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Biodegradation of hydrocarbons in constructed wetlands: evidence and qualification with ^{13}C labelling methods

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Abstract/Kurzfassung

Abstract

Petroleum hydrocarbon (PHC) pollution happens frequently in our modern fuel- dependent civilization. Controlled biodegradation with constructed wetlands (CWs) aims at an environmental friendly remediation. In CWs many factors determine the microbial consortia and their biodegradation potential, which we tried to recapitulate in an extensive literature review. Furthermore, we tried to give impulses for future research to enhance biodegradation approaches. The experimental aim of our study was to find out which microbial groups are involved in biodegradation in a certain CW setup.

For this purpose we investigated the degradation of hydrocarbons (HCs), in this case Hexadecane (C_{16}), in an “in vitro” experiment under poor nutrient conditions. Substrate, in this case sand, as well as groundwater samples were taken from the constructed wetland (CW) which was installed at a diesel contaminated site. Samples were incubated in 20 gastight microcosms (MCs) under amendment free conditions with 12°C. Additionally to the groundwater pollution, 10 MCs were contaminated with 30 µg of ^{13}C labelled (δ= 100‰) Hexadecane (C_{16}). First of all, samples from the gaseous phase of 5 MCs were taken after a determined schedule (after 0, 6, 11, 24, 48, 96, 192, 312, 504 hours) in order to prove degradation. These samples were injected by hand into a Gas Chromatograph Combustion Isotope Ratio Mass Spectrometer (GC-C-IRMS) in order to determine the isotopic change of the CO_2 regarding time. Furthermore, after the gas measurements were finished, the other 15 MCs were used for phospholipid fatty acid analyses with stable isotope probing (PLFA-SIP). They were freeze dried after 0, 2 and 19 days, extracted and measured with an GC-C-IRMS to identify which MO groups show label incorporation.

The isotopic CO_2 measurements revealed a significant increase in the δ value. Consequently, the CW microorganism (MO) consortium was viable for biodegradation, even under poor nutrient conditions. PLFA- SIP analyses revealed that the most promising HC-degrading microbial groups were gram negative bacteria and fungi.

In conclusion PLFA- SIP offers a viable method to determine the most efficient biodegradation setup of different CW compositions.

Kurzfassung

Verschmutzungen mit Mineralölprodukten passieren häufig in unserer von fossilen Brennstoffen abhängigen Gesellschaft. Kontrollierter biologischer Abbau mit Pflanzenkläranlagen zielt auf eine umweltfreundliche Lösung für dieses Problem. In Pflanzenkläranlagen, eine Vielzahl an Faktoren bestimmen die mikrobielle Zusammensetzung und deren biologisches Abbaupotential. Wir haben versucht diese Faktoren in einer erweiterten Literaturarbeit zusammen zu fassen. Außerdem wollten wir Ansätze für die zukünftige Forschung geben um den kontrollierten biologischen Abbau zu verbessern. Der experimentelle Teil der Arbeit zielte auf die Bestimmung der mikrobiellen Gruppen in einer Pflanzenkläranlage die für den biologischen Abbau von Verschmutzungen verantwortlich sind.

Für diesen Zweck haben wir den Abbau von Kohlenwasserstoffen, in diesem Fall Hexadecan, in einem Laborexperiment ohne Nährstoffzusätze getestet. Füllmaterial und Grundwasserproben wurden dabei entnommen von einer Pflanzenkläranlage, die an einem mit Diesel kontaminierten Standort installiert wurde. Die Proben wurden in 20 gasdichten Mikrokosmen, bei 12 °C und wie erwähnt, ohne Nährstoffzugabe inkubiert. Zusätzlich zur Dieselkontamination des Grundwassers, wurden in 10 Mikrokosmen noch 30µg von ^{13}C markiertem (δ= 100‰) Hexadecan (C_{16}) dazugegeben. Als erstes wurden aus 5 Mikrokosmen CO_2 Proben entnommen, nach 0, 6, 11, 24, 48, 96, 192, 312 und 504 Stunden um den biologischen Abbau anhand vom Einbau in das veratmete CO_2 nachzuweisen. Dafür wurden Gasproben per Hand in einen Gas Chromatograph Combustion Isotope Ratio Mass Spectrometer (GC-C-IRMS) injiziert. Weiters wurden dann PLFA Extraktionen mit den anderen 15 Mikrokosmen durchgeführt, wobei dann auch das Isotopenverhältnis (PLFA-SIP) mit der GC-C-IRMS gemessen wurde. Die Proben wurden gefriergetrocknet nach 0, 2 und 19 Tagen, extrahiert und gemessen um festzustellen welche Mikroorganismengruppen am meisten ^{13}C eingebaut haben.

Die CO_2 Messungen zeigten das es einen signifikanten Anstieg des δ Wertes gab, was darauf hinweist das die Mikroorganismen auch unter nährstoffarmen Verhältnissen fähig sind Hexadecan biologisch abzubauen. Die PLFA- SIP Analysen offenbarten das die Mikroorganismengruppen gram negative Bakterien und Pilze am meisten ^{13}C assimiliert haben.

Abschließend bleibt noch zu erwähnen, dass PLFA- SIP Analysen eine sehr geeignete Methode zur Bestimmung des effizientesten Pflanzenkläranlagen- Setups ermöglichen.

Keywords and abbreviations

Keywords: bioremediation, biodegradation, Phospholipid fatty acids, constructed wetland, diesel, petroleum hydrocarbons, contamination, willow

Abbreviation	Explanation	Explanation on page
HC	hydrocarbons	2
PHCs	petroleum hydrocarbons	2
MC	microcosm	2
MO, MOs	microorganism, microorganisms	2
CW	constructed wetland	2
PLFA	phospholipid fatty acids	2
δ	delta- value	2
BTEX	benzene toluene ethylbenzene xylenes	8
PAH	polyaromatic hydrocarbons	9
NAPL	nonaqueous phase liquids	10
LP	longstanding pollution	11
pH	potentia hydrogenii	21
N	nitrogen	22
P	phosphor	22
K	potassium	22
Fe	iron	22
MTBE	methyl-*tert*-butylether	32
H- value	Henry coefficient	32
KOC value	carbon partition coefficient	33
S	sulfur	33
O	oxygen	33
D	deuterium	33
CSIA	compound specific analyses	35
SIP	stable isotope probing	35
app.	approximately	32
REPMIX	representative mixture for diesel	37
LOWMO	adapted extraction method for low MO content	48
FAME	fatty acid methyl esters	49
FID	flame ionization detection	49

Table of content

Tables and figures

Tables:

Figures:

1.Introduction

1.1 Environmental pollution with PHCs

1.1.1 General aspects of environmental pollution with PHCs

The origin of petroleum oil or petroleum hydrocarbons (PHCs) is the geochemical formation of hydrocarbons (HCs). Thereby, more and more complex organic compounds are formed by reactions with high pressure and temperature of buried biomass over geological periods. The reserves of organic carbon in oil is estimated with ~0.23 * 10^{12} tonnes of carbon (Tissot and Welte, 1984).

For our industrialized civilization, the availability of HCs as fuels and starting compounds for a vast range of chemical syntheses is of great importance (Widdel and Rabus, 2001). Furthermore, they play an important role in providing energy for transportation, power generation, industrial growth, agricultural production and other basic human needs (Basha et al., 2009). For instant, in the energy production, petroleum is the second most used source after coal and 94 % of total recovered petroleum is used as fuel (Ball and Truskewycz, 2013). The world oil consumption of petroleum liquids in 2009 was 84.5, in 2020 it will rise to 97.6 and in 2040 even to 119.4 million barrels per day (Sieminski, 2014).

This dependence creates a huge petroleum logistic network, which leads to spills and seepages, hence to environmental contaminations which are complicated and long lasting. These potentially cause adverse human health effects, safety hazards, ecological and aesthetic impacts and more (Bowers and Smith, 2014). For instant, most of the marine oil spills are massive, for example the Exxon Valdez spill in 1989 released more than 11 million gallons of oil at the Alaskan shores (Short et al., 2004).

1.1.2 Characteristics and toxicity of PHCs

Chemically, HCs are exclusively made of carbon and hydrogen; most of them are nonpolar and exhibit low reactivity at room temperature, because of the lack of functional groups. The occurrence, type and arrangement of unsaturated bonds (π- bonds) determine their reactivity. According to their bonding feature, HCs are differentiated into four groups: the alkanes (saturated HCs), alkenes, alkynes and aromatic hydrocarbons. The three non-aromatic i.e. aliphatic groups can be further categorized in straight chain, branched chain and cyclic compounds. Combinations between aliphatic and aromatic compounds are also possible, for example as alkylbenzenes (Widdel and Rabus, 2001).

If nitrogen, sulphur or oxygen are incorporated in the HCs, they can also have a polar character (Tissot and Welte, 1984).

The weak chemical reactivity of PHCs is caused by the high homolytic and heterolytic dissociation energies of their C-C bonds and C-H. Therefore only super acids are capable of the protonation of pure alkanes, otherwise they do not participate in acid- base reactions. Addition reactions occur at alkenes and alkynes at their double and triple- bonds, but aromatics won't react that way. Redox reactions work at relatively mild conditions, for instance the reduction of alkenes to alkanes by catalytic hydrogenation. Moreover, methyl or methylene groups at aromatic rings can be oxidized catalytic to the corresponding carboxyl or carbonyl- groups. Finally, the most common reaction is the combustion of fuel with oxygen to CO_2 and water (Heider et al., 1998).

PHCs are complex mixtures which are altered by the chemical, physical and biological reactions in the soil. Therefore the behaviour and the toxicity is nearly unpredictable (Lundegard and Johnson, 2006). Generally the most toxic petroleum compounds are the most volatile and soluble ones (Bowers and Smith, 2014). That is the fraction of the volatile aromatic hydrocarbons and the major compounds of this fraction is called BTEX (Periago et al., 1997). BTEX abbreviates the 4 monoaromatic main forms benzene, toluene, ethylbenzene, and xylenes. Benzene for example, is considered to be the most hazardous compound for humans; long terms exposures can cause haemotoxicity, genotoxicity, immunological and reproductive effects as well as various types of cancer (World Health Organization, 2010).

The next dangerous fraction belongs to the PAHs (polyaromatic hydrocarbons). They belong to the recalcitrant constituents of petroleum oil and are of fundamental importance in economic growth due to their vast number of applications in the energy and industrial sectors (Okoh, 2006). Additionally there are many anthropogenic sources of accidental PAH pollution, e.g. the burning of fossil fuels and wood, the production of coke and charcoal, metal smelting, petroleum refining and petroleum spills (Billiard et al., 2008).The detrimental effects of PAHs are their toxicity, mutagenicity and carcinogenicity (Wu et al., 2011).

The true extent of the potential toxicity of PHCs is difficult to determine due to the variability of fuel compounds, the chemical changes after weathering, the generation of metabolites with distinctive hazard profiles, site specific differing physical conditions and the uncertainties in toxicity of potential risk drivers, i.e. fuel constituents for which toxicity criteria are established and which are relatively mobile in the environment (Bowers and Smith, 2014).

The other major concern about PHC contamination is the longevity of the higher molecular weight organic molecules like the PAHs. Their contamination can persist for up to 50 years as a nonaqueous phase liquid (NAPL) (Hamed, 2005, Johnston et al., 2007). During this phase, the NAPL degrades continuously and releases step by step smaller and soluble organic constituents into the environment via dissolution (Kim and Corapcioglu, 2003), volatilization (Davis et al., 2005) and degradation (Atlas, 1981). Therefore, a long termed intoxication, although in small doses, is given.

1.1.3 Organic pollution in Austria

Organic pollution is also a problem in well-developed countries as Austria, due to the dependency on different organic chemicals. In the most cases, they were caused by leaks in storage tanks. In table 1 all polluted sites in Austria, their status, the remediation method and the contaminants are listed. 60% of the pollution is caused by PHCs, therefore also, or maybe even more in industrial countries, sustainable bioremediation strategies are extremely important.

Table 1: HC contaminated sites in Austria (Austrian Environment Agency, 2016)

Identification	Type	Province	District	Municipality	Status	Pollution-site number	Status remediation	Remediation method	Pollutants
Tanklager Lobau	Longstanding pollution (LP)	W	Donaustadt	Wien,Donaustadt	LP-remediated	12	secured	Barrier wells, Partial closure	Mineral oil
SHELL – Pilzgasse	Longstanding pollution	W	Floridsdorf	Wien,Floridsdorf	LP	7	Securing in progress	Partial closure, Barrier wells	Mineral oil
Mobil	Longstanding pollution	W	Donaustadt	Wien,Donaustadt	LP-remediated	6	secured	Barrier wells, partial closure, Funnel-Gate	Mineral oil
Industriegelände Moosbierbaum - Teilfläche Nord	Longstanding pollution	NÖ	Tulln	Zwentendorf an der Donau	LP	64			Mineral oil, HC
Säureteerablagerung Unterlanzendorf	Old deposit	NÖ	Wien-Umgebung	Lanzendorf	LP	26			HC, Mineral oil, Sulfate
Flatschacherstraße-Lastenstraße	Longstanding pollution	Ktn	Klagenfurt	Klagenfurt	LP-remediated	4	remediated	Part- stripping	Mineral oil
ÖMV-Raffinerie Schwechat	Longstanding pollution	NÖ	Wien-Umgebung	Schwechat	LP-remediated	18	secured	Barrier wells	Mineral oil
Aral-Flyggen/St. Bartlmä	Longstanding pollution	T	Innsbruck	Innsbruck	LP-remediated	4	remediated		Mineral oil
Schwermetallsilos	Old deposit	OÖ	Wels-Land	Aichkirchen	LP-remediated	5	remediated		metals, Mineral oil, Chlorinated CH
Mineralöllände Hafen Freudenau I	Longstanding pollution	W	Leopoldstadt	Wien,Leopoldstadt	LP	14	Securing in progress	Part- stripping	Mineral oil
AGIP/St. Michael	Longstanding pollution	St	Leoben	Sankt Michael in Obersteiermark	LP-remediated	9	remediated		Mineral oil
Raffinerie Vösendorf	Longstanding pollution	NÖ	Mödling	Vösendorf	LP-remediated	20	secured	Partial closure, groundwater remediation	Mineral oil, PAH
Lackfabrik Eisenstädter - Teilbereich Ost	Longstanding pollution	NÖ	Mödling	Vösendorf	LP	60			Mineral oil
PAM/Troppacher	Longstanding pollution	T	Innsbruck	Innsbruck	LP-remediated	6	remediated	Soil vapour extraction, Barrier wells, Part-stripping	Mineral oil, 111-Trichlorethane
Spattgrube	Old deposit	OÖ	Linz-Land	Enns	LP	48	Securing in progress		Chlorinated CH, Mineral oil, 111-Trichlorethane, Phenole, PAH, Pesticides
ÖMV Tanklager St. Peterstraße	Longstanding pollution	Ktn	Klagenfurt	Klagenfurt	LP-remediated	8	remediated		Mineral oil
ÖCW Weißenstein	Longstanding pollution	Ktn	Villach Land	Weißenstein	LP	13	Securing in progress		Mineral oil
Teerfabrik Rütgers – Angern	Longstanding pollution	NÖ	Gänserndorf	Angern an der March	LP	53			PAH, 111-Trichlorethane, Mineral oil
BP-Tanklager Flatschacherstraße	Longstanding pollution	Ktn	Klagenfurt	Klagenfurt	LP	24			Mineral oil

Jarosik	Longstanding pollution	OÖ	Gmunden	Vorchdorf	LP-remediated	18	remediated		Chlorinated HCs, Mineral oil
Rohrbacher Lederfabrik	Longstanding pollution	OÖ	Rohrbach	Rohrbach-Berg	LP-remediated	33	remediated	Stripping	Chlorinated HCs, Mineral oil
Schwellenimprägnierung Schneegattern	Longstanding pollution	OÖ	Braunau am Inn	Lengau	LP	50	Securing in progress	Stripping	PAH, Mineral oil
Reindlmühl	Longstanding pollution	OÖ	Gmunden	Altmünster	LP	37			Mineral oil
Linoleumfabrik Brunn am Gebirge	Longstanding pollution	NÖ	Mödling	Brunn am Gebirge	LP-remediated	28	secured	Partial remediation, Funnel-Gate	PAH, Mineral oil
ELAN-Tanklager Raiffeisenstraße	Longstanding pollution	Ktn	Klagenfurt	Klagenfurt	LP-remediated	19	remediated	Stripping	Mineral oil
Zwidl Grube	Old deposit	OÖ	Wels-Land	Steinhaus	LP-remediated	49	remediated		Metals, Mineral oil, PAH
Waggonreparaturwerkstätte Deutsch-Wagram	Longstanding pollution	NÖ	Gänserndorf	Deutsch-Wagram	LP-remediated	43	remediated	Stripping	Mineral oil, Chlorinated HCs
Tanklager Mare	Longstanding pollution	NÖ	Korneuburg	Korneuburg	LP	46			Mineral oil
Tankstelle Lorenzoni	Longstanding pollution	St	Südoststeiermark	Fehring	LP	24			Mineral oil, Benzene, BTX
Wilhelmsburger Eisenwerke	Longstanding pollution	NÖ	St. Pölten Land	Wilhelmsburg	LP-remediated	47	remediated	Part- stripping, Partial closure	metals, Mineral oil
Deponie Gusswerkstraße	Old deposit	OÖ	Steyr-Stadt	Steyr	LP-remediated	53	secured	Part- stripping, Barrier wells	Mineral oil, metals, Chrome
Retentionsbecken Gusswerkstraße	Longstanding pollution	OÖ	Steyr-Stadt	Steyr	LP-remediated	54	secured	Part- stripping, Barrier wells	Mineral oil, metals
BP-Tanklager Linz 1 alt - Schadensfall SF2A	Longstanding pollution	OÖ	Linz	Linz	LP	67	Securing in progress		Mineral oil, HC
Deponie Bachfeld	Old deposit	NÖ	Gänserndorf	Schönkirchen-Reyersdorf	LP	75			Mineral oil
Frachtenbahnhof Praterstern - Bereich Werkstätte	Longstanding pollution	W	Leopoldstadt	Wien,Leopoldstadt	LP	26	Securing in progress		Mineral oil, HC
Mineralölkontamination Riedgasse Dornbirn	Longstanding pollution	Vbg	Dornbirn	Dornbirn	LP	3			Mineral oil, HC

1.1.3.1 Guidelines for the determination of PHC pollution in Austria

In Austria the basic guidelines for the measurement of PHC pollutions are ÖNORM EN 14039: 2005 01 01 and in Germany DIN EN ISO 16703:2011-09. Both guidelines determine hydrocarbons quantitatively in the carbon atom range between C10 and C40 by TPH analyses (Total Petroleum Hydrocarbon) with gas chromatography. Volatile hydrocarbons cannot be detected with these methods.

