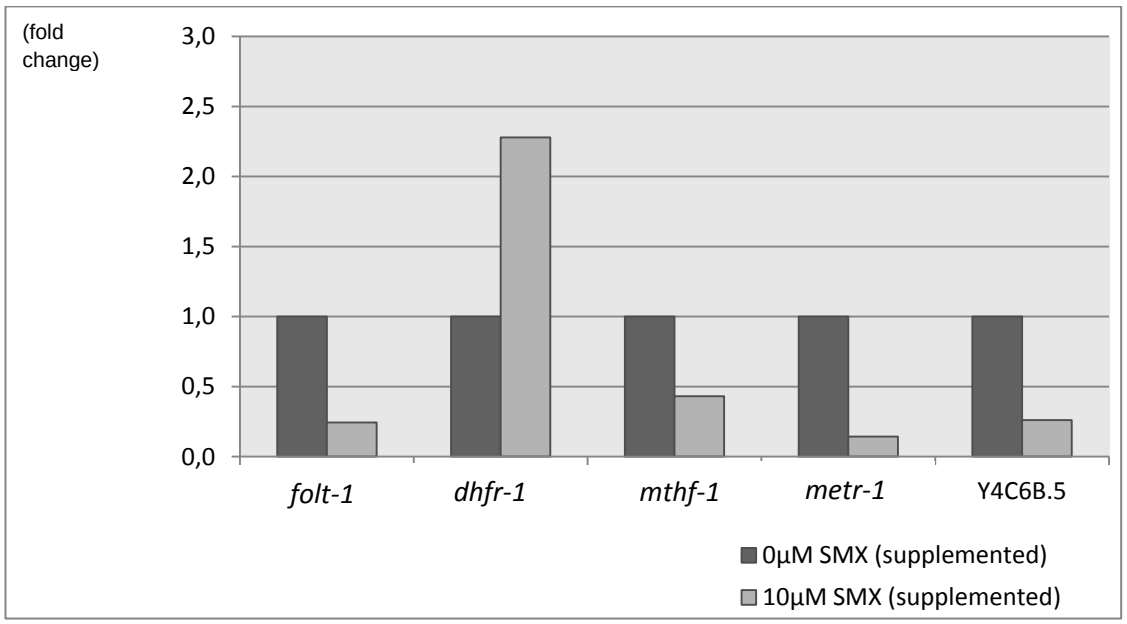

Figure 28: Fold change of supplemented N2 fed with 0 µM treated OP50 bacteria (dark) compared to supplemented N2 fed with 10 µM treated OP50 bacteria (light). Only the gene dhfr-1 is higher expressed in those nematodes being fed with 10 µM treated OP50 bacteria. In all other cases is the expression lower.

2. Remaining metabolic profiles of stored and non-stored samples

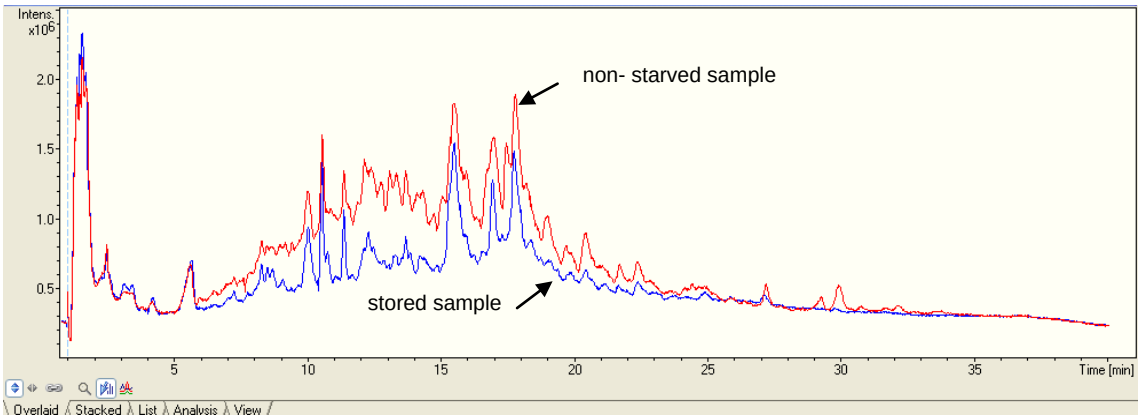

Figure 29 Metabolic profile of non-starved versus starved wild type nematodes. Metabolic profile of starving (blue) and non-starving (red) N2 var. Bristol wild type. (mixed population)