This quantitative determination is selectively too imprecise as far as human and environmental hazards are concerned. Diesel for example, contains 2000 to 4000 different hydrocarbons (Gallego et al., 2001). Significant differences in the toxicity of crude oil compounds and important factors like bioavailability, age of the contaminant, soil properties and sensitivity of various organisms are not considered in these measurements (Erlacher, 2008). Therefore, e.g. Erlacher (2008) suggests an alternative method which separates the PHCs in fractions which is more viable for the determination of the true hazard.

Table 2: Table with screening and intervention values in Austria for TPH analyses (Erlacher, 2008)

	Guideline	Screening value[a] [mg/kg]	Intervention value[b] [mg/kg]
Total Petroleum Hydrocarbons in soil	ÖNORM S2088- 1	100[1]	500[1]/1000[2]/2000[3]
TPH in Elutriates in areas protected by water law	ÖNORM S2088- 1	1[4]	5[5]
TPH in Elutriates in areas non-protected by water law	ÖNORM S2088- 1	2[4]	5[5]
TPH in soil of land use with high risk of oral uptake of contaminated soil (e.g. playground, garden)	ÖNORM S2088- 2	50[6]	-[7]
TPH in soil land use: agricultural or horticultural and non-agricultural	ÖNORM S2088- 2	200[6]	-[7]

[a] Values which, if exceeded, result in further site investigation. If value falls below, usually no hazard is given.

[b] Value which, if exceeded, usually result in safeguard and remediation measures.

[1] Impact due to petroleum hydrocarbon mixtures of boiling points over 160 °C. Analysis according to ISO 16703 (GC- FID)

[2] Impact due to petroleum hydrocarbon mixtures of low mobility (e.g. Lubricating oil) and main part of impact is caused by petroleum hydrocarbons of boiling points over 300 °C (e.g. alkanes $>C_{17}$), respectively. Analysis according to ISO 160703 (GC- FID).

[3] Impacts due to petroleum hydrocarbon mixtures of low mobility (e.g. Lubricating oil, hydraulic oil) at sides with high retention capacity outside of important water supply regions may be considered with an intervention value of 2 mg/kg, if it is proven that no contamination of groundwater exists or may be expected.

[4] Impacts due to petroleum hydrocarbon products of medium mobility (e.g. diesel to fuel oil extra light) or low mobility (e.g. lubricating oil) and the main part of impact is caused by petroleum hydrocarbons of boiling points over 160 °C (e.g. alkanes C_{10}- C_{40}), respectively. Analysis according to ISO 9377- 2 (GC- FID).

[5] Impacts due to petroleum hydrocarbon products of boiling points ranging from 30°C to 181 °C (motor gasoline and benzene, respectively). Analysis according to ÖNORM S2120 (IR- Spectroscopy).

[6] Analysis according to ÖNORM S2120 (IR- Spectroscopy).

[7] Individual evaluation, depending on soil type.

1.1.3.2 Characteristic of the PHC fraction "diesel" and implications for degradation

Generally, diesel is a complex hydrocarbon mixture of thousands of individual components. The exact composition is variable depending on the origin of the crude oil, the refining process and the mixtures added by the refiner for final formulation (Penet et al., 2004).

In order to emphasize the more complex relations in microbial diesel degradation, a detailed description of diesel content shall be given. Basically, the diesel fraction ranges with domestic fuel oil in the medium distillation range of crude oil between 150 and 370°C. Diesel is a mixture of hydrocarbons with a carbon atoms range from C_9 - C_{26}, approximately (Großmann et al., 2005).

Zeschmann (1993) determined a density of 0.820 to 0.860 kg / L and water solubility from 5 to 20 mg / L. Diesel contains approximately 45 % alkanes, 25 % cycloalkanes and 28 % aromatic compounds depending on the quality of the fuel (Großmann et al., 2005). The cetane number determines the combustibility i. e. the quality of the diesel. Generally, a high cetane number stands for a high n-alkanes content and hence a high quality diesel. On the contrary, a high content of aromatics decreases combustibility and subsequently the quality (Großmann et al., 2005). Therefore, the lower the cetane number, the worse is the biodegradability.

But there is a certain variation in the diesel components depending on origin, refining process etc. which also changes its characteristics. Liang et al., (2005) e.g. determined a ratio from 27.90% n-alkanes, 53.87% branched alkanes, 7.72% saturated cycloalkanes, 0.26% PAHs, 3.70% alkylated PAHs and 6.55% alkylbenzenes, which is different compared to Großmann in the preceding paragraph.

Sjögren et al. (1995) compared 10 different diesel fuels with significantly different compound percentages, e.g. total aromatics content (vol.%) varied from 1.8 to 25.1 %. Due to this broad variation in the different compound content there is no unique recipe to perform a successful diesel remediation, hence every diesel contamination should be primarily analysed.

To offer a foretaste of the degradability of diesel, Das and Chandran, (2010) described a susceptibility gradient of hydrocarbons from easy to hard: linear alkanes > branched alkanes > small aromatics > cyclic alkanes> PAHs. Moreover high molecular compounds as PAHs may not be degraded at all (Das and Chandran, 2010). In order to give a more detailed view on the diesel, the composition which was determined by Liang shall be given.

To compare diesel with combusted diesel particulate matter Liang et al., (2005) identified approximately 70 % (on mass basis) of a diesel fraction. The diesel was a low sulfur diesel fuel from the Steve Krebs Oil Company, Inc. with a sulfur content of 433 ppm. The composition was determined by gas chromatography/ mass spectrometer analyses. The aromatics made up approximately 4% of total mass volume.

Table 3: Compounds and contents in diesel (Liang et al., 2005)

Compounds	Diesel fuel composition/µg g^{-1}	Percentage		Compounds	Diesel fuel composition/µg g^{-1}	Percentage	
n-Alkanes				*PAHs*			
n-Decane (C10)	12115	1.77	%	Naphthalene (Nap)	753	0.11	%
n-Undecane (C11)	11271	1.65	%	Acenaphthylene (Acy)	159	0.023	%
n-Dodecane (C12)	17149	2.51	%	Acenaphthene (Ace)	85	0.012	%
n-Tridecane (C13)	28834	4.22	%	Fluorene (Flu)	100	0.015	%
n-Tetradecane (C14)	25604	3.74	%	Phenanthrene (Phe)	247	0.036	%
n-Pentadecane (C15)	27660	4.04	%	Anthracene (Ant)	7,5	0.001	%
n-Hexadecane (C16)	23965	3.50	%	Pyrene (Pye) 5.0	5	0.001	%
n-Heptadecane (C17)	26082	3.81	%	Biphenyl	437	0.064	%
n-Octadecane (C18)	8727	1.28	%	**Sum of PAHs**	**1793**	**0.262**	**%**
n-Nonadecane (C19)	4988	0.73	%				
n-Eicosane (C20)	2193	0.32	%	*Alkylated PAHs*			
n-Heneicosane (C21)	1092	0.16	%	1-Methylnaphthalene (1-MN)	585	0.086	%
n-Docosane (C22)	756	0.11	%	2-Methylnaphthalene (2-MN)	2291	0.335	%
n-Tricosane (C23)	220	0.03	%	1,2-Dimethylnaphthalene (1,2-DMN)	373	0.055	%
n-Tetracosane (C24)	107	0.02	%	1,4-Dimethylnaphthalene	1540	0.225	%
Sum of n-alkanes	**190763**	**27.90**	**%**	1,6-Dimethylnaphthalene	1807	0.264	%
Branched alkanes				1,7-Dimethylnaphthalene	2548	0.373	%
Norfarnesane (C14)	11469	1.68	%	2,6-Dimethylnaphthalene	1224	0.179	%
Farnesane (C15)	9719	1.42	%	2,7-Dimethylnaphthalene	1837	0.269	%
Norpristane (C18)	7992	1.17	%	Trimethylnaphthalene (TMN)	12327	1803	%
Pristine (C19)	5871	0.86	%	1-Methylphenanthrene (1-MPh)	242	0.035	%
Phytane (C20)	4775	0.70	%	2-Methylphenanthrene (2-MPh)	528	0.077	%
Other branched alkanes	328578	48.05	%	**Sum of alkylated PAHs**	**25302**	**3.7**	**%**
Sum of branched alkanes	**368404**	**53.87**	**%**	*Alkylbenzenes*			
Saturated cycloalkanes				Toluene	1377	0.201	%
Heptylcyclohexane (C13)	13144	1.92	%	C2-Benzenes	12932	1.891	%
Octylcyclohexane (C14)	11467	1.678	%	C3-Benzenes	10003	1.463	%
Nonylcyclohexane (C15)	10582	1,547	%	C4-Benzenes	9724	1.422	%
Decylcyclohexane (C16)	9135	1.336	%	C5-Benzenes	9724	1.422	%
Undecylcyclohexane (C17)	6207	0.908	%	C6-Benzenes	5222	0.764	%
Dodecylcyclohexane (C18)	2073	0.303	%	**Sum of alkylbenzenes**	**44796**	**6.55**	**%**
Tridecylcyclohexane (C19)	165	0.024	%				
Tetradecylcyclohexane (C20)	25	0.004	%		683856	100	%
Sum of saturated cycloalkanes	**52798**	**7.721**	**%**				

1.2 Biodegradation of PHCs

The source materials of fossil fuels are ancient plants and animals. Over millions of years under pressure and heat, they were converted into organic compounds. Fossil oil also consists of mineral compounds which are not derived from animals or plants, hence it is a very complex mixture (Ediki and Owan, 2014).

The dependency on fossil fuels for the humanity is enormous, therefore environmental friendly remediation e.g. bioremediation strategies are essential for the future. Bioremediation is the controlled improvement of biodegradation. Generally, biodegradation is the biochemical process for the complete mineralization of pollutants or the transformation into smaller compounds by living organisms (Bento et al., 2005). In practical bioremediation we have the following approaches (Sylvia et al., 2005) :

- Natural attenuation: In order to degrade contaminants, natural attenuation uses autochthone MOs without external modifications of the environment, which is especially useful in sensitively balanced habitats (Mills et al., 2003).
- Biostimulation: It is based on the stimulation of native MOs by supplying them with additional nutrients or substrates for increasing their degradation capacity (Riser-Roberts, 1998).
- Bioventing: Bioventing stimulates the natural biodegradation by supplying the contaminated soil with oxygen through air injection (Cangialosi et al., 2004).
- Bioaugmentation: This approach includes the inoculation of the appropriate contaminant degrading MOs into the appropriate environment (Vogel, 1996). Due to strong selection and a metabolic succession, indigenous species from an oil contaminated environment adapt their metabolic capacities to biodegrade all intermediate compounds of PHCs (Kostka et al., 2011). Therefore, they are an excellent choice for bioaugmentation approaches with PHC contamination.
- Landfarming: Hereby, the controlled application and dispersion of organic bioavailable waste on the contaminated surface, as well as the incorporation in the upper soil zone by tilling should enhance biodegradation. The content of the used waste should be more or less determined (Genouw et al., 1994).
- Composting: It relies also on organic wastes which are add to contaminated material and get mixed with it. Composting provides a broad range of MOs and available nutrients, furthermore it improves structure and water retaining capacity of the feedstock. On the contrary to landfarming it is mostly operated ex situ (Castelo-Grande et al., 2010).
- Phytoremediation: This biological technology process uses natural plant processes to increase degradation and removal of contaminations in soil or groundwater. Hereby, plant metabolism, plant uptake, volatilization through evapotranspiration, root absorption and biodegradation in the rhizosphere are involved in the contaminant removal (Kamath et al., 2004).

In CWs, phytoremediation is the main process which is utilized for contaminant removal. However, it can be combined with biostimulation and bioaugmentation, if you add nutrients or MOs to the support matrix of the CW.

1.2.1 The mechanism of microbial hydrocarbon degradation

In order to optimize biodegradation of PHCs it is necessary to understand the mechanism of biodegradation. The aerobic pathway is the most investigated and most efficient way in bioremediation, although the anaerobic has also its benefits, e.g. in sites with limited access to air (Heider et al., 1998). Therefore the practical optimum lies maybe in between in form of a combination. Nonetheless, the first condition is that the microorganisms (MOs) may reach the pollutant.

1.2.1.1 The accessibility of the pollutant

The first condition for effective biodegradation is the accessibility of the biological catalyst to the pollutant (Fritsche and Hofrichter, 2009). Due to the hydrophobicity of most of the organic pollutants, certain metabolic mechanisms are necessary. As far as long chain alkanes are concerned there are two possibilities: the direct contact with the bacterial cell i.e. interfacial accession, and the biosurfactant- mediated uptake (Bouchez-Naïtali and Vandecasteele, 2008).

In order to connect the pollutant to the microbial cell, certain MOs possess the metabolic ability to produce biosurfactants. For example *Rhodococcus equi Ou 2* are able to produce biosurfactants which increase the accessibility of the pollutant. In this case the surfactants produced by strain Ou2 were able to pseudosolubilize and emulsify hexadecane. Pseudosolubilization is the formation of micelles, a mechanism well suited for hydrocarbon transfer to hydrophilic strains since the hydrophobic compounds contained in the micelles are surrounded by the hydrophilic outer layer formed by the biosurfactant. Emulsification increases the surface area of the hydrocarbon phase (Bouchez-Naïtali and Vandecasteele, 2008).

1.2.1.2 The availability of the pollutant

Organic pollutants which are in prolonged contact to the soil could show reduced bioavailability and subsequently biodegradation possibility. This phenomenon is called sequestration. Due to their interaction with humic acid or fulvic acid polymer layers they get adsorbed to the solid phase and are inaccessible to the fluid phase. On the one hand it is disadvantageous because the pollutant undergoes a limitation to the MOs accessibility, on the other hand it benefits the detoxification due to the fixation of the pollutant on the soil particles (Haritash and Kaushik, 2009). The extent of these limitation is depending on pollutant characteristics like tendency of organic carbon to bind to soils (K_{oc} value) and on soil parameters like clay content, Cation Exchange Capacity (CEC) and, in particular, organic matter (Nocentini et al., 2000). These bioavailability drawbacks have to be considered for a successful bioremediation approach.

1.2.1.3 Aerobic degradation

For the microbial degradation of the majority of organic pollutants aerobic conditions are most efficient. Therefore, the characteristics of the aerobic pathways should be mentioned (Fritsche and Hofrichter, 2009).

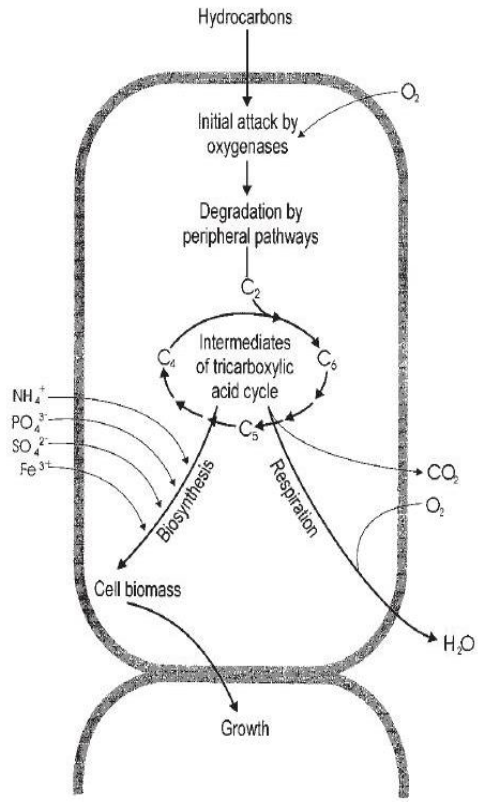


Figure 1: Schema of the aerobic degradation pathway (Fritsche and Hofrichter, 2009)

As mentioned above the accessibility of the enzymes to the water- insoluble pollutants is the first condition. Afterwards, the oxidative process of the initial intracellular attack of organic pollutants will follow. Thereby, the activation and incorporation of oxygen is the enzymatic key reaction, which is catalysed by oxygenases and peroxidases. Subsequently, peripheral degradation pathways convert organic pollutants step by step into intermediates of the central intermediary metabolism, e.g. the tricarboxylic acid cycle. Finally, cell biomass will be synthesized from the central precursor metabolites, into intermediates of the tricarboxylic acid cycle. Afterwards cell biosynthesis could happen with the needed nutrients, in other words the cell is growing. Also gluconeogenesis will be supplied, for the sugars which are required for growth and various biosynthesis (Fritsche and Hofrichter, 2009).