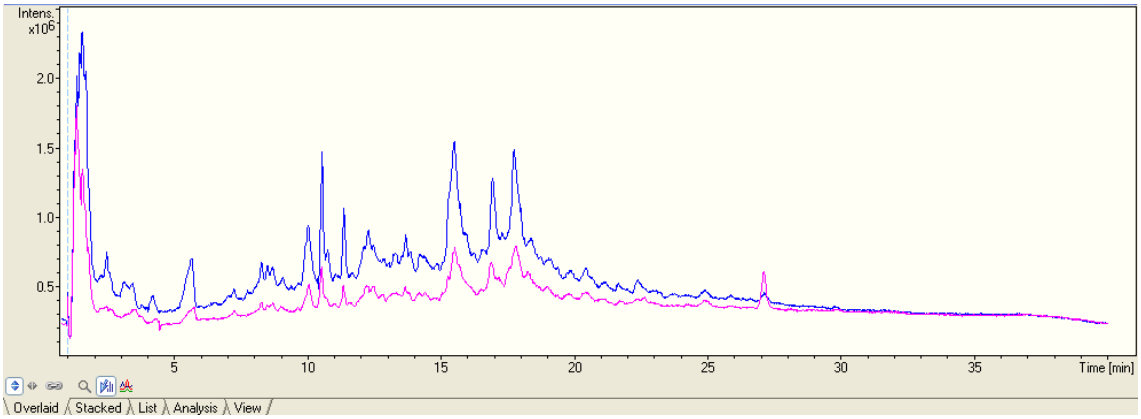

Figure 30: Metabolic profile of starved wild type nematodes. Two metabolic profiles of starving N2 var. Bristol wild type nematodes fed with OP50 bacteria to see if the developed gradient can produce repeatable results. (mixed population)

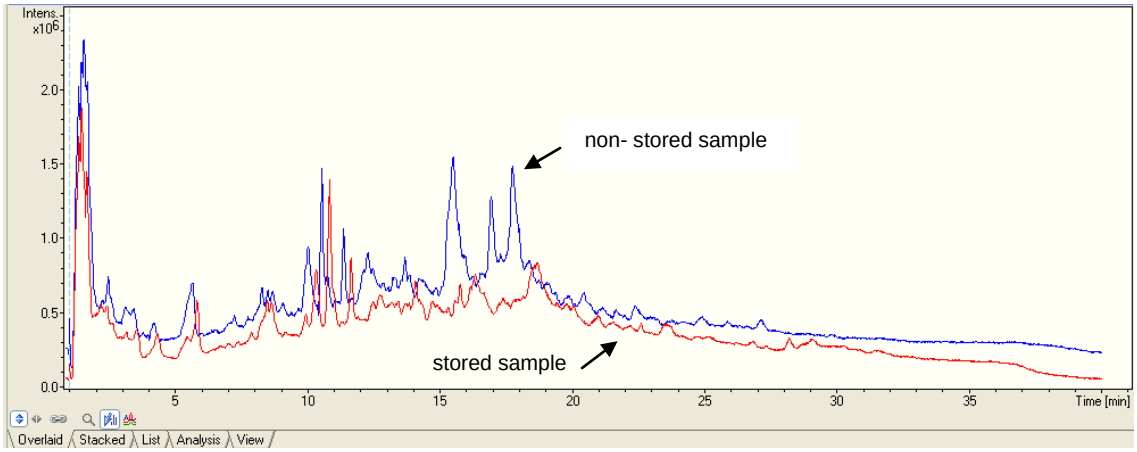

Figure 31: Metabolic profile of starved and stored wild type nematodes. Metabolic profile of N2 var. Bristol wild type nematodes fed with OP50 bacteria. Before they were stored at -20°C, they have had to starve for 17h over night. The blue graph shows the profile of the sample without storage. The red graph shows the stored sample. (mixed population)

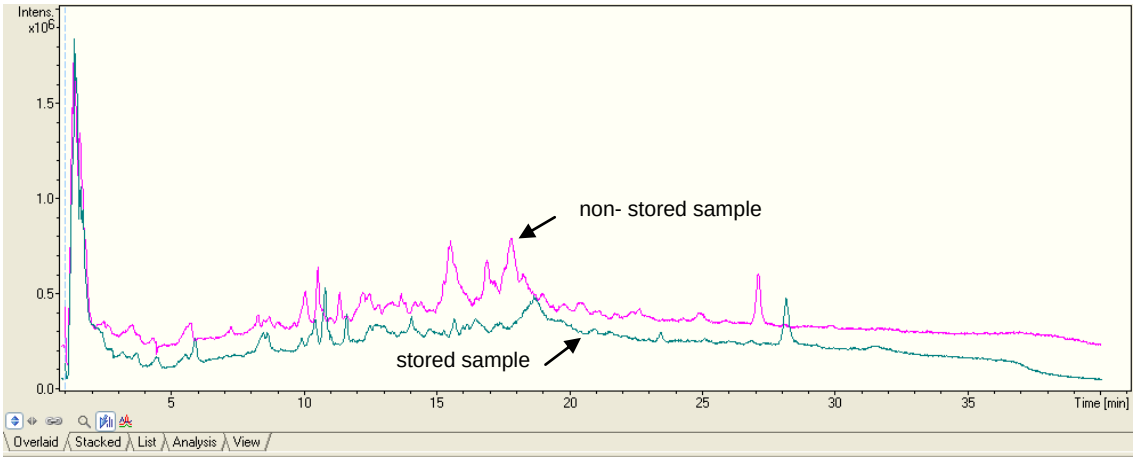

Figure 32: Second metabolic profile of starved and stored wild type nematodes. Metabolic profile of N2 var. Bristol wild type nematodes fed with OP50 bacteria.Before they were stored at -20°C, they have had to starve for 17h over night. The pink graph shows the profile of the sample without storage. The green graph shows the stored sample. (mixed population)