Biodegradation approaches often concern weathered and old contaminations, where the volatile fractions are already vanished and the recalcitrant part is left. This part mostly concerns the fraction of the PAHs, therefore some further description of its degradation shall be given.

PAHs exist in all phases, as vapors in the air, solutions in water, sorbed by solid bodies and as water- immiscible liquids (Alexander, 2000). Bacteria, fungi and plants are capable of PAH biodegradation. Most eukaryotes are only transforming PAH molecules in the reactions with cytochrome P450 (Baboshin and Golovleva, 2012). Generally, PAH biodegradation is more efficient under aerobic conditions, than under anaerobic (Mihelcic and Luthy, 1988). Another difficulty is that incomplete degradation could produce intermediates which may inhibit PAH biodegradation. Moreover, they could be stable and environmentally hazardous. Therefore a broad consortium would increase the possibility of a complete degradation (Kazunga and Aitken, 2000).

On the other side, the presence of other PAHs in the soil matrix could also have an activating effect on the degradation of a certain PAH (Baboshin and Golovleva, 2009). This effect may result from cross induction, an increase in the biomass production and cometabolism (Bouchez et al., 1995).

1.2.1.4 Anaerobic degradation

In fact, anaerobic processes are usually slower and less efficient than aerobic. Therefore, anaerobic applications are restricted to sites with limited access of air, for example in groundwater aquifers (Hunkeler et al., 1995).

Heider et al., (1998) stated that aliphatic alkenes and alkanes with chain lengths of 6-20 carbon atoms, monocyclic alkylbenzenes, such as toluene, ethylbenzene, propylbenzene, p-cymene, xylene- and ethyltoluene- isomers, as well as benzene and naphthalene can be degraded anaerobically.

For instance some bacteria employ oxygen- independent radical reactions to assimilate hydrocarbons. Besides, the few anaerobic initiation reactions which are known are surprisingly diverse. In contrast, aerobic pathways always start with an oxygenation reaction (Heider et al., 1998).

In chemotrophic reactions, a part of the hydrocarbon is used for catabolism or energy conservation and the other part is assimilated into cell mass (figure 2). Anaerobic pathways are completely different compared to the aerobic mechanisms. Novel hydrocarbon activation mechanisms are used which are indicated by jagged arrows in figure 2 (Widdel and Rabus, 2001). These mechanisms are denitrifying, ferric- or iron- reducing, sulfate-reducing and proton reducing which is a syntrophic association with methanogens.

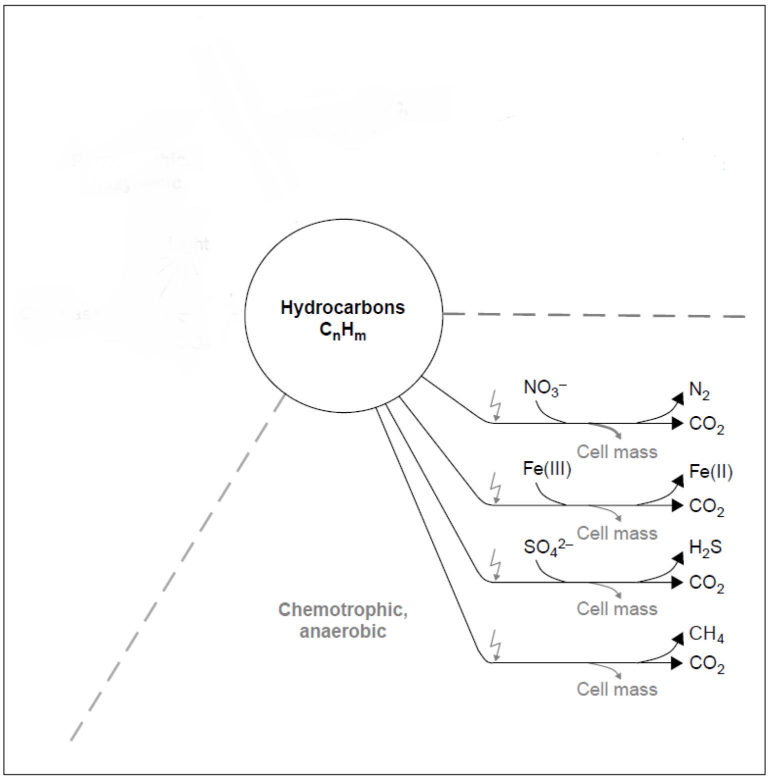


Figure 2: anaerobic degradation mechanisms (Widdel and Rabus, 2001)

1.2.1.5 PHC degrading MOs

Many microorganisms (bacteria, filamentous fungi and yeasts) are able to degrade PHCs, using them as carbon source (Throne-Holst et al., 2007). Bacteria and yeast use mainly the aerobic and anaerobic pathways. The bioremediation potential of basidomyceteous fungi which cause white rod in wood arises from their powerful extracellular enzymes. They are known as peroxidases and they can attack a broad array of organic compounds (Yateem et al., 1998).

White rod basidiomycetes are also capable of PHC biodegradation in a considerable extent. E.g. the white rod fungus *Punctularai strigosozonata* degraded 99 % of C_{10} alkanes after 20 days of growth. Although, the mechanism of the degradation of more complex oil compounds remained obscure,still it showed potential for bioremediation approaches (Young et al., 2015). Lignolytic basidiomycetes can mineralize PAHs in their reactions, catalysed by laccases and peroxidases (Cerniglia, 1997).

1.2.1.6 Co- metabolic biodegradation

Cometabolism can be defined as the metabolism of an organic compound in the presence of a growth substrate which is used as the primary carbon and energy source" (Fritsche and Hofrichter, 2009). Synergistic interactions between different MO consortia increase the biodegradation versatility. The secretion of important degradative enzymes, growth factors or biosurfactants of certain MOs might benefit the degradation ability of others. Mukherjee and Bordoloi (2011) showed that a consortium of three bacterial strains degraded PHCs more efficiently than each of them alone.

The exact definition of cometabolism could vary, e.g. Al-Isawi et al., (2015) defined it as the simultaneous degradation of two compounds, where the first compound which was diesel enables the degradation of the second compound which was root exudate.

Cometabolism is especially important for the degradation of high molecular PAHs like benzo[*a*]pyrene (Juhasz and Naidu, 2000).

1.2.2 Environmental factors influencing PHC degradation

The most important factors are availability of oxygen as electron acceptor, temperature, pH (potential hydrogenii) value, nutrient availability and moisture. Furthermore, salinity of the environment, physical state and concentration of the contaminant are also considerable.

Oxygen availability

For a complete degradation of the majority of organic pollutants aerobic conditions are important. Oxygen is needed as co-substrate in reactions catalyzed by oxygenases and peroxidases. These two are mainly responsible for aerobic degradation of organic pollutants (Karigar and Rao, 2011).

Vieira et al., (2009) showed that intermittent loading in CWs with an aeration interval of 33 hours showed the best PHC degradation. He compared it to experiments with constant aeration and without aeration. That is maybe also a compromise between aerobic and anaerobic conditions.

Temperature

Firstly, temperature influences the activity of the microorganisms. The range of possible activity is very broad; however Okoh and Trejo-Hernandez (2006) mentioned an optimum of 30 to 40°C for microbial degradation in soil environments. Secondly, temperature also has effects on the viscosity, following the degree of distribution and the diffusion rates of the pollutant in the environment. As far as the reactivity is concerned, the higher the temperature, the smaller are the boundary layers, hence the recalcitrance of the organic pollutant (Margesin and Schinner, 2001, Müller et al., 1998).

pH- Value

The optimum for hydrocarbon degradation is a pH of 7.0, although some MOs like acidophiles and alkaliphiles are also capable of degrading hydrocarbons in acidic (pH= 2-3) and basic (pH= 9- 10.5) environments (Margesin and Schinner, 2001).

Nutrients and humidity

Nutrient addition including nitrogen (N) and phosphor (P) is standard practice for increasing hydrocarbon degradation. The most essential nutrients are N, P, potassium (K), and iron (Fe). Their lack could hinder the breakdown process, or lead to an incomplete breakdown (Atlas and Bartha, 1986).

The C/N and C/P ratio in the substrate should be as close as possible to the bacterial requirements. Mills and Frankenberger, (1994) reported that the diesel biodegradation depended on the P- availability.

After Bossert and Bartha, (1986) the water activity of soils ranges between 0.0 and 0.99. This is a very broad range and thus causes problems in efficient biodegradation, because a constant and optimal level is required.

1.2.3 Degradation of diesel

As mentioned, diesel is from the middle- distillate fraction of petroleum separation. Firstly, diesel in the substrate could reduce the oxidation- reduction potential, hence the substrate becomes more anaerobic (Lin and Mendelssohn, 2009).

As far as volatility is concerned, in diesel we have on the one site BTEX compounds and on the other side PAHs. The changes in the BTEX compounds, characterized with a high vapor pressure and aqueous solubility, is caused mainly by evaporation and dissolution. Benzene and toluene primarily dissolve in the groundwater, therefore ethylbenzene and the xylenes are relative resistant to biodegradation compared to benzene and toluene (Kaplan et al., 1997).

With a low solubility and a recalcitrant characteristic, the PAHs are the least affected fraction of weathering (Mariano et al., 2008). There are three reasons for the PAH- recalcitrance:

1. The chemical attack of aromatic rings requires high activation energy
2. Restricted accessibility of PAHs
3. PAHs as well as other hydrocarbons e.g. BTEX, show toxicities for bacteria

PAHs tend to sorb on hydrophobic surfaces and this tendency is increasing with the number of their aromatic rings. The sorption and the low water solubility cause inaccessibility of the major fraction of PAHs for MO degradation. Furthermore, it is assumed presently that adsorbed PAHs, solid PAH crystals or hydrocarbons dissolved in NAPLs remain unavailable to biodegradation (Mariano et al., 2008).

Diesel released into soil is altered by biotic and abiotic weathering reactions in the soil/groundwater matrix. All these reactions act more or less together, depending on different factors e.g.: fuel composition, temperature, moisture, nutrients and oxygen contents (Kaplan et al., 1997). The major chemical reactions are: hydrolysis, dehydrogenation, oxidation and polymerization (Lyman et al., 1992). The major physical reactions are evaporation, dissolution, dispersion, oil- sediment aggregation, sedimentation and the biotic mechanisms include microbial uptake and metabolic degradation (Baughman et al., 1981). Weathering is termed as the combination of those processes that affect the composition of spilled oil in the environment.

Table 6: Mariano et al., (2008) compared commercial and weathered diesel oil by chromatographic analyses (Figure 4). The weathered diesel was collected from a petrol station where the leakage occurred approximately ten years ago.

BTEX compounds decreased and PAHs became enriched in the weathered diesel, due to the fact that the other compounds were primarily degraded.

Table 4: Comparison of wheathered and commercial diesel (Mariano et al., 2008)

BTEX concentration in diesel oils.

	commercial	weathered
	μg/kg	
Benzene	<DL	<DL
Toluene	84.90	<DL
Ethylbenzene	171.71	69.78
m,p-Xylene	565.24	112.86
o-Xylene	321.41	14.52
Total	1143.26	197.16

DL (detection limit) = 1.58 μg/kg.

PAH concentration in diesel oils.

	commercial	weathered
	μg/kg	
Naphthalene	578.31	4276.21
Acenaphthylene	<DL(1)	<DL(2)
Acenaphthene	257.32	822.63
Fluorene	534.84	1221.17
Phenanthrene	257.65	2024.41
Anthracene	13.88	<DL
Fluoranthene	<DL	<DL
Pyrene	<DL	<DL
Benzo[a]anthracene	<DL	<DL
Chrysene	<DL	<DL
Benzo[b]fluoranthene	<DL	<DL
Benzo[k]fluoranthene	<DL	<DL
Benzo[a]pyrene	<DL	<DL
Indeno(1,2,3-cd)pyrene	<DL	<DL
Dibenz[a,h]anthracene	<DL	<DL
Benzo[g,h,i]perylene	<DL	<DL
Total	1641.99	8344.42

DL (detection limit) = (1) 3.16 μg/kg; (2) 31.9 μg/kg.

Penet et al. (2004) reported by a diesel biodegradation experiment with activated sludge, that branched alkanes and aromatics belong to the more recalcitrant compounds in diesel. Linear alkanes were degraded after 2 days, but branched alkanes like farnesane, pristane and phytane were still detectable after 28 days (figure 3).

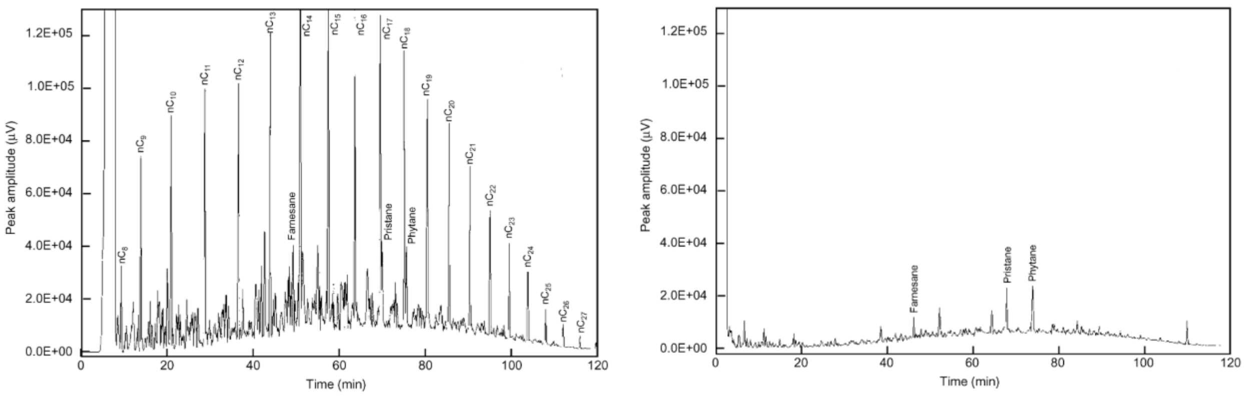


Figure 3: comparison of the GC patterns of commercial (left) and weathered (right) diesel (Penet et al., 2004)

1.3 Biodegradation of PHCs with constructed wetlands (CWs)

A constructed wetland (CW) can be defined as “a designed and man-made complex of substrates, emergent and submergent vegetation, animal life, and water, that simulates natural wetlands for human use and benefits” (Hammer, 1989).

CWs are engineered biological remediation systems based on natural wetlands, which take advantage of their decomposing ability. Therefore, viable macrophytic vegetation and an appropriate filter material are provided. The bioremediation performance of that system is mainly achieved by a complex microbial community. The main benefit of the CW compared to natural wetlands is the controlled optimization of the microbial activity. For this purpose the composition of the support matrix (i.e. the filter body), the vegetation and the water regime can be adapted to the local environment and the contaminant in order to maximize the biodegradation success.

1.3.1 General design of modern vertical flow constructed wetlands

The main focus is to create synergistic effects between the support matrix the plantation and the MOs, to guarantee ideal conditions for biodegradation. Therefore, biological, chemical and physical characteristics of the whole system and the interactions of the participants have to be well reconsidered.

In this study the main focus is on the vertical flow CWs. Our CW works with vertical intermittent loading. Hereby, the pollutant flow passes the filter from the top with the plantation and the microbial active zone, to the bottom with the drainage in certain intervals.

This system allows the filter pores to fill up with air between the loadings and creates mainly aerobic conditions in the support matrix. These conditions enable currently the highest removal rates for many HC contaminants (Eke, 2008).

1.3.1.1 Support matrix

Generally the support matrix has to provide a suitable habitat for the biota, which is depending on pH, toxicity of the pollutant, porosity, surface area, availability of nutrients and organic matter content. These conditions are mainly influenced by the granulometric and hydraulic properties, mineralogical composition, acid- base and surface charge properties, content of organic matter, sorptive properties and the contaminant (Dordio and Carvalho, 2013).

For an effective support matrix the aim is to reach an equilibrium which should allow sufficient retention of the pollutant and simultaneously prevent clogging, i.e. appropriate hydraulic properties. Retention means in this case, sorption of non-polar organic pollutants onto suspended solids and onto the support matrix in order to make them accessible to the MOs (Reddy and DeLaune, 2004).

Traditionally, a mixture of sand with gravel, like in our CW, has shown appropriate hydraulic loading without clogging (Tietz et al., 2007), although that is also depending on the contaminant. However, these mixture acts simply as filter for larger particles and as support for the development of the biota. As far as sorption of organic pollutants is concerned, their capacity is negligible. Therefore, numerous other materials, natural and artificial, have been tested for their retention ability (Dordio and Carvalho, 2013).