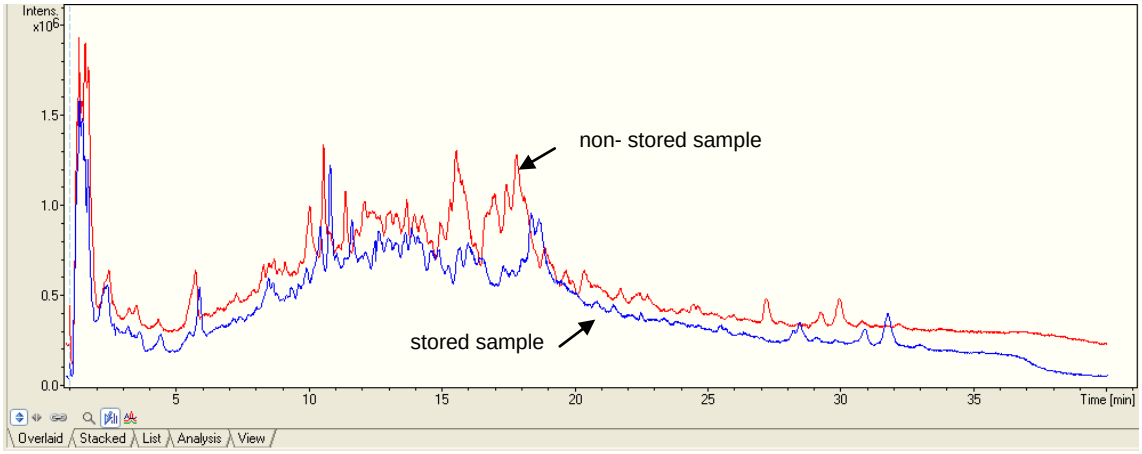

Figure 33: Metabolic profile of non-starved and stored wild type nematodes. Metabolic profile of N2 var. Bristol wild type nematodes fed with OP50 bacteria. The red graph shows the profile of the sample without storage. The blue graph shows the storedsample

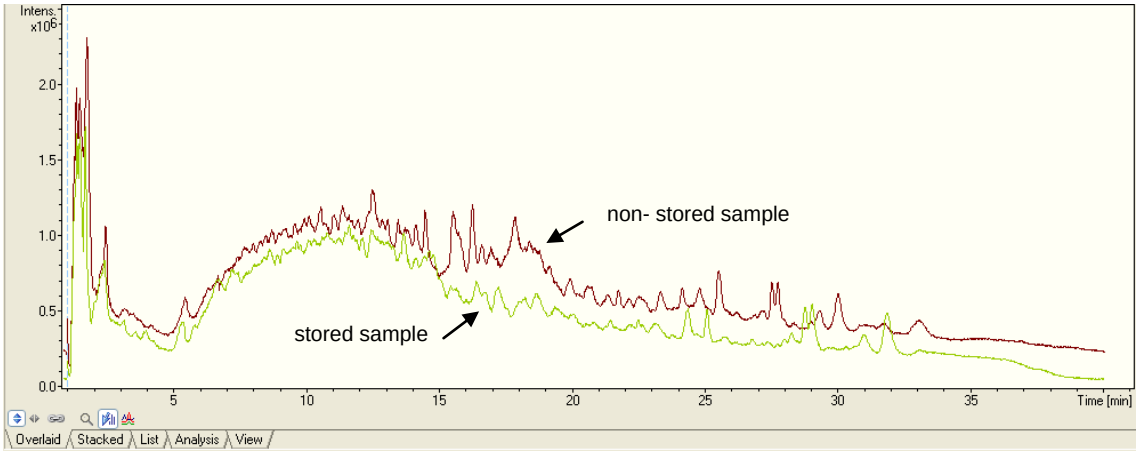

Figure 34 Metabolic profile of supplemented and stored wild type nematodes fed with OP50 bacteria. The brown graph shows the profile of the sample without storage. The light green graph shows the stored sample.