Activated carbon (i.e. charcoal with enhanced surface by thermal and chemical treatment) is currently one of the most efficient sorbents. Therefore, it would be an excellent pollutant sorbent in the support matrix. In fact due to high costs for the production, it is too expensive for the use in CWs (Dordio and Carvalho, 2013). A more economical and ecological alternative would be biochar. Biochar is a newly constructed scientific term, which is defined as “a carbon (C)-rich product when biomass such as wood, manure or leaves is heated in a closed container with little or unavailable air” (Lehmann and Joseph, 2009). Biochar is also a carbonaceous sorbent with medium to high surface areas (Cao et al., 2011). Furthermore, biochar contains a non- carbonized fraction that may interact with soil contaminants. Especially, the extent of oxygen- containing carboxyl, hydroxyl and phenolic surface functional groups in biochar could effectively bind soil contaminants. Thus, biochar shows potential as a very effective sorbent for organic and inorganic contaminants in soil and water (Uchimiya et al., 2011).

1.3.1.2 Plantation

Plants are very important for CWs due to their capability to adsorb, absorb, concentrate or metabolize organic xenobiotics and enhance microbial activity. Many plants used in CWs have structural mechanisms to avoid root anoxia (Table 6). These mechanisms are possible by the evolution of air spaces (aerenchyma) in roots and stems that enable the diffusion of oxygen from the aerial portions of the plants into the roots. Jung et al. (2008) determined different forms of aerenchyma, maybe the form has an influence on the phytodegradation ability. The oxygen flow is apparently large enough not only to supply the roots but also to diffuse out and support the adjacent soil with oxygen(Armstrong and Armstrong, 1990).

Table 5: Adapted from commonly used plant species in CWs from Tietz et al., (2007)

				Aerenchyma pattern		
Scientific name	**Common English name**	**Aerenchyma**	**Habit**	**Root**	**Shoot**	**Reference**
Phragmites australis	Common reed	Yes	Aem	RL	HW+LA	(Jung et al., 2008)
Typha angustifolia	narrow- leaved cattail	Yes	Aem	RL	HW+LA	(Jung et al., 2008)
Typha latifolia	broad leaved cattail	Yes	Aem	RL	HW+LA	(Jung et al., 2008)
Scirpus sp.	bulrushes	Yes	Aem	TL	LA	(Jung et al., 2008)
Juncus sp.	rushes	Yes	W	RL	HW	(Jung et al., 2008)
Carex sp.	sedges	Yes	Aem	TL	HA	(Jung et al., 2008)
Glyceria maxima	reed sweet grass	Yes	n.a.	n.a.	n.a.	(Smirnoff and Crawford, 1983)
Cyperus sp.	flat sedge	Yes	Aem	n.a.	n.a.	(Jung et al., 2008)
Iris pseudacorus	yellow flag iris	Yes	W	I	LA	(Jung et al., 2008)
Phalaris arundinacea	reed canarygras	Yes	n.a.	n.a.	n.a.	(Smirnoff and Crawford, 1983)
Schoenoplectus lacustris*	common club- rush	Yes	Aem	TL	HA+ LA	(Jung et al., 2008)
Caltha palustris*	marsh marigold	Yes	n.a.	n.a.	n.a.	(Visser et al., 2000)
Alisma sp.*	water plantain	Yes	Aem	na	HA+HW	(Jung et al., 2008)
Acorus calamus*	sweet flag	Yes	Aem	HA	HA+LA	(Jung et al., 2008)
Salix sp.*	willow	Yes	n.a.	n.a.	n.a.	(Kuzovkina and Quigley, 2005)
Alnus sp.*	alder	Yes	n.a.	n.a.	n.a.	(Machacova et al., 2013)
Rumex conglomeratus *	clustered docks	Yes	W	HA	WA	(Jung et al., 2008)
Rumex alpinus**	alpine docks	no	-	-	-	(Šťastná et al., 2010)

* used rather seldom

** used only for CWs on a higher sea level

Habit abbreviations: Aem, emergent aquatic; W, wetland

Pattern abbreviations: HA, honeycomb aerenchyma; TL, tangential lysigeny; RL, radial lysigeny; WA, wheelshaped aerenchyma; HW, hollow aerenchyma; LA, leafy aerenchyma; I, intercellular air space, or non-aerenchyma;

'+' indicates a casewhere two or more types are observed in a species

n.a. data not available

The most important benefits of the plantation:

- Supply of the surface area for development of microorganisms and to stimulate their growth aided by exudates released through the roots (Brix, 1994)
- Transport and release of oxygen through the roots for enhancing aerobic degradation in the rhizosphere (Brix, 1993)
- Diminishing of the wastewater pollutants load by adsorption, phytodegradation and absorption (Susarla et al., 2002)
- Promotion of hydraulic conductivity of the support matrix by their extensive roots and rhizomes in order to help prevent clogging (Kadlec and Wallace, 2008)
- The vegetation cover protects the surface from erosion. Furthermore, litter provides an insulation layer on the wetland surface to ensure operation during winter (Haberl et al., 2003).

The action of plants on the contaminants is variable: they can be immobilized, stored, volatilized, transformed and mineralized or a combination of them. The extent of these phytodegradation processes is depending on the specific compound, environmental conditions and the involved plant genotype (Campos et al., 2008).

A promising example for plants in diesel degrading CWs would be reed. Wang et al., (2011) found out that reed was tolerant to diesel concentrations until 20,000 mg/kg soil, though the growth was disturbed.

1.3.2 The viability of constructed wetlands

Many studies have proven that CWs are an effective system for sustainable degradation of pollutants, although it has some weaknesses. Following, there is a list of the advantages and issues of CWs, adapted from Tietz et al., 2007.

Advantages of vertical flow CWs

- CWs enable effective and environmental- friendly remediation with low input (energy, equipment, supervision)
- CWs have a relative broad tolerance ratio concerning constituents and concentration of pollutants
- Compared to mechanical treatment the construction has lower costs for the operation and the maintenance with the same effluent quality
- The aerobic conditions in vertical flow CWs allow an effective degradation of organic pollutants
- CWs can remediate the whole year, with some restrictions in cold periods
- There is no access to the polluted water for animals or humans
- The plantation of the CWs may provide a valuable wildlife habitat
- The reliability of this system is relative high, due to the low technical requirements
- Wetlands are able to tolerate fluctuations in the flow

Issues of CWs

- Constructed wetlands are land intensive option compared to mechanical treatment.
- Persistent pollutants may accumulate in the support matrix, unaltered.
- The biodegrading MOs may produce toxic metabolites that are not further degraded
- High organic loads might diminish the permeability of the filter material until total clogging and surface run- off. A reconstitution of the support matrix would be very costly.

- There is a lack of information about the longevity of CWs.
- In cold climates low temperatures may create fluctuations in the degradation rates.

1.3.3 Comparison of CW remediation systems

Currently modern CWs can be differentiated in 4 types (Dordio and Carvalho, 2013) (Figure 4) :

1. free water surface (FWS) or surface flow (SF) wetlands
2. horizontal subsurface flow wetlands (HSSF)
3. vertical subsurface flow wetlands (VSSF)
4. hybrid systems

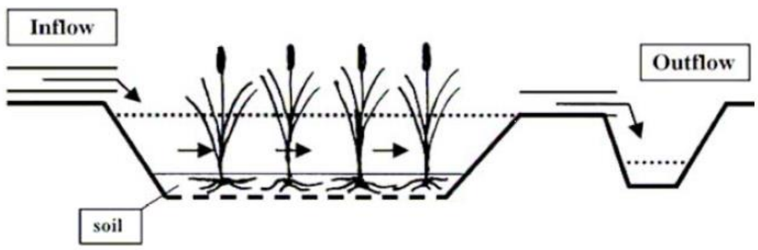
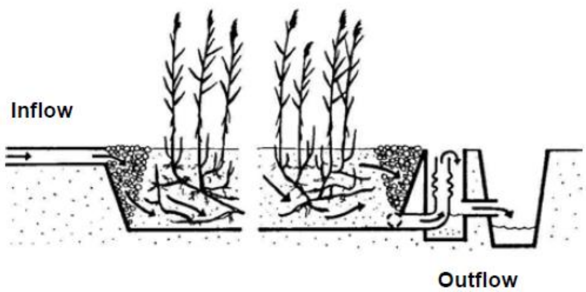
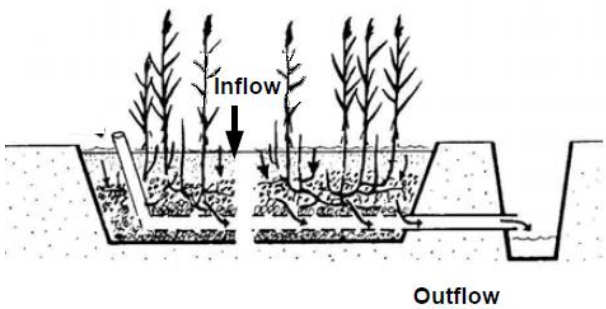
1. free water surface (FWS) or surface flow (SF) wetlands	
2. horizontal subsurface flow wetlands (HSSF)	
3. vertical subsurface flow wetlands (VSSF)	
4. hybrid systems	Combinations of type 1, 2 and 3 Single cell, dual cell, multi cell

Figure 4: the 4 different types of CWs (Dordio and Carvalho, 2013)

Ad 1: free water surface (FWS) or surface flow (SF) wetlands

The water level is on the surface like in natural wetlands. The disadvantage is that the contaminated water is accessible to humans and animals. They also may provide breeding areas for mosquito larvae and produce odour problems. In hydrocarbon bioremediation these type is not used very often. Al-Baldawi et al., (2013) compared free surface flow (FSF) and subsurface flow (SSF) system and concluded that the FSF system had a greater efficiency and performance in the removal of lower diesel concentrations (up to 1% diesel), while the SSF system were better at higher concentrations.

Ad 2: horizontal subsurface flow wetlands (HSSF)

This type is typically constructed as a bed or a channel containing appropriate media (like coarse rock, gravel or sand). The water level is below the surface and the inlet and outlet is constructed in order to create a horizontal flow where adequate plants are inserted. In contrast horizontal filters are normally loaded continuously and the support matrix is permanently water-saturated, hence aerobic and anaerobic processes occur (Tietz et al., 2007).

Ad 3: vertical subsurface flow wetlands (VSSF)

As described previously, due to the intermittent loading this type exhibits majorly aerobic conditions which guarantee the best biodegradation performance. Additionally, when using natural hydraulic gradients this system requires less maintenance and technical equipment than HSSF- systems (Tietz et al., 2007).

Moreover, the vertical-flow constructed wetlands or soil filters are gaining popularity due to their greater oxygen transfer capacity and smaller size as compared to the horizontal-flow wetland systems (Kadlec and Wallace, 2008).

A disadvantage compared to the other systems may be the higher possibility of clogging. Due to the finer material- hence the lower pore size- a high hydraulic loading rate could lead to clogging of the support matrix. The recovery of such a malfunction would be very costly.

Ad 4: hybrid systems

Hybrid systems can be various combinations of vertical flow and horizontal flow systems, e.g. single cell, double cell or multi cell. Therefore aerobic biodegradation as well as anaerobic conditions are possible, depending on the combinations (Eke, 2008).

These combinations can fulfil different purposes, e.g. Chen et al. (2012) stated that for the removal of highly chlorinated hydrocarbons like PCE (perchlorinated ethylene) an initial anaerobic step is needed, for further microbial mineralization.

1.3.4 Description of the most important removal processes in CWs

Numerous processes occur in CWs and the knowledge of each of them is essential. For a better understanding the most important removal mechanisms should be described. They are: phytoremediation, microbial degradation, volatilisation, sorption and sedimentation. (Haberl et al., 2003).

Phytoremediation

Phytoremediation is the umbrella term of all synergistic relationships between plant, MOs and the environment, which remove, transfer, stabilize or destruct contaminants. These processes are defined as phytodegradation, phytovolatilization, phytoextraction, phytostabilization, rhizofiltration and rhizodegradation. Although these processes are defined individually, they may interact. For instance, the contaminant is co- metabolized by the microbes in the soil, which are nurtured by the root exudates of the plant. Subsequently, the plant enzymes are degrading the contaminant further, until the final mineralization by microbes to carbon dioxide and water (Bragg-Flavan, 2009).

- Phytodegradation is the intra- or extra cellular degradation of contaminants by plant exudates.
- Phytovolatilization is the transfer of volatile contaminants from soil to the air, by the plants evapotranspiration.
- Phytoextraction which is also known as phytoaccumulation, describes the uptake of contaminants by plant roots and their accumulation and/or translocation into plant tissues (Bragg-Flavan, 2009).
- During Phytostabilization chemical compounds are produced by plants, which are able to immobilize contaminants at the interface of roots and soil.
- Rhizofiltration is the adsorption of contaminants by the roots and rhizodegradation is the enhancement of the MOs in the rhizosphere by the plant root exudates (Frick et al., 1999).

Microbial degradation

- Aerobic respiration

In bioremediation the aerobic respiration guarantees the fastest and most efficient biological process to degrade the majority of organic pollutants (Das and Chandran, 2010). The key factor is oxygen, which is needed for the enzymatic key reactions of aerobic biodegradation. These reactions are oxidations catalyzed by oxygenases and peroxidases. In vertical flow CWs with intermittent loading, the pore spaces could be refilled with oxygen, in between the influent charges. Therefore, aerobic conditions are provided in the majority of the support matrix.

- Anaerobic respiration

Anaerobic degradation is respiration of MOs with other electron acceptors than O_2. These MOs aquire their electron acceptors under nitrate-, iron-, manganese-, or sulphate reducing conditions, as well as under methanogenic conditions (Haberl et al., 2003). They grow in syntrophic cocultures with other anaerobics or grow by anoxygenic photosynthesis (Widdel and Rabus, 2001). Many studies reported about high anaerobic HC degradation potentials, especially of more recalcitrant compounds like PAHs.

Anaerobic hydrocarbon biodegradation studies lack field studies. In most of anaerobic degradation studies they examined the fate of individual hydrocarbons by taking advantage of the resultant hydrocarbon-degrading enrichment cultures and isolates. Such studies are surely adequate for examining the underlying physiology and metabolism of anaerobic hydrocarbon metabolism, but in many respects these experiments are ecologically unrealistic. The selective pressure exerted by a single substrate at high concentration is not comparable to the low concentrations of multicomponent contamination associated with petroleum spills (Townsend et al., 2003).

Nonetheless, anaerobic respiration is viable of hydrocarbon degradation, especially for those who have high water solubility like BTEX. These are the most water- soluble aromatic hydrocarbons (saturation concentrations at 25 °C are 1800 mg/L for benzene , 580 mg/L for toluene, app. 200 mg/L for xylene and 125 mg/L for ethylbenzene) and spread most easily. Therefore a concept for augmented bioremediation would be to offer electron acceptors in water at higher concentrations than dissolved oxygen from air (8.6 mg/L at 25°C).Hence, the anaerobic degradation of BTEX would be increased. Nitrate and Sulphate have a higher solubility than oxygen, for example (Widdel and Rabus, 2001).

Volatilization

Volatilization is the direct contaminant emission from the water phase to the atmosphere. Additionally, volatile contaminants can also evaporate through plant tissues like roots or the aerenchyma. Many HCs are volatile, like BTEX or MTBE (Methyl-*tert*-butylether), depending on the Henry coefficient (H- value). BTEX has a high H- value (H=272- 959) and is therefore very volatile (Hong et al., 2001). Diesel on the contrary, has a medium range distillation temperature (180- 350°C), therefore the vaporizing content is minimal depending on the differing content.

Sorption and sedimentation

Generally, adsorption and ion exchange happens on the surfaces of plants, substrate and litter. Sorption is the physical or chemical adhesion of the organic pollutant to the surface of a solid body. For the evaluation of the sorption capability of support matrix material, the carbon partition coefficient (K_{OC}) is an appropriate parameter. It is defined as the ratio of contaminant mass adsorbed per unit weight of organic carbon in the soil to the concentration in solution (Haberl et al., 2003).

Additionally, an empirical relationship exists between the K_{OC} and the atomic hydrogen/oxygen ratio in natural organic matter (Grathwohl, 1990). Thus, the extent of sorption depends on the compound’s hydrophobic characteristics as well as on the organic carbon content, the chemical structure and composition of soil organic matter.

Sedimentation occurs when the contaminant is bound in particulate organic matter (POM). In this case the POM could settle into the CW or may get mechanically retained in it. In contaminated waters containing high amounts of POM, a mechanical filtration of the influent would be most vital for the approach, in order to prevent clogging (Thurston, 1999).

1.4 The role of isotopes in biodegradation assays of PHCs

For the composition of petroleum three points are important: the nature and composition of the parent material, the mode of accumulation of the organic material and the reactions which transformed the material into the end product. As mentioned earlier, PHCs are complex mixtures mainly composing hydrocarbons with varying amounts of heterocompounds like sulfur (S), nitrogen (N), oxygen (O) or metallo- organic molecules (Hoefs, 2008).