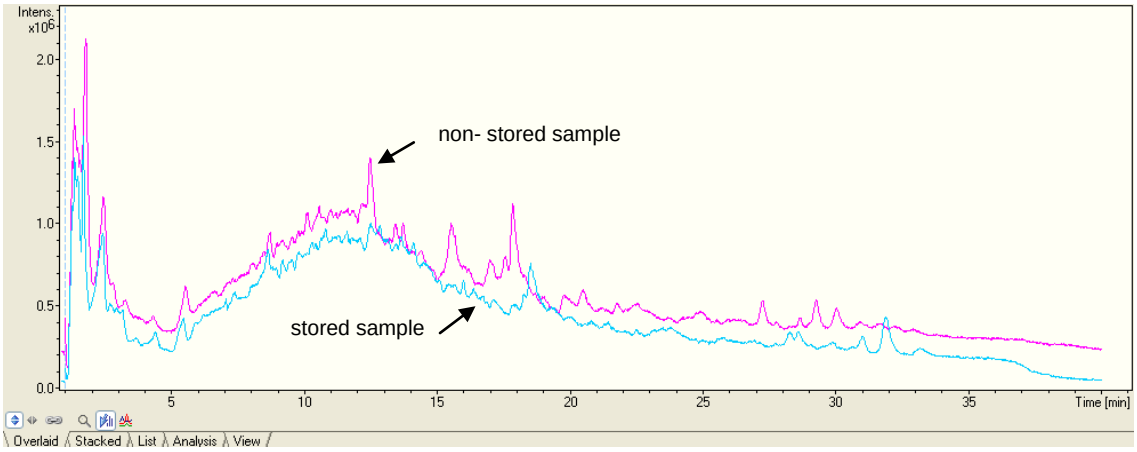

Figure 35: Metabolic profile of non-supplemented and stored wild type nematodes fed with OP50 bacteria. The pink graph shows the profile of the sample without storage. The light blue graph shows the stored sample. (aged synchronized population, L4-larvae)

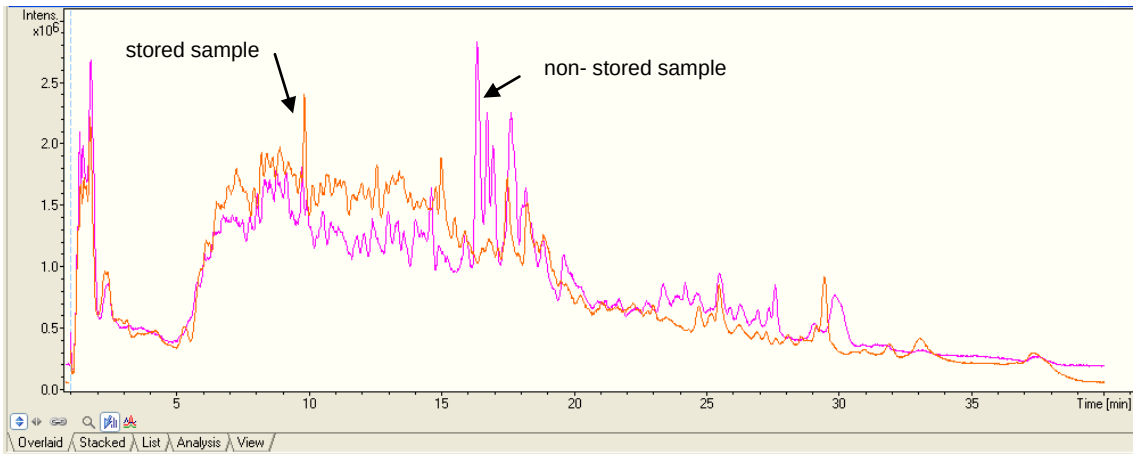

Figure 36: Metabolic profile of supplemented and stored RB755 nematodes fed with OP50 bacteria. The pink graph shows the profile of the sample without storage. The orange graph shows the stored sample. (aged synchronized population, L4-larvae)