Many of the compounds have useful stable and not stable isotopes. Thereby, the source material or more specifically the type of kerogen and the sediments in which it has been formed, determine mainly the isotopic composition and subsequently the origin of the material. Biodegradation, water washing and migration have only minor effects on the isotope ratio. These isotopes enable many analytical possibilities, e. g. the combined stable isotope analyses with ^{13}C, Deuterium (D), ^{34}S, ^{15}N, which is a powerful tool in petroleum analyses (Schoell, 1984; Sofer, 1984; Stahl, 1977).

In order to validate natural attenuation and engineered environmental remediation systems, effective tools for monitoring and verifying contaminant removal processes have to be used. These systems purpose is to delineate between abiotic mass- removal processes and requested biodegradation (Cowie et al., 2010).

A very viable parameter for biodegradation is the isotope variation in the $^{13}C/^{12}C$ ratio. In crude oil the different compounds show small but characteristic $\delta^{13}C$ differences. In the present, many artificially enriched isotopic compounds are available, in order to measure the degradation of these compounds by analyzing their isotopic composition. We will describe the utility of ^{13}C and ^{14}C enriched compounds.

Isotopic measurements with the stable $^{13}C/^{12}C$ ratio exhibit high reproducibility and sensitivity. Moreover, it is the best method for the quantification of mineralization (Bahr et al., 2015).

1.4.1 ^{13}C in PHC biodegradation

Overall, approximately 1.1 % of total carbons content is ^{13}C isotope. The international standard for $^{13}C/^{12}C$ introduced in 1957 was the internal calcite structure of the fossil Belemnita Americana from the Cretaceous Pee Dee Formation in South Carolina, in many publications referred to as the Pee Dee Belemnite (or PDB). The short notation $\delta^{13}C_{PDB}$ refers to this standard. It had an abundance ratio $R_{standard}$ $^{13}C/^{12}C$ of 0.011237. The IAEA (International Atomic Energy Agency) in Vienna subsequently defined the hypothetical VPDB scale (Vienna PDB, considered as identical to the PDB) as reference to stable Carbon analysis (Craig, 1957).

1.4.1.1 Fractionation between $^{12}C/^{13}C$

Chemical reactions which create equilibrium like the dissolution of CO_2 in water also create equilibrium isotope effects. Unidirectional reactions like biodegradation on the contrast create often kinetic isotope effects. Therefore, biological reactions create $^{12}C/^{13}C$ isotope fractionation, i.e. shifts in the isotopic ratio. The reason is that chemical bonds with the heavier isotope are stronger to a minute extent and the required energy for their cleavage is higher. Subsequently, in biological systems usually the lighter isotopes are preferentially reacted. For instance, fractionation occurs in CO_2 fixation by photosynthetic organisms or in methane formation from CO_2 (Meckenstock et al., 2004).

Following, biodegradation with MOs causes isotope fractionation. Mostly the lighter isotopes of a contaminant are metabolized earlier than the heavier ones. Subsequently, there is an increase in the concentration of the heavier isotope, which could be used in natural abundance studies (Richnow et al., 2003).

The variation in fractionation is caused by the MOs enzymes. Worsey and Williams, (1975) found out that oxygenase reactions of P. putida strain mt-2 , which were catalysing a C–H bond cleavage of xylene exhibits significant carbon isotope effects. Olsen et al., (1994) assessed a similar reaction of Ralstonia picketii strain PKO1 with monooxygenase, but this time it showed a less significant isotope fractionation. In conclusion, in some cases the extent of isotope fractionation can be related to the enzyme reactions involved, but it cannot be generalized that an enzymatic reaction always lead to the same fractionation (Meckenstock et al., 2004).

Nevertheless, in combination with other measurements like hydrogen isotope fractionation it could be useful for in situ quantification of bacterial activity in biodegradation (Morasch et al., 2002). The kinetic isotope fractionation can also be calculated using the Rayleigh equation (Fischer et al., 2004; Rayleigh, 1896).

The measurement method which is based on fractionation of single compounds (e.g. contaminants is Compound Specific Isotope Analysis (CSIA).

CSIA is viable for site characterization e.g. to find out if biological or abiotic degradation occurred. Furthermore, it can identify how much and where the biodegradation happened. In addition it is also feasible for gaining information about the right remediation approach and subsequently for the monitoring of the respective approach (Diagnostics, 2011).

1.4.1.2 Natural abundance vs. artificial labelling studies

Natural abundance studies rely on a naturally difference in the isotopic ratio. A proper example for natural abundance studies is the detection of methanotrophic bacteria. The methane that is used for growth shows a high depletion compared to other carbon substrates of – 50 to -100 ‰. Furthermore, methanotrophs fractionate against ^{13}C in their metabolism which adds another 0- 20 ‰ depletion (Jahnke et al., 1999).

Artificial labelling, or stable isotope probing (SIP) artificially creates the differences in the isotopic ratio. It offers interesting possibilities to separately study the activities of different MO- groups and their biodegradation ability. Additionally, it can provide information that ranges from identification of broad groups of MOs to the identification of specific organisms, genes or enzymes. In summary, it can confirm the biodegradation ability or the effectiveness of existing remediation approaches or aid in the design of remediation setups. Until now there is a huge amount of SIP applications for contaminations like PAHs, pestizides or gasoline constituents (Diagnostics, 2011).

Stable isotope biomarkers are not limited by legal restrictions and health concerns like radioisotopes and can be used directly in the field. There are many options to use stable isotope labels, like the linking of population structure with specific microbial processes by the labelling with specific ^{13}C compounds. Basically, the added isotope tracer will be incorporated into the biomass of the metabolically active population. Subsequently the tracer can be detected in biomarkers like PLFAs, which reveals numerous information (Boschker and Middelburg, 2002).

1.4.2 ^{14}C in PHC biodegradation

The use of ^{13}C- ranges has also disadvantages, e.g. petroleum hydrocarbons ($\delta^{13}C_{PHC}$ = -18 to -35‰) interfere with the limited range of natural organic matter ($\delta^{13}C_{NOM}$ = -24 to -34‰ for C3 plants). Therefore it is difficult to distinguish between the degradation of PHC carbon and other carbon sources (Faure, 1986).

In contrast, due to radioactive decay over millions of years, PHCs contain no detectable ^{14}C ($\Delta^{14}C_{PHC}$=-1000‰) and shows a great difference compared to modern carbon sources e.g. recently fixed carbon from the atmosphere ($\Delta^{14}C_{atm}$≈+55‰) (Turnbull et al., 2007). Hence, a negative shift in $\Delta^{14}C$, e.g. in PLFAs of the MOs show directly and without labelling the uptake and metabolism of PHCs (Slater et al., 2006).

The use in in situ experiments with radioactive markers such as ^{14}C is usually restricted; they are almost exclusively applied in laboratory conditions (Chapelle et al., 1996).

1.4.3 Representative fractions of diesel for isotopic labelling

It is difficult to create a laboratory experiment with diesel for isotopic analyses. The main problem is the simulation of in situ conditions caused by the plethora of different parameters-from the environmental conditions to the microbial community. Furthermore, due to the vast complexity of the diesel compounds it is nearly impossible to create measurable and comparable conditions or an identical diesel mixture which is labelled. That mixture would be too costly and the 100 % determination of every compound in the diesel bulk is also not realizable.

Therefore, a mixture of characteristic compounds which reflect the characteristic and recalcitrance of diesel is suggested for further biodegradation experiments. Based on measurements of diesel from (Liang et al., (2005) and Sjögren et al., (1995) we would suggest these composition in order to produce a comparable and measureable diesel substitute. Here is the representative mixture for diesel (REPMIX) we suggest (Table 6):

Table 6: REPMIX for diesel biodegradation studies

PHC	Fraction	content
Hexadecane	alkanes	app. 30 %
Toluene	monoaromatics	app. 7 %
Decylcyclohexane	cycloalkanes	app. 7 %
Naphtalene	PAHs	app. 6%
Pristane	branched alkanes	app. 50 %

The most important fractions to label would be the more recalcitrant with naphthalene and pristane, which are responsible for the long-time contamination. It is depending on the budget how much of the different fractions could be add as labelled chemical, because labelled material is relative expensive. The normal chemicals for REPMIX are available at certain chemical producers, e.g. Sigma Aldrich for affordable prizes.

Pelz et al., (2001) used ^{13}C labelled toluene in their experiments. Feisthauer et al., (2010) used n-hexadecane as a model aliphatic hydrocarbon, although for anaerobic oil degraders. Nevertheless, e.g. in the groundwater saturated zones they are of significant importance. In this study n- hexadecane was used as well as labelled pollutant. Morasch et al., (2007) used naphthalene as ^{13}C labelled substrate to determine the intrinsic biodegradation potential of aromatic pollutants under oxic and under anoxic conditions.

Naphtalene is within the diesel content with a significant amount, furthermore it belongs to the 16 priority PAHs designated by the United States Environmental Protection Agency (US-EPA; http://www.epa.gov/) and to the 33 priority substances recently defined in the EU Water Framework Directive (Liang et al., 2005).

1.5 Approaches to prove biodegradation

To prove the MOs biodegradation with sufficient information for a practical approach to plan remediation strategies is a difficult task.

On the one hand under field conditions the source zone could contain toxic concentrations of the contaminant or exhibit nutrient depletion which will inhibit the biodegradation. On the other hand in laboratory experiments the non- cultivability of the degrading MOs or the absence of them in the place of the sampling will cause the same inhibition.(Bahr et al., 2015)

Hence, there is no single standard procedure that can prove biodegradation of organic environmental pollutants in contaminated environments, due to the fact that every method has its advantages and limitations. Therefore two or more individual approaches can be combined (Morasch et al., 2007).

Here are some examples of possible approaches to prove biodegradation of contaminants:

- The evidence of concentration decrease of contaminants over time and distance (Wiedemeier, 1999)
- enrichment of heavy stable isotopes in the remaining fraction of organic contaminants (Hunkeler et al., 2002)
- ^{14}C radiotracer studies (Bianchin et al., 2006)
- succession of redox zones in the field (Vroblesky and Chapelle, 1994)
- accumulation of signature metabolites (Beller, 2002)
- investigation of the intrinsic microbial biodegradation potential in microcosm studies (Ambrosoli et al., 2005)
- characterization of the bacterial community by molecular techniques (Bakermans et al., 2002)
- tracing ^{13}C in fatty acid profiles of bacteria (Geyer et al., 2005)
- detection of bacterial enzymes (Heinaru et al., 2005)
- In situ microcosm (Bactrap ®) with stable isotope labelling (Bahr et al., 2015; Bombach et al., 2010)

1.5.1 PLFA approaches

Due to their rapid decomposition of a few days outside the living cell, PLFAs are useful biomarkers for active microbial biomass (Dey and Guha, 2007). Therefore, they can be distinguished to the remains of dead organisms that have accumulated over time. PLFAs are found in bacteria and eukaryotes, they show a range of 30 to 50 different compounds and several of these can be used as specific biomarkers (Pelz et al., 2001).

Originally, the purpose of the PLFA analyses was more like a fingerprint of different microbial species. Many qualitative analyses were assessed, where fatty acid patterns characterize bacterial species (Dunlap and Perry, 1967; Makula and Finnerty, 1968; Wilkinson et al., 1972). However, in a consortium of unknown constituents it remains difficult to determine specific species by their PLFA patterns due to the ubiquity of the PLFA in related species.

For instant, *Micrococcus cerificans* has been shown to synthesize significant amounts of 15:0 and 17:0 acids when grown on undecane, tridecane, pentadecane and heptadecane (Makula and Finnerty, 1968). In another experiment several bacterial strains were grown on propionate, which also led to an increased synthesis of 15:0 and 17:0 acids (Vestal and Perry, 1971). Therefore, 15:0 and 17:0 fatty acids cannot be considered as potential markers of hydrocarbon- degraders (Aries et al., 2001).

The occurrence of a broader pattern could indicate more or less the presence of PHC degraders, e.g. Aries et al., (2001) considered the simultaneous appearance of iso-, anteiso- and mid-chain branched PLFAs, odd-numbered straight chain MUFAs and branched MUFAs as a potential bio-indicator.

In any case, a qualitative applicability is given by the possibility of differentiating between major groups of MOs like bacteria, fungi and algae with some further details within these groups (Boschker and Middelburg, 2002).

Quantitatively, PLFAs offer a plethora of information about changes in communities and the biomass. Therefore, PLFAs are more often used as a qualitative and quantitative combination in the presence.

Another disadvantage is that PLFA analyses are relatively slow in the measurement. Normally, the preparation of a small batch with 20 to 24 samples takes appr. 1.5 to 3 days depending on the laboratory equipment. Buyer and Sasser, (2012) developed a high throughput method which enabled the preparing 96 soil samples and blanks in 1.5 days, a 4- to 5-fold increase in throughput, but their results were not compareable to those of the normal PLFA extraction.

Similarities concerning the C- chainlength of PLFAs and the hydrocarbon substrate were often monitored if it were alkanes, e.g. when labelled hexadecane was added as substrate their was an enrichment in the 16:0 PLFA (Rodgers et al., 2000). Maybe the PLFA pattern depends more on the substrate than on the species, as was expected.

Greenwood et al., (2009) considered with their quantitative and qualitative PLFA data that hydrocarbon degrading microbe concentrations increased with repeated hydrocarbon treatment. In conclusion, PLFAs are sensitive to community shifts, but have the disadvantage of a poor taxonomical resolution (Watzinger, 2015).

A statistical tool for PLFA analyses may be the hydrocarbon degradation activity index (HDAI). It was developed by Aries et al., (2001), and it should be a tool to reveal the development of hydrocarbon degrading strains in oil-contaminated sediments with PLFAs. Thereby, a certain consortium of PHC degrading bacteria was cultivated on two different media, on defined ammonium acetate medium (AAM) and on a Blend Arabian Light petroleum medium (BALM). The variation of the PLFA-percentages in the AAM and BALM of the saturated fatty acids (SFAs), the straight chain mono unsaturated fatty acids (MUFAs) and the branched MUFAs should show a correlation by the following equation:

HDAI=(odd numbered straight chain SFAs % + branched SFAs % + odd-numbered MUFAs % +branched MUFAs %)/even-numbered MUFAs %

For the AAM cultures the HDAI values were less than 0.1, whereas for the culture on BALM 1.57 was reached, hence the higher the HDAI, the more possible is PHC biodegradation. Therefore it might be a potential tool to evidence bacterial growth at the expense of a complex hydrocarbon mixture such as crude oil (Aries et al., 2001). The experiment based on marine sediments, it wasn’t applied with biodegradation on land. Furthermore the limitations of the HDAI are also described, e.g. the index does not describe exactly the quantitative variations for the different PLFA groups of the consortium (Aries et al., 2001).

1.5.2 Viability of PLFA analyses combined with SIP to indicate changes in PHC degrading MO- community structures

Stable isotope analysis with PLFA biomarkers provides a powerful approach. This combination combines the unique possibility to directly connect the microbial identity (PLFA as microbial group biomarker), the biomass (concentration of the biomarker) and activity (isotope concentration in the PLFAs) (Boschker and Middelburg, 2002).

In artificial labelling approaches it is recommended not to use too highly labelled substrate because of 3 reasons (Watzinger, 2015):

1. Highly labelled substances are expensive
2. Analytical problems might arise with the IRMS
3. They might influence the bacterial metabolism

1.6 Hypothesis, aims and used methods

After the extended literature review the question was how to determine the viability of a CW for the degradation of weathered diesel. Therefore, we tried to simulate the conditions of the

CW in the laboratory with material of the support matrix and the contaminated groundwater. Subsequently, ^{13}C enriched hexadecane was added as label and the MO community was determined by PLFA analyses. A CO_2 analyse was also performed as pretest, to find out if biodegradation is happening with this setup.

The hypotheses were:

- The autochthone MO community is viable for PHC biodegradation.
- PLFA analyses are a proper method to determine the best variation of a CW setup.
- PLFA analyses allow the characterization of the PHC degrading community.

2.Materials and Methods

2.1 Overview

The main objective was to determine the viability of the autochthone microorganisms from the constructed wetland (CW) to degrade hydrocarbons. Therefore, the $^{13}C/^{12}C$ isotopic ratio of a labelled Hexadecane as contaminant was determined for detection and quantification. This ratio was measured for the respired CO_2 and the incorporated PLFAs.

2.2 Characterization of the constructed wetland plant

Our CW was a vertical flow system with 4 beds (Figure 5 and 6). Each bed had a length of 4 meter, a width of 3 meter and a depth of 1.5 meter. In each of those beds was a different filter body: bed 1 contained 0/4 sand, bed 2 contains 1/4 Liapor expanded clay , bed 3 contains 0/4 Sand plus 3%(vol) Biochar and bed 4 also 0/4 Sand. The plantations of the CW were *Salix viminalis* plants.