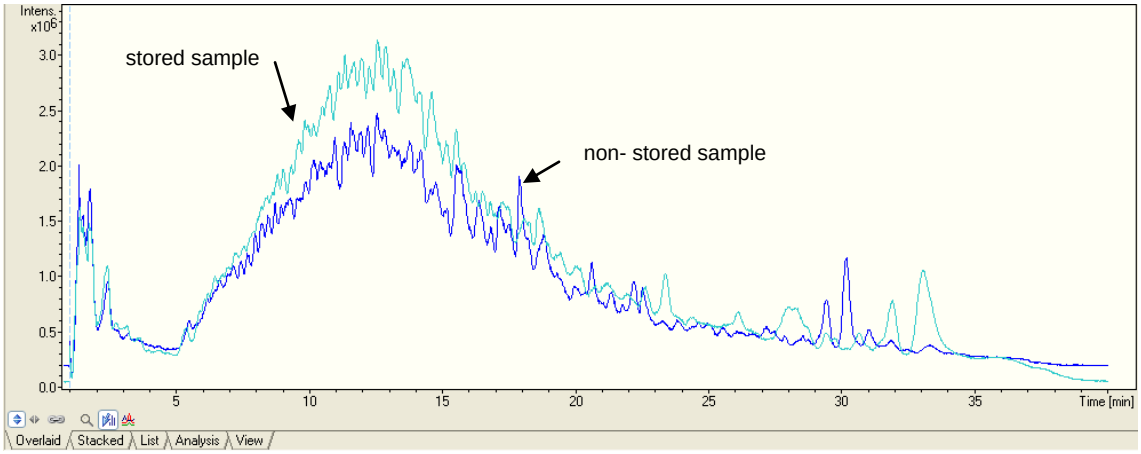

Figure 37: Metabolic profile of non-supplemented and stored RB755 nematodes fed with OP50 bacteria. The dark blue graph shows the profile of the sample without storage. The light blue graph shows the stored sample. (aged synchronized population, L4-larvae)

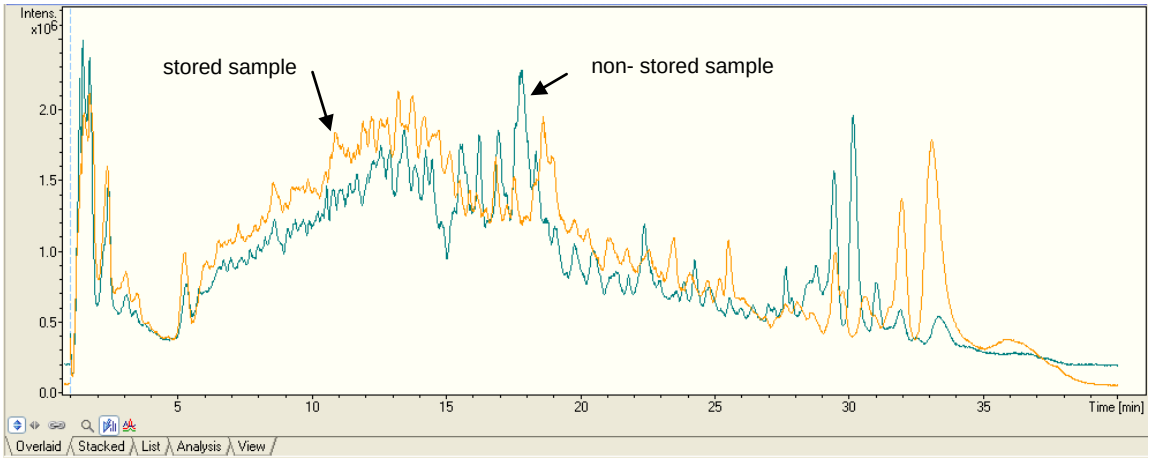

Figure 38: Metabolic profile of supplemented and stored wild type nematodes fed with MH828 bacteria. The green graph shows the profile of the sample without storage. The orange graph shows the stored sample. (aged synchronized population, L4-larvae)

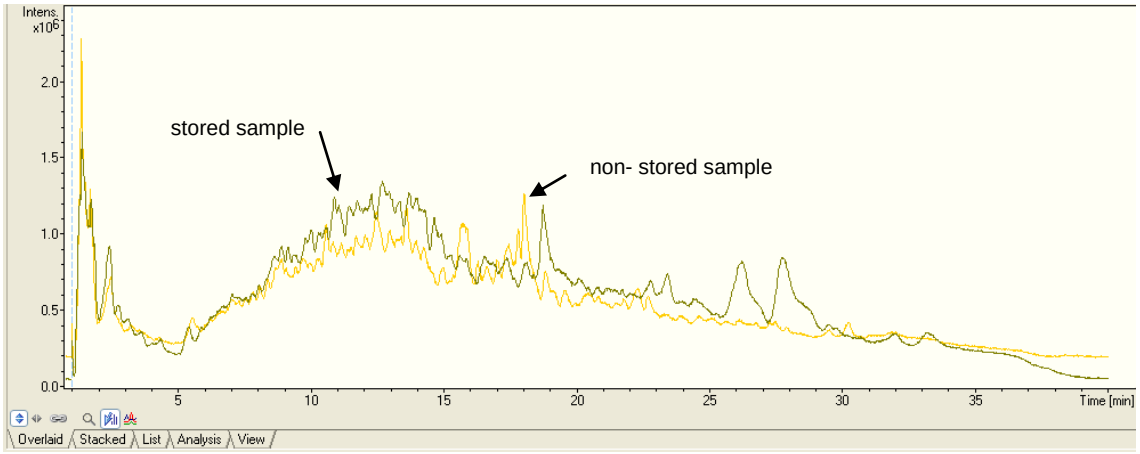

Figure 39: Profile of non-supplemented and stored wild type nematodes fed with MH828 bacteria. The yellow graph shows the profile of the sample without storage. The green graph shows the stored sample. (aged synchronized population, L4-larvae)