Figure 5: bed 1 from the constructed wetland (Paul Kinner ©)

Figure 6: plantation: Salix viminalis (Paul Kinner ©)

Table 7: support matrix profile of the CW (adapted from Paul Kinner ©)

	layer diameter	bed 1	bed 2	bed 3	bed 4
surface layer	10 cm	Gravel 4mm/8mm washed	Gravel 4/8 washed	Gravel 4/8 washed	Gravel4/8 washed
main layer	100 cm	sand 0/4 washed	sand 0/4 washed	Liapor HD 1/4 (expanded clay round tight)	sand 0/4 washed + Biochar (upper 80 cm) sand 0/4 washed (lower 20 cm)
transition layer	10 cm	gravel 4/8 washed	gravel 4/8 washed	gravel 4/8 washed	gravel 4/8 washed
drainage layer	20 cm	gravel 16/32 washed	gravel 16/32 washed	gravel 16/32 washed	gravel 16/32 washed

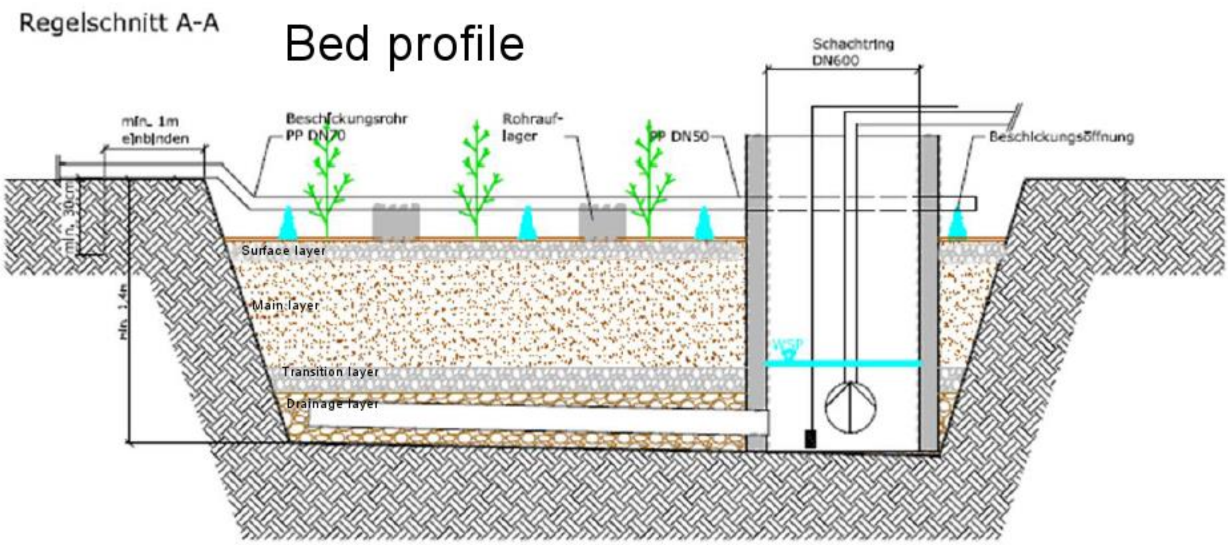


Figure 7: Support matrix profile of the CW (adapted from Paul Kinner ©)

Willows were selected because of their physiological characteristics. They show high rates of evapotranspiration, efficient nutrient uptake, tolerance of flooded conditions and high biomass productivity. Furthermore, they possess the ability to transport oxygen down to the root zone through aerenchyma formation, which is contributing to better conditions for bacterial growth (Kuzovkina and Quigley, 2005). The species *Salix viminalis* in particular, is capable of high growth rates and high adaptability to new climate conditions (Labrecque and Teodorescu, 2003).

The possible measurements with the instruments of the CWs are:

- O_2- content (mg/l and %(vol))
- Water temperature (°C)
- Electronic conductivity (µS/cm)
- pH- value
- ambient temperature (°C)
- plot temperature (°C)

2.3 Microcosm design

In order to simulate similar conditions as in the CW, we prepared 20 microcosms (MCs). Therefore, the solid phase was flooded with groundwater from the CW (table 8). The groundwater sample showed an increased iron and manganese content. No amendments were added, similar to the en situ conditions. The containers we used were 250 ml Boston round bottles with Mininert ® valves, which enable gastight sampling for CO_2 analyses. They were filled up with 50 g (dry mass- water content was measured before) of the wetland substrate and 100 ml of the diesel contaminated groundwater. Subsequently, they were stored at 12°C into a temperature chamber. Finally each MC was labelled with 30 µl of the label, except the 5 MCs which were freeze dried after 0 days (MC 16- 20).

Table 8: Groundwater characteristics (Paul Kinner ©)

Dissolved oxygen	~0 mg/l
Temperature	~12 °C
pH	6.9
electr. conductivity	~1600 µS/cm
Fe	1.4 mg/L
Mn	0.75 mg/L
Hydrocarbon- concentration	**~1.5 mg/l**

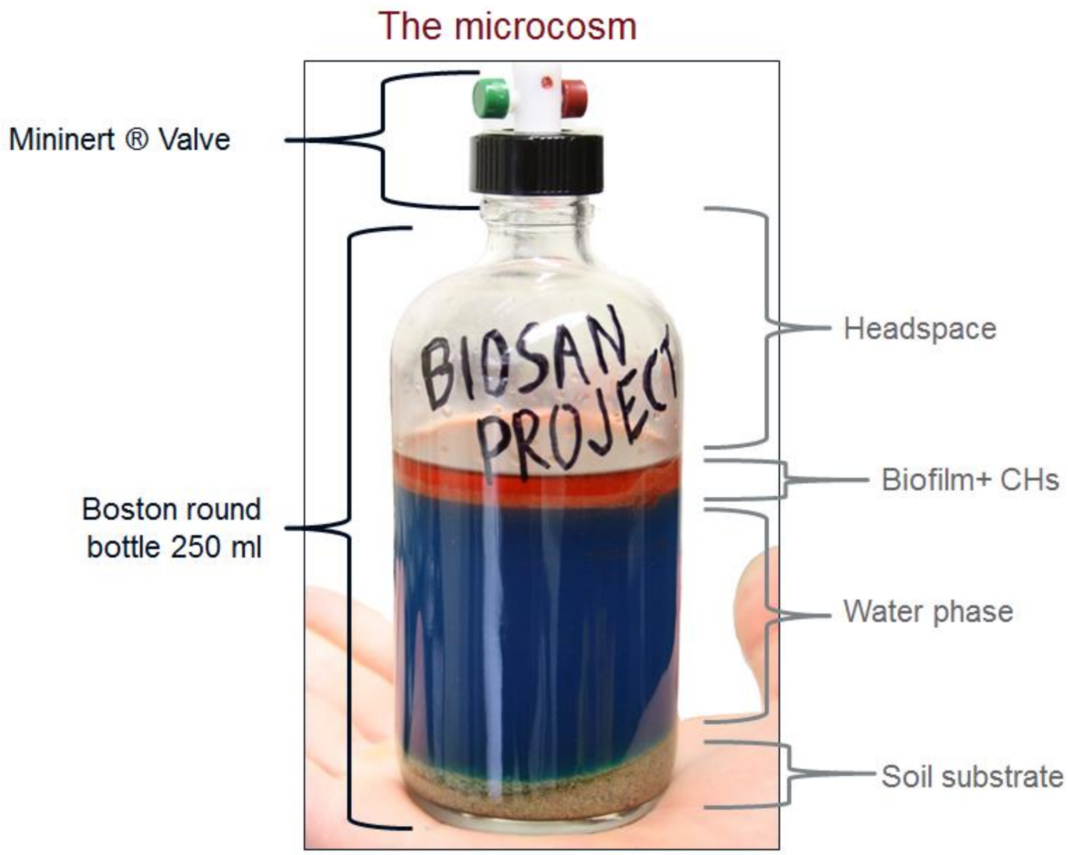


Figure 8: Boston round bottle which were used as MC with the different layers in colour for visualization

The labelled HC was Hexadecane (C_{16}), it is an alkane and therefore easily degradable by MOs. The labelled ^{13}C atoms were at the 1st and the 2nd position of the hexadecane molecule (Figure 9).

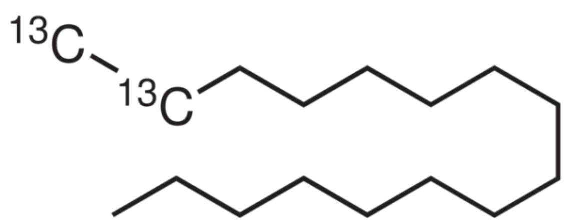


Figure 9: ^{13}C label (Sigma Aldrich)

This label we acquired from Sigma Aldrich had a total weight of 100 mg, was sealed in a gastight glass ampule and had a purity of more than 99%. The delta of the label was δ= 10074 ‰ which is 12.44% ^{13}C content.

The intended delta value we determined as appropriate was δ=100 ‰. With a HPLC hexadecane from Sigma Aldrich which had a measured delta of -32.31 ‰ the enriched C_{16} was diluted. Subsequently, we generated 10 ml of C_{16} label with δ=98,75 ‰ which is 1.23 % ^{13}C content.

2.4 Measurement plan

20 MCs were prepared, each one was labelled with 30 µl ^{13}C labelled C_{16} label, 5 MCs were sterilized as control and 15 were freeze dried. Afterwards 5 MCs were used for CO_2 measurement and 15 were used for PLFA analyses (Table 9).

Table 9: measurement schedule for all 20 MCs

MC Name	Purpose	measurement schedule	Comment
S1 Control	CO2	after 0, 6, 11, 24, 48,96,192,312,504 hours	sterilized
S2	CO2	after 0, 6, 11, 24, 48,96,192,312,504 hours	
S3	CO2	after 0, 6, 11, 24, 48,96,192,312,504 hours	
S4	CO2	after 0, 6, 11, 24, 48,96,192,312,504 hours	
S5	CO2	after 0, 6, 11, 24, 48,96,192,312,504 hours	
S6 Control	PLFA	after 18 days	sterilized
S7	PLFA	after 18 days	
S8	PLFA	after 18 days	
S9	PLFA	after 18 days	
S10	PLFA	after 18 days	
S11 Control	PLFA	after 2 days	sterilized
S12	PLFA	after 2 days	
S13	PLFA	after 2 days	
S14	PLFA	after 2 days	
S15	PLFA	after 2 days	
S16 Control	PLFA	after 0 days	sterilized
S17	PLFA	after 0 days	
S18	PLFA	after 0 days	
S19	PLFA	after 0 days	
S20	PLFA	after 0 days	

2.5 CO_2 measurements

The gas samples for measuring $^{13}C/^{12}C$ ratio of CO_2 were directly taken from the MCs. Afterwards they were injected by hand into the GC of the Delta V IRMS. Therefore, we used 100 mml gastight Hamilton syringes to withdraw the gas sample of the microcosm through the Mininert® valve.

The advantage of these valves is that they allow gastight withdrawal of the headspace air by switching it to “green”. At each withdrawal the syringes were flushed 5 times to homogenize the sample. After sampling the valves were switched back to starting position, which is “red”. Now the perforated septum can be exchanged for the next sampling.

The withdrawn volume was refilled with synthetic air (80% Helium, 20% O_2- prepared with a 2 L gastight syringe), to compensate the vacuum, which was caused by the withdrawal. Besides, the microcosm was provided with sufficient oxygen to prevent anaerobic conditions.

During the injections at the Delta V, the MCs were stored in a cooling box with cooling pads to prevent an influencing drift in temperature while measuring.

External CO_2 standards were prepared with different CO_2 contents in order to create a calibration curve. For that reason, different CO_2 concentrations (0.5 %, 1%, 2%, 4%, 5% v/v) were mixed in a 2 L gastight syringe with Helium.

The measurement device was a Trace GC Ultra with Combi PAL Autosampler with GC Isolink and a ConFlow IV interface for gas sample fractionation. Afterwards the samples were oxidized in a capillary combustion reactor at 1030°C. Subsequently, the isotopic ratio was measured in a Delta V Advantage Mass Spectrometer. The Injection per syringe was carried out into a SSL (Split/Splitless) Injector.

2.6 PLFA Analysis

2.6.1 Qualitative analyses

The composition of the MOs was investigated by phospholipid fatty acids (PLFAs) analyses. Basically, the procedure was the same as Bligh and Dyer (1959), adapted by Watzinger et al. (2014). Due to the low content of MOs in the soil, an adapted extraction method for low MO content had to be assessed which we called LOWMO. Through a preliminary PLFA tests (pretest) we found out that we had to use all 50 g of substrate in the MCs for each sampling. 16 PLFAs which were contained in a relevant amount were analysed and arranged in five groups.

The samples were freeze dried, in order to eliminate the water content subtraction of the citrate puffer. Firstly, a proper approach with this large sample volume and the available laboratory equipment, for the Bligh and Dyer extraction had to be found.

Consequent upon this LOWMO approach, we added 200 ml Bligh and Dyer solution into the microcosms with 50 g soil. In order to optimize the extraction, the samples were put onto a shaker for 30 minutes with 300 rpm, afterwards they were stored overnight. Every extraction was limited to 4 samples (3 samples+ 1 blank), limited by the laboratory equipment capacity.

On the next day, the liquid phase with the B&D and the dissolved PLFAs was separated from the solid phase into a 250 ml Schott flask. In order to get the maximum output we filtrated the two phases through a filter paper on a glass funnel. Subsequently, the filter paper with the solid phase was discarded and 20ml of Chloroform with a volumetric pipette were added through the glass funnel to rinse PLFA rests from the funnel. After that, 20 ml distilled water were add to separate the polar phase from the chloroform phase. For an optimal phase separation, the flasks were put onto the shaker for 15 min with 300 rpm, afterwards they rested for another 15 min to separate and tranquilize.

For avoiding rests of the polar water/methanol phase in the SPE extraction the whole phase was pipetted with a 100 ml volumetric pipette and discarded. The lower chloroform phase (app. 70 ml) was separated into 6 12 ml centrifuge vials per sample or microcosms, retrospectively. These chloroform vials, in total 60 ml, were put onto the heating block at 40°C and constant N_2 flow, in order to combine them into one vial. Before combining two vials the one we emptied, was vortexed for 10 seconds. When vaporization of the one left vial with the whole amount of sample was done, the PLFAs were re-dissolved in 500 µl Chloroform and we continued with the SPE extraction.

Table 10: Comparison of the LOWMO to the normal PLFA extraction method

Normal method (Watzinger et al., 2014)	LOWMO
1 • 2g soil sample into 12 ml centrifuge tubes	1 • Freeze drying of the 50 g soil
2 • + 8 ml B&D	2 • + 200 ml B&D
3 • Vortex (2 x 10 s) stored darkly overnight	3 • Shaker 30 min, 300 rpm
4 • Vortex (2 x 10 s) Centrifuge: 1500 g, 10 min	4 • Stored darkly overnight
5 • Supernatant in new 12 ml tubes	5 • Liquid phase separation into 250 ml Schott bottle
6 • +2 ml B&D in the 12 ml tubes with soil	6 • +20 ml $CHCl_3$ and 20 ml H_20
7 • Vortex (2 x 10 s) Centrifuge: 1500 g, 5 min	7 • Shaker 15 min 300 rpm and 15 min waiting
8 • Supernatants are brought together in 12 ml tubes	8 • Pipetting Supernatant
9 • + 1.5 ml $CHCl_3$ and 1.5 ml H_2O	9 • Vaporizing the 60 ml CHCL3 phase 40°C , under N_2 flow
10 • Vortex (2 x 10 s) Centrifuge: 2000 g, 5 min	10 • Combining the 6 vials into 1 and complete vaporization
11 • 3.6 (2 x 1.8 ml) $CHCl_3$- phase in centrifuge tubes	11 • + 500 µl $CHCl_3$
12 • 40°C, N_2 – drying + 500 µl $CHCl_3$	

The interpretation of PLFA biomarkers was modified after Paul (2014):

- Gram positive bacteria: i14:0, i15:0, a15:0, i16:0, a17:0,i17:0 (Brennan, 1988)
- Actinomycetes: 10Me16:0, 10Me17:0, 10Me18:0, 12Me18:0 (White et al., 1997)
- Gram negative bacteria: 16:1ω7c, cy17:0, 17:1ω8,18:1ω7c, cy19:0 (Wilkinson, 1988); (Moss and Daneshvar, 1992); (Waldrop et al., 2000)
- Fungi: 16:1ω5c,18:2ω6.9, 18:1ω9c (Zak et al., 1994);(Frostegård and Bååth, 1996);(Olsson et al., 1995)
- unspecific fatty acids: (14:0, 15:0, 16:1ω6c, 16:0, 17:0, 18:0, 19:1)

The PLFAs were measured on a gas chromatgraph (GC): Hewlett Packard 5890m II, equipped with flame ionization detector (FID), Agilent 7890A.

2.6.2 ^{13}C label incorporation analyses

The reason why PLFAs are derivatised to their fatty acid methyl esters (FAMEs) prior to analysis is to make them measureable for GC.

Hence, a mixture of FAMEs is injected into the instrument prior to the GC column, by passing through the GC, they are separated into a series of individual FAME peaks.

Afterwards the FAME peaks enter the oxidation column and get burned down to series of CO_2 peaks. By the sensible mass spectrometer columns these CO_2 peaks can be separated into $^{12}CO_2$ (mass 44) and $^{13}CO_2$ (mass 45). Finally these $\delta^{13}C$ values determined by GC-c-IRMS are therefore those of the methyl esters ($\delta^{13}C$ FAME) (Yao et al., 2015).

In this work, the $\delta^{13}C$ values of the FAMEs, in relation with Vienna Pee Dee Belemnite (VPDB) with a ratio of $^{13}C/^{12}C= 0.01123272$ were measured on a gas chromatograph combustion isotopic ratio mass spectrometry (GC-c-IRMS) Agilent GC 7890A to Delta V Advantage IRMS (Thermo Fisher) with a CuO/NiO/Pt combustion oven and a CTC PAL autosampler and Gerstel PTV injector in Seibersdorf.

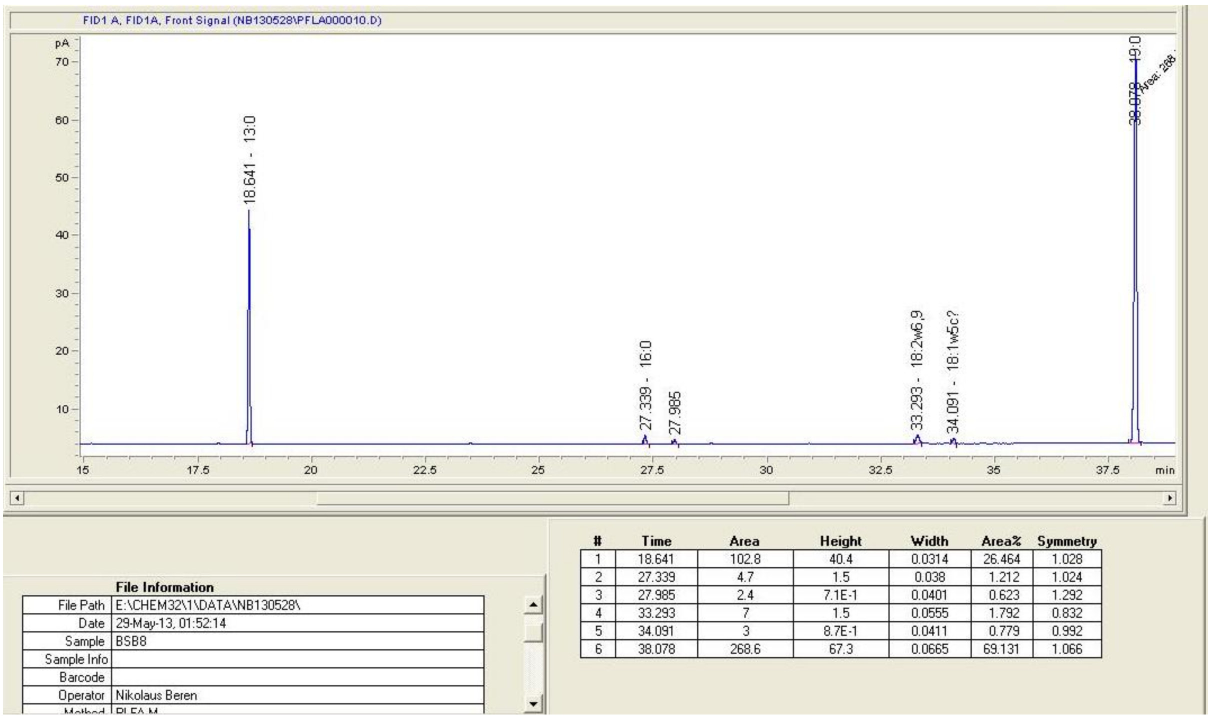
A mass balance equation is used to account for the one carbon added in the methyl group during the derivatisation process (Esperschütz et al., 2009) to determine the isotope ratio of the PLFAs themselves ($\delta^{13}C$ PLFA).

3. Results & Discussion

3.1 Pretests

3.1.1 PLFA measurements

First of all, pretests were assessed, on the one hand to train the PLFA extraction and on the other hand to learn more about the amount of PLFA biomass in the CW substrate. Therefore, samples of the sand from the same bed 2 were taken and measured after the standard method with 2 g sample.



#	Time	Area	Height	Width	Area%	Symmetry
1	18.641	102.8	40.4	0.0314	26.464	1.028
2	27.339	4.7	1.5	0.038	1.212	1.024
3	27.985	2.4	7.1E-1	0.0401	0.623	1.292
4	33.293	7	1.5	0.0555	1.792	0.832
5	34.091	3	8.7E-1	0.0411	0.779	0.992
6	38.078	268.6	67.3	0.0665	69.131	1.066

Figure 10: GC- FID chromatogram of the PLFA pretests

The flame ionization detection (FID) results of the Pretests showed us that the MOs content with a 2 g substrate sample of the CW was too low to get significant results (Figure 10). The WP was build 3 month ago before sampling therefore the microbial biomass in the support matrix was small. With the FID measurements, a peak height of 17 pA was determined as minimum for a feasible result and as can be seen the only peaks which exceed this limit are the two external standards. Subsequently to obtain sufficient biomass for the PLFA analyses the LOWMO method was developed as described in the Materials & Methods part.

As mentioned in the Introduction part, PLFA extraction is costly and difficult to handle. In the future, approaches to optimize the extraction e.g. the high throughput method from Buyer and Sasser (2012) could be assessed. Furthermore there are still fatty acids of MOs which are unknown (Watzinger, 2015), so there is still much room for improvement for the PLFA approaches.

3.1.2 CO_2 measurements

In order to prove incorporation of the hexadecane- label and subsequently biodegradation, we assessed CO_2 measurements with the MCs S1, S2, S3, S4 and S5. The sterile S1 MC showed as anticipated no CO_2 production; therefore we have only results from S2- 5 (Figure 11).

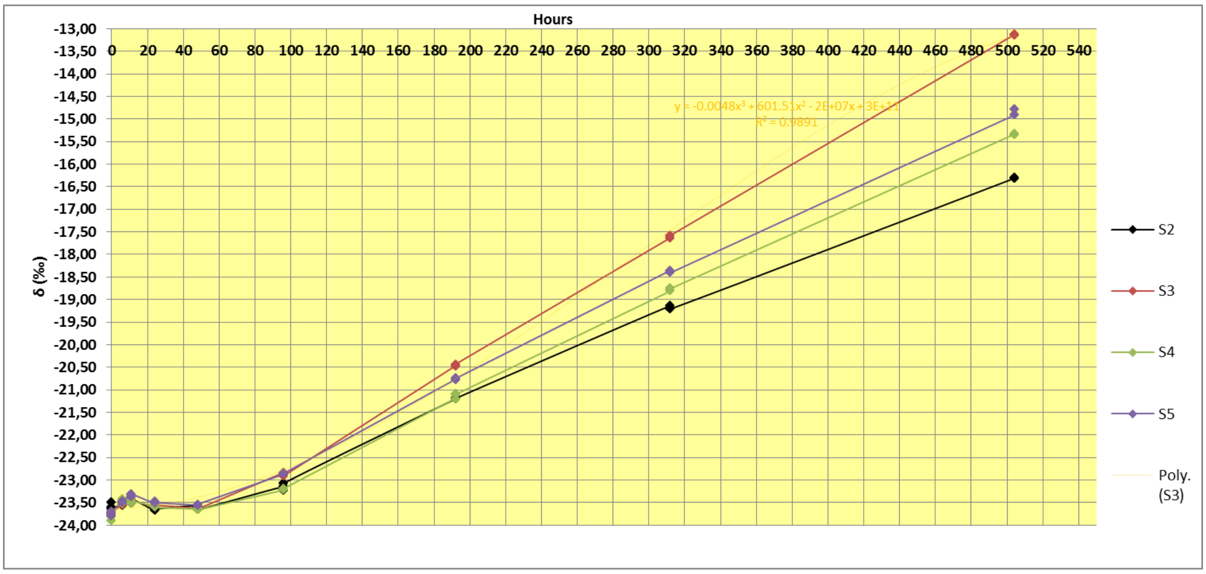


Figure 11:CO2 results of the Delta V

Each of the 4 MCs showed definitely an increase in the delta value, therefore even under poor nutrient conditions there is biodegradation measureable. Hence, in our MO consortium are HC degraders, because it was almost the only carbon source.

One of the major problems of bioremediation research is that laboratory conditions are hardly comparable to field conditions. Due to a huge variation in number of factors in the field such as weather, it is difficult to create similar conditions. Therefore, further research should be also carried out in the field, in order to be able to deliver practically relevant results (Dadrasnia and Agamuthu, 2014).

3.2 Main experiment

3.2.1 PLFA analyses

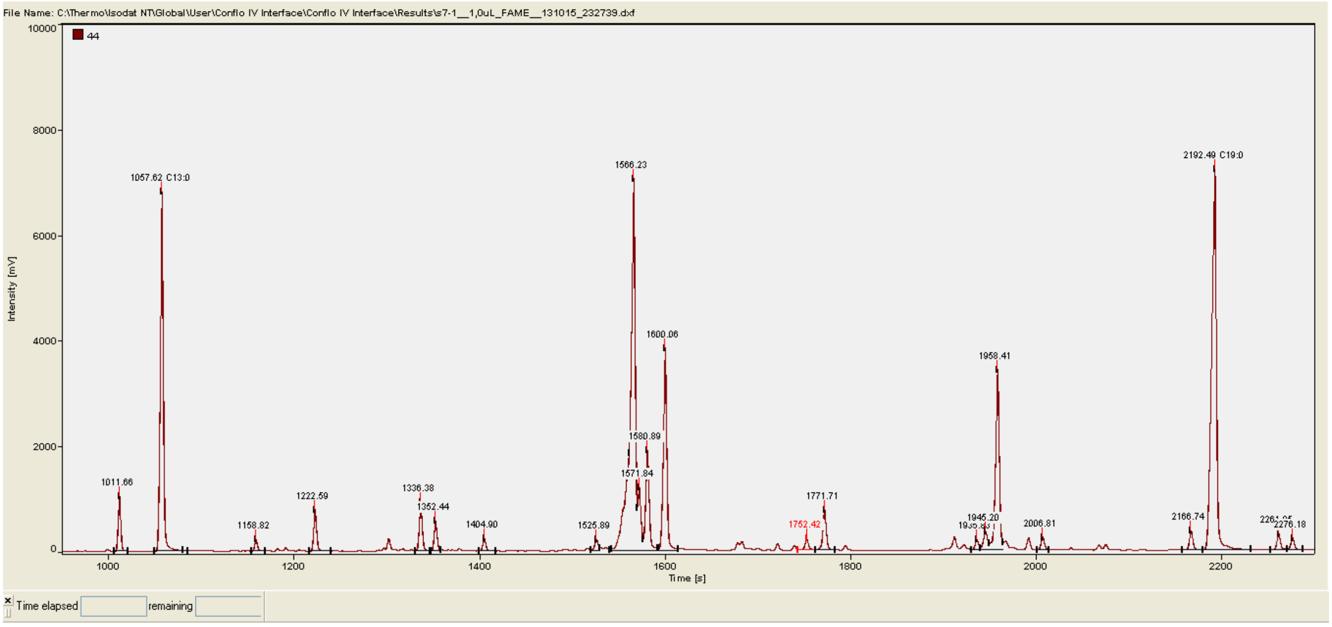


Figure 12: PLFA GC- IRMS chromatogram of MC 7 with the LOWMO method

As can be seen on the chromatogram from the GC- IRMS the LOWMO method brought sufficient peak heights (Figure 12). The first peak (1012) is the remnant of the undegraded Hexadecane label which is easily to prove because its delta value is +92,1‰. The peaks of 16:1ω7 (1566.23) and 16:1ω6 (1571.84) were inseparable; therefore their areas were add together, however the major part, as can be seen, belongs to 16:1ω7.

Table 11: Identification of the PLFAs with retention time

Retention Time rounded (Sec)	1012	1058	1159	1223	1336	1352	1405	1526	1566	1572	1581	1600	1752	1772	1936	1945	1957	2007	2167	2192
PLFA		13:0	i14:0	14:0	i15:0	a15:0	15:0	i16:0	16:1ω7	16:1ω6	16:1ω5	16:0	17:1ω6	cy17:0	18:2ω6,9	18:1ω9c	18:1ω7c/9t	18:0	19:1	19:0
Comment	C_{16}	Std																		Std

Due to the lack of experience with the LOWMO method in the laboratory, the significance of the results is very low (Table 12). The standard deviation of the individual PLFA measurement repetitions was over every reasonable significance level; hence no statistical analyses would make sense. Therefore, only qualitative results will be shown, described and discussed.

Table 12: GC- FID PLFA results of the 15 MCs after 0 days (red), 2 days (blue) and 19 day (green)

MC	Freezing date	extraction date	Comment	Total biomass (µg/g)	Average biomass	Std Dev
S6	06.08.2013	29.08.2013	Sterile	0,0731		
S7	06.08.2013	21.08.2013	20 ml CHCl	2,1251		
S8	06.08.2013	22.08.2013	20 ml CHCl	1,2667	**1,4528**	**0,4511**
S9	06.08.2013	27.08.2013		1,2627		
S10	06.08.2013	03.09.2013	30 ml CHCl	1,1566		
S11	20.07.2013	03.09.2013	Sterile	0,4297		
S12	20.07.2013	27.08.2013		0,3562		
S13	20.07.2013	29.08.2013		0,1950	**1,4584**	**1,4111**
S14	20.07.2013	03.09.2013		2,2134		
S15	20.07.2013	04.09.2013		3,0687		
S16	18.07.2013	04.09.2013	Sterile	2,2222		
S17	18.07.2013	27.08.2013		2,6399		
S18	18.07.2013	27.08.2013		1,9358	**1,9982**	**1,2542**
S19	18.07.2013	29.08.2013		0,2707		
S20	18.07.2013	04.09.2013		3,1466		

It is important to run an adequate set of pretests. On the one hand in order to train the PLFA extraction and the measurement and on the other hand to get important information about the biomass content. This is especially important when the sample material is not natural soil.

In natural soils for obtaining a representative PLFA content, 1- 3 g of soil should be sufficient (Frostegård et al., 1991). However, environmental samples as compost, landfill leachate or waste material may need a larger sample size and also special treatments like freeze drying to get proper results (Mellendorf et al., 2010; Watzinger et al., 2008). Subsequently with a sample amount of 50 g it is vital that the preparations, as far as laboratory, equipment, chemicals, extraction method, are sophisticated and as identically operated as possible.

3.2.2 ^{13}C isotope analyses

From measured 15 MCs the three most representative and reproducible MCs were chosen, we took MC 20 as the microcosm after 0 days, MC 15 after 2 days and MC 9 after 19 days (Figure 13). They showed the best results, as far as the comparison to the external standards and peak heights are concerned. Due to the LOWMO method there are fluctuations in the different repetitions of the results which make the evaluation of the results difficult.

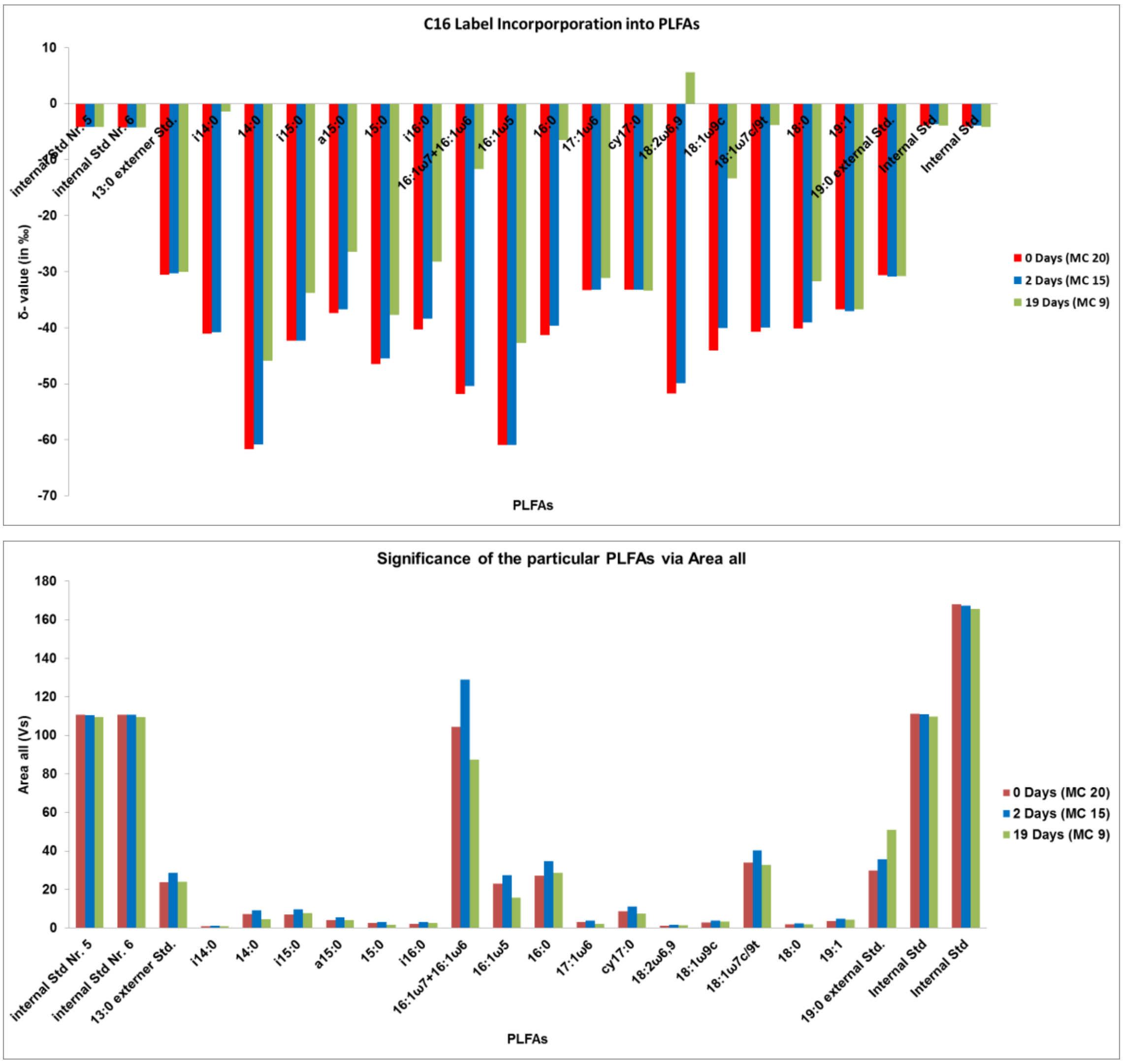


Figure 13: GC- amount and $\delta^{13}C$ values of the PLFAs on day 0, 2, 15 after ^{13}C- hexadecane addition

On table 13 there are all relevant values, the area all values from GC- IRMS and the delta values. The area all values show the amount and following the significance of the different PLFAs. With the delta values the label incorporation can be seen, the more positive the values, the more label was incorporated in the PLFA of that certain MO. The results were arranged in descending order, to see which ones were most abundant. Due to diffusion processes between the water phase and the headspace, there were probably aerobic conditions.