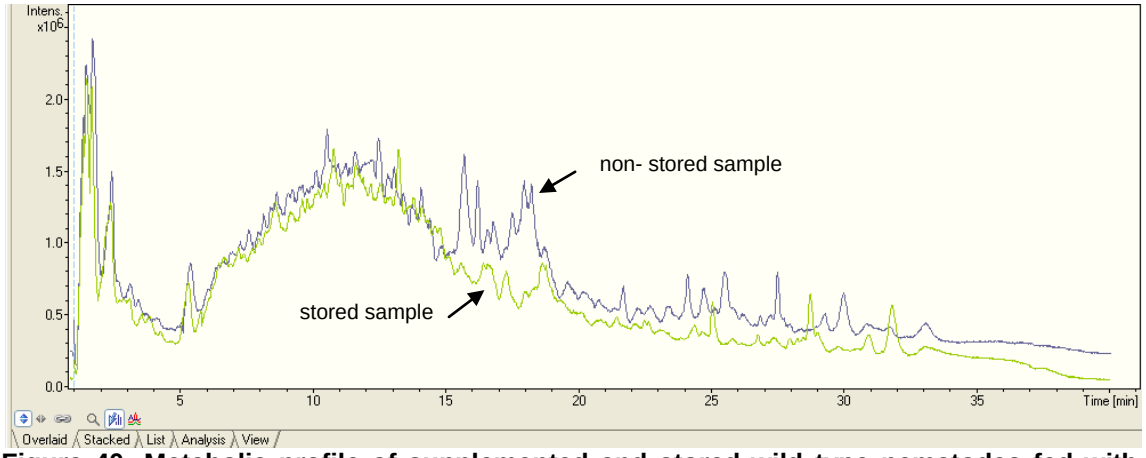

Figure 40: Metabolic profile of supplemented and stored wild type nematodes fed with MH951 bacteria. The blue graph shows the profile of the sample without storage. The green graph shows the stored sample. (aged synchronized population, L4-larvae)

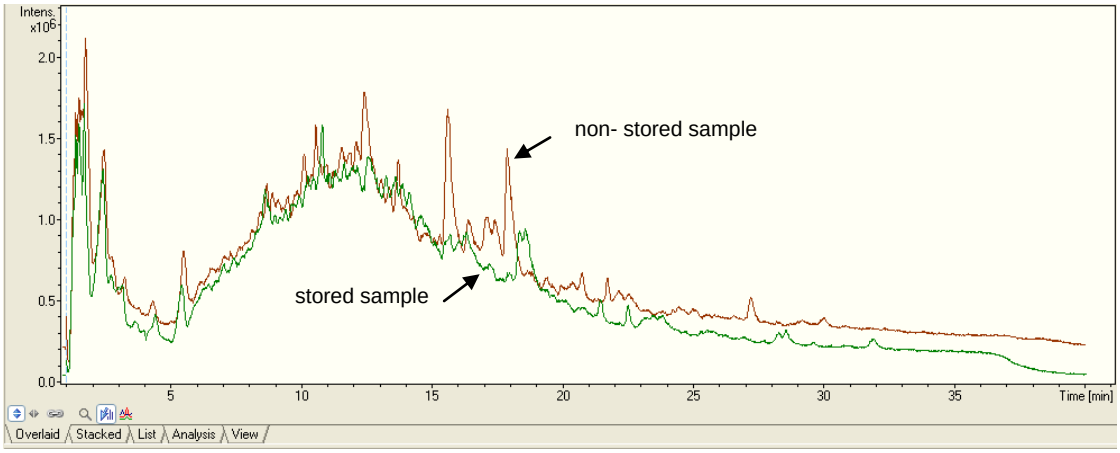

Figure 41: Metabolic profile of non-supplemented and stored wild type nematodes fed with MH951 bacteria. The brown graph shows the profile of the sample without storage. The green graph shows the stored sample. (aged synchronized population, L4-larvae)

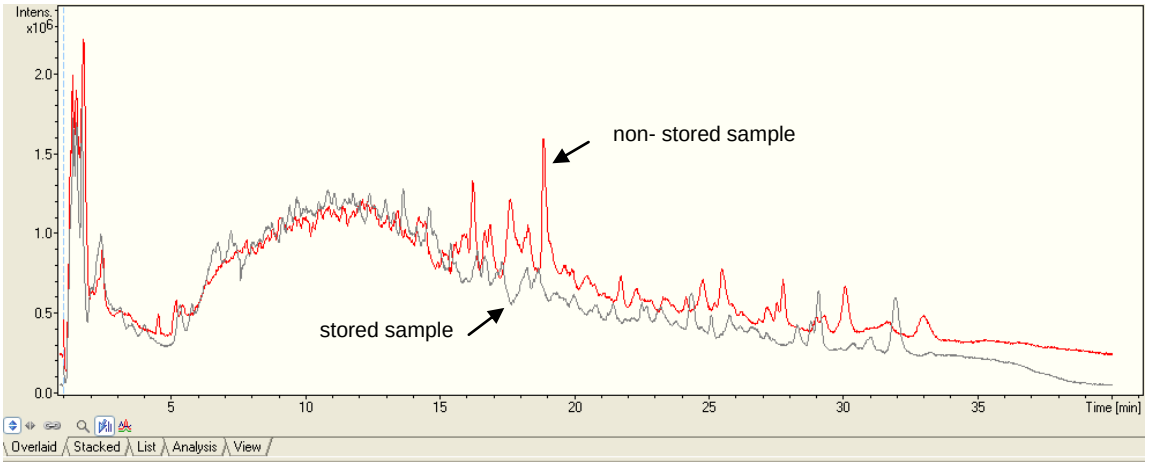

Figure 42: Metabolic profile of supplemented and stored wild type nematodes fed with OP50 bacteria, which were grown in minimal media and to which 0µM sulfamethoxazol were added. The red graph shows the profile of the sample without storage. The grey graph shows the stored sample. (aged synchronized population, L4-larvae)

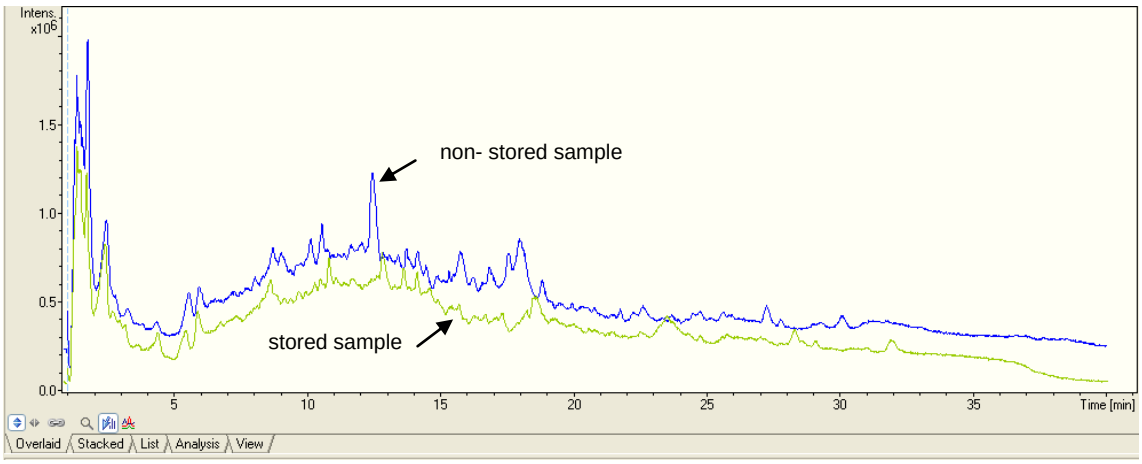

Figure 43: Metabolic profile of non-supplemented and stored wild type nematodes fed with OP50 bacteria, which were grown in minimal media and to which 0µM sulfamethoxazol were added. The blue graph shows the profile of the sample without storage. The green graph shows the stored sample. (aged synchronized population, L4-larvae)

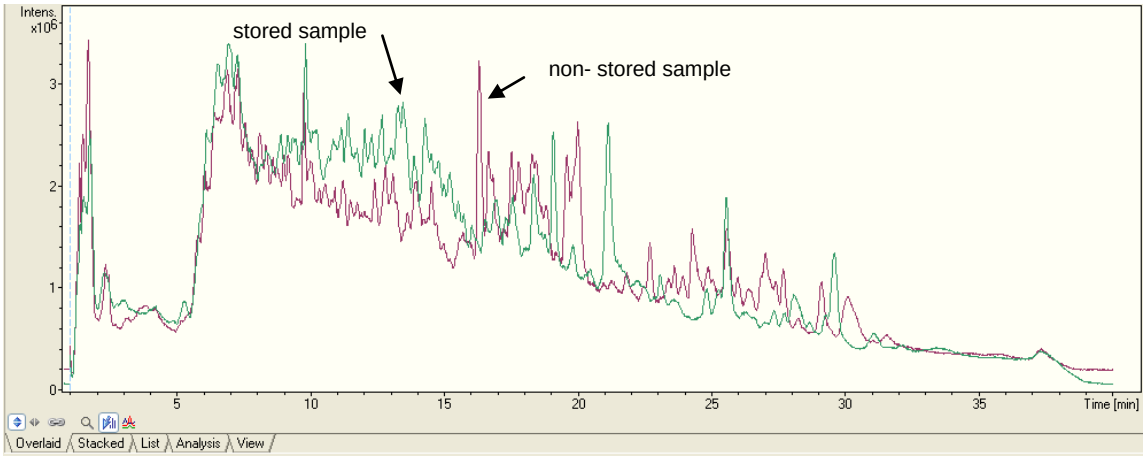

Figure 44: Metabolic profile of supplemented and stored wild type nematodes fed with OP50 bacteria, which were grown in minimal media and to which 10µM sulfamethoxazol were added. The purple graph shows the profile of the sample without storage. The green graph shows the stored sample. (aged synchronized population, L4-larvae)

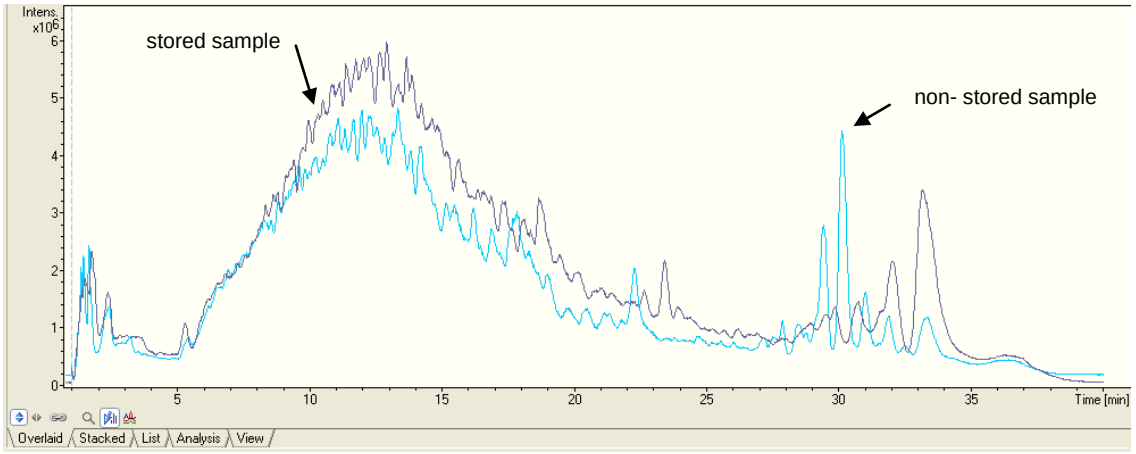

Figure 45: Metabolic profile of non-supplemented and stored wild type nematodes fed with OP50 bacteria, which were grown in minimal media and to which 10µM sulfamethoxazol were added. The light blue graph shows the profile of the sample without storage. The dark blue graph shows the stored sample. (aged synchronized population, L4-larvae)

3. Abbreviations

%	percent
°C	degree Celsius
µg	microgram
µl	microliter
µm	micrometer
CAN	acetonitrile
C. elegans	Caenhorhabditis elegans
CaCl2	calcium chloride
CGC	Caenorhabditis Genetic Center
csq1	calsequestrin1
CT-values	cycle threshold
DEPC	diethyl pyrocarbonate
dH2O	distilled water
dhfr	Dihydrofolate reductase
DYT	medium for bacteria to grow in
EtOH	ethanol
folt-1	gene coding for a folate transporter
g	gram
gDNA	genomic deoxyribonucleic acid
Glu	glucose
h	hour
HC	homocysteine
HPLC	high pressure liquid chomatography
HKG	housekeeping gene
K2PO4	potassium phosphate
KH2PO4	potassium dihydrogen phosphate
KOH	potassium hydroxide

L1	first of overall four larvae stages
L4	last of overall four larvae stages
LB	Lysogeny broth
LC	liquid chromatography
M	molarity
M9	name of a buffer solution
mdh2	Primer (target gene)
MgSO4	magnesium sulfate
MH828	bacteria strain (food source for the nematodes)
MH951	bacteria strain (food source for the nematodes)
ml	milliliter
MS	mass spectrometer
ms	Primer (target gene)
mthfr	Primer (target gene)
N2	*C. elegans* Bristol N2 wild-type
Na2HPO4- 7H2O	Disodium phosphate heptahydrate
NaCl	sodium chloride
NaOCl	sodium hypochlorite
NGM-agar	natural growth medium- agar
NH4Cl	ammonium chloride
NMR	Nuclear magnetic resonance
OP50 *E. coli*	food for *C. elegans*
PCA	principal component analysis
PCR	poly chain reaction
pH	pH- value
pmp3	Primer (housekeeping gene)
ppb	parts per billion
qPCR	Real time quantitative PCR

RB755	*C. elegans* strain, methionin synthase deficient
RFC	reduced folate carrier
RNA	ribonucleic acid
rpm	rounds per minute
SD	standard deviation
SMX	Sulfamethoxazole
UPLC	Ultra Performance Liquid Chromatography
Vol.	volume
Y4C6B.5	Primer (target gene)
Y45F10D.4	Primer (housekeeping gene)

4. List of figures

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Lebenslauf

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