Table 13: GC- IRMS Seibersdorf results of the 15 MCs PLFA extractions ordered after area all

	Relevance via area all					C13 Shift				MO group
PLFA	**0 Days (MC 20)**	**2 Days (MC 15)**	**19 Days (MC 9)**	**Sum**		**0 days**	**2 days**	**19 days**	**C13 Shift 0-19**	
16:1ω7+16:1ω6	104,384	128,973	87,393	320,75		-51,791	-50,421	-11,665	-40,126	gram negative
18:1ω7c/9t	34,051	40,294	32,761	107,106		-40,75	-39,99	-3,847	-36,903	gram negative
16:0	27,141	34,67	28,695	90,506		-41,268	-39,667	-6,514	-34,754	ubiquitous
16:1ω5	23,092	27,477	15,783	66,352		-60,897	-60,888	-42,711	-18,186	fungi
cy17:0	8,781	11,104	7,464	27,349		-33,199	-33,195	-33,417	0,218	gram negative
i15:0	6,933	9,664	7,654	24,251		-42,32	-42,286	-33,758	-8,562	gram positive
14:0	7,356	9,105	4,657	21,118		-61,694	-60,813	-45,875	-15,819	gram positive
a15:0	4,222	5,586	4,148	13,956		-37,426	-36,753	-26,418	-11,008	gram positive
19:1	3,702	4,832	4,404	12,938		-36,724	-37,044	-36,725	0,001	ubiquitous
18:1ω9c	2,894	3,898	3,423	10,215		-44,056	-40,056	-13,351	-30,705	fungi
17:1ω6	3,132	3,852	2,259	9,243		-33,3	-33,236	-31,142	-2,158	gram negative
i16:0	2,155	3,09	2,54	7,785		-40,333	-38,414	-28,216	-12,117	gram positive
15:0	2,625	3,247	1,746	7,618		-46,502	-45,442	-37,678	-8,824	ubiquitous
18:0	1,964	2,457	1,99	6,411		-40,136	-39,041	-31,701	-8,435	ubiquitous
18:2ω6,9	1,288	1,634	1,482	4,404		-51,776	-49,865	5,586	-57,362	fungi
i14:0	0,876	1,193	1,018	3,087		-41,084	-40,803	-1,4	-39,684	gram positive

These results show that mostly gram negative bacteria incorporated of the labelled hexadecane, followed by fungi. Gram positive bacteria also contributed to the biodegradation. The highest delta value belongs to fungi although the amount was relative small.

Since there are probably weathered rests of diesel contained in the groundwater, we can assume there are more recalcitrant rests of HCs dissolved. Unfortunately we can’t say if those were degraded because the only labelled HC was the Hexadecane. It would make sense to label only the most recalcitrant compound in the HCs to get a better impression of the bioremediation viability.

Johnsen et al., (2002) which researched the degradation of PAHs at industrial sides, had also gram negative bacteria (a combination of *Sphingomonas* and *beta-proteobacterium*) as the most efficient degraders. On the contrary he found out that fungi didn’t contribute significantly to the PAH degradation metabolism. Adam et al., (2015) stated in their results that complex microbial degrader consortia are more viable of PAH (in this case pyrene) degradation than single key players in organic amended soils. Additionally, gram negative bacteria made up the major part of pyrene degradation followed by gram positive bacteria and actinomycetes and fungi showed also label incorporation.

Sutton et al., (2013) investigated the microbial community composition and diversity at a long termed diesel contamination at a railway site by pyrosequencing of bacterial 16S rRNA gene fragments. In the samples with higher contamination Chloroflexi and Firmicutes were most abundant. The phylum Chloroflexi stains were mostly gram negative and there are aerobic and anaerobic isolates (Sutcliffe, 2010). In our MCs it could have been also possible that partially anaerobic conditions occured. The only oxygen was from the air of the headspace, in the groundwater nearly no oxygen was dissolved. It has to be considered that in the nature at contaminated sites, aerobic and anaerobic conditions could prevail both.

Andreolli et al. (2016) also investigated the microbial community of a diesel contaminated site, in this case the surrounding soil of a diesel storage tank leakage. By PCR–DGGE analyses they found out that the bacteria Chloroflexi and the fungi Ascomycota were the most abundant microbes in the contaminated sites. These results are also similar to our own.

3.2.3 Theoretical approaches for future works

One approach is to enhance the diesel biodegradation MO community with the addition of HC compounds itself i.e. a hydrocarbon- biostimulation. The theory is the MOs will adapt and select itself towards HC degradation and build up a viable bioremediation community. However, it should be assessed as lab study firstly in order to avoid additionally contamination in the field. E.g. Nie et al., (2011) found out that diesel degrading bacteria became more active with increasing diesel concentration in the rhizosphere of Phragmites australis, which would partially agree to these theory.

Secondly, to cover the range of diesel compounds we suggest to use the diesel mixture REPMIX from chapter 1.4.3 is used, it would be similar to diesel conditions but easier to handle, control and to measure, because every fraction and it´s concentration is known.

Still, the REPMIX has to be analysed for its comparability to diesel as far as physical properties, e.g. fluidity or miscibility of the mixture, are concerned. Furthermore, with an in vitro experiment, a biodegradation study may be assessed in comparison to other diesel degradation rates although those comparisons are difficult because of many differences in the experimental setups. Also isotopic analyses of the different compounds in the REPMIX could be assessed in order to find out if a certain compound shows significant differences in its $^{13}C/^{12}C$ ratio, which can be used for isotopic analyses and which may spare high costs for artificial labelled substances.

After a theoretical comparability approve of the REPMIX it could be used for lab diesel biodegradation studies. A MO community from a long termed diesel contamination may be cultivated and “fertilized” with the REPMIX diesel mixture and a classic biostimulation which should guarantee the NPK and iron supply. On the one hand to compare different setups of CWs for example, or on the other hand the determination of efficient PHC biodegradation MO consortia. The advantage would be to determine the degraders of the different fractions of diesel.

4.Conclusion and recommendations

4.1 Reviews and perspectives of PLFA analyses

The purpose of this study was to determine if PLFA-SIP analyses are suited to compare different bed compositions of a CW. Due to the low microbial content this purpose had to be adapted to a method for the extraction of a sufficient amount of PLFAs for analyses. Therefore, as mentioned in the R&D part, it is very important to run a reasonable number of pretests. This should be done in order to improve the approach and the measurement timetable, because the access to measurement devices is in the most laboratories limited.

If the method is determined and the handling with the lab- utensils well trained, PLFA-SIP analyses would be a very effective method to compare different treatments for biodegradation efficiency. The suitability lies in the high sensibility for changes in microbial communities and the fact that it is relatively cheap and easy to execute (Watzinger, 2015). Subsequently, it can be combined with other methods which guarantee a better taxonomical resolution, in order to identify the microbial biodegradation community.

The comparability of PLFA results of different scientists or scientific institutions is hardly given, because there are numerous differences in the extraction process (Watzinger, 2015). These differences have more or less influence on the results which make a direct comparison to other scientific works difficult. We have the ISO/TS 29843-2:2011 which is the current norm of the international organization for standardization (ISO) for PLFA analyses. Unfortunately it lacks on the range of applicability, like in this work it wouldn't have been appropriate due to the low MO content. Therefore, a broad ranged international PLFA standard procedure should be developed to guarantee exact, prompt and comparable results.

4.2 Defining the diesel contaminations

In every PHC bioremediation strategy it is important to have sufficient information about the contamination. With diesel e.g. the content of the recalcitrant part of the diesel fraction is important to know, in order to choose the right remediation solution. Moreover, as mentioned by Sjögren et al., (1995) (chapter 1.1.3.2) the composition of diesel varys depending on the origin of the crude oil, the refining process and the mixtures added by the refiner for final formulation (Penet et al., 2004). Additionally the weathering effects are influenced by numerous factors. Therefore, diesel degradation studies of different regions are hardly comparable and for each contamination, contaminant analyses should be assessed.

It is impossible to measure all delta values of the thousands of hydrocarbons in the diesel. Therefore, we suggest assessing a representative mixture, which should reflect the characteristics of diesel. If there is a big pollution it would be wise to analyse the diesel exactly in order to create an adequate bioremediation strategy.

4.3 Possible trouble with CWs in the near future

The main threat of CWs in PHC bioremediation in the near future will be clogging. If the support matrix is not able any more to supply the main layer with sufficient oxygen and contaminant flux, the bioremediation viability will decrease fast.

For example Eke and Scholz, (2008) revealed that filters subjected to diesel contamination showed higher suspended solids concentration and turbidity than those without hydrocarbons, especially in the top layer. That also proves that HC compounds accumulate in the upper layers of support matrixes.

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7.Raw data

CO_2 measurements

Datum	Hours	Area all (Vs)	Average (Vs)	$\delta^{13}C$ (‰)
19.07.2013	0	64.046		-23.50
19.07.2013	0	69.547	70.365	-23.63
19.07.2013	0	77.502		-23.61
19.07.2013	6	73.320	72.661	-23.57
19.07.2013	6	72.001		-23.53
19.07.2013	11	71.274		-23.50
19.07.2013	11	73.714	72.811	-23.51
19.07.2013	11	73.445		-23.42
20.07.2013	24	78.704	78.322	-23.67
20.07.2013	24	77.940		-23.64
21.07.2013	48	80.184		-23.56
21.07.2013	48	80.453	79.369	-23.60
21.07.2013	48	77.471		-23.66
23.07.2013	96	80.642		-23.14
23.07.2013	96	83.020	82.954	-23.23
23.07.2013	96	85.200		-23.07
27.07.2013	192	98.449	97.768	-21.20
27.07.2013	192	97.086		-21.19
31.07.2013	312	73.355	73.042	-19.14
31.07.2013	312	72.729		-19.21
08.06.2013	504	88.833	87.931	-16.315
08.06.2013	504	87.029		-16.297
19.07.2013	0	63.439		-23.75
19.07.2013	0	64.310	63.276	-23.79
19.07.2013	0	62.079		-23.71
19.07.2013	6	69.291		-23.54
19.07.2013	6	69.783	70.703	-23.53
19.07.2013	6	73.036		-23.48
19.07.2013	11	71.525		-23.48
19.07.2013	11	73.114	70.722	-23.41
19.07.2013	11	67.527		-23.45
20.07.2013	24	79.246	78.689	-23.58
20.07.2013	24	78.132		-23.56
21.07.2013	48	79.752	79.118	-23.63
21.07.2013	48	78.483		-23.64
23.07.2013	96	74.265		-22.83
23.07.2013	96	78.256	77.560	-22.87
23.07.2013	96	80.160		-22.90
27.07.2013	192	57.083	57.326	-20.47
27.07.2013	192	57.569		-20.43
31.07.2013	312	34.156	33.320	-17.63
31.07.2013	312	32.484		-17.58
08.06.2013	504	18.147	18.296	-13.14
08.06.2013	504	18.445		-13.129
19.07.2013	0	75.835		-23.89
19.07.2013	0	75.747	76.102	-23.81
19.07.2013	0	76.723		-23.74
19.07.2013	6	73.043		-23.43
19.07.2013	6	80.946	77.887	-23.51
19.07.2013	6	79.671		-23.50

19.07.2013	11	67.685		-23.51
19.07.2013	11	73.921	73.134	-23.40
19.07.2013	11	77.796		-23.46
20.07.2013	24	83.280	83.664	-23.57
20.07.2013	24	84.048		-23.60
21.07.2013	48	84.472		-23.63
21.07.2013	48	83.450	83.519	-23.65
21.07.2013	48	82.634		-23.65
23.07.2013	96	84.802	84.920	-23.21
23.07.2013	96	85.038		-23.19
27.07.2013	192	98.957		-21.19
27.07.2013	192	95.912	97.284	-21.10
27.07.2013	192	96.983		-21.10
31.07.2013	312	69.159	69.385	-18.80
31.07.2013	312	69.611		-18.76
08.06.2013	504	64.502	65.788	-15.332
08.06.2013	504	67.074		-15.346
19.07.2013	0	60.780		-23.74
19.07.2013	0	62.200	62.015	-23.80
19.07.2013	0	63.064		-23.70
19.07.2013	6	65.538		-23.48
19.07.2013	6	66.632	65.669	-23.48
19.07.2013	6	64.838		-23.49
19.07.2013	11	62.426		-23.31
19.07.2013	11	60.534	62.272	-23.37
19.07.2013	11	63.857		-23.31
20.07.2013	24	68.397		-23.52
20.07.2013	24	68.575	68.511	-23.47
20.07.2013	24	68.560		-23.50
21.07.2013	48	70.018	70.160	-23.56
21.07.2013	48	70.302		-23.54
23.07.2013	96	70.387	71.628	-22.86
23.07.2013	96	72.869		-22.85
27.07.2013	192	84.317	84.545	-20.77
27.07.2013	192	84.773		-20.74
31.07.2013	312	65.242	64.861	-18.36
31.07.2013	312	64.480		-18.40
08.06.2013	504	61.419	61.376	-14.914
08.06.2013	504	61.333		-14.778

PLFA FID measurements

Microcosm	Freezing time	Extraction time	14:0|RT	14:0|Area	Amount in µg/g	Amount in nmol/g	% of Total (µg/g)
S6	06.08.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S7	06.08.2013	21.08.2013	21.5031	24.1866	0.0297	0.1225	4.19
S8	06.08.2013	22.08.2013	21.5039	13.2523	0.0169	0.0699	3.96
S9	06.08.2013	27.08.2013	21.5044	19.6304	0.0393	0.1619	3.11
S10	06.08.2013	03.09.2013	21.5030	7.0129	0.0230	0.0948	3.64
S11	20.07.2013	03.09.2013	21.5025	5.3570	0.0180	0.0742	4.18
S12	20.07.2013	27.08.2013	21.5070	3.5435	0.0126	0.0519	3.53
S13	20.07.2013	29.08.2013	21.5063	2.5988	0.0087	0.0359	4.46
S14	20.07.2013	03.09.2013	21.5030	37.6247	0.0851	0.3510	3.84
S15	20.07.2013	04.09.2013	21.5039	33.3307	0.0982	0.4051	3.20
S16	18.07.2013	04.09.2013	21.5014	13.1994	0.0652	0.2688	2.93
S17	18.07.2013	27.08.2013	21.5033	37.6414	0.0749	0.3090	2.84
S18	18.07.2013	27.08.2013	21.5055	66.4382	0.0776	0.3200	4.01
S19	18.07.2013	29.08.2013	21.5035	2.0086	0.0096	0.0395	3.54
S20	18.07.2013	04.09.2013	21.5033	44.4749	0.1078	0.4447	3.43

Microcosm	Freezing time	Extraction time	i15:0|RT	i15:0|Area	Amount in µg/g	Amount in nmol/g	% of Total (µg/g)
S6	06.08.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	**0.00**
S7	06.08.2013	21.08.2013	23.4726	30.7565	0.0377	0.1472	**5.33**
S8	06.08.2013	22.08.2013	23.4709	20.9797	0.0268	0.1046	**6.27**
S9	06.08.2013	27.08.2013	23.4728	32.4136	0.0648	0.2528	**5.13**
S10	06.08.2013	03.09.2013	23.4701	11.6311	0.0381	0.1486	**6.04**
S11	20.07.2013	03.09.2013	0.0000	0.0000	0.0000	0.0000	**0.00**
S12	20.07.2013	27.08.2013	23.4716	4.9693	0.0176	0.0687	**4.95**
S13	20.07.2013	29.08.2013	23.4705	3.3443	0.0112	0.0436	**5.73**
S14	20.07.2013	03.09.2013	23.4720	43.7743	0.0990	0.3860	**4.47**
S15	20.07.2013	04.09.2013	23.4714	36.3829	0.1072	0.4180	**3.49**
S16	18.07.2013	04.09.2013	23.4672	14.1084	0.0696	0.2716	**3.13**
S17	18.07.2013	27.08.2013	23.4705	43.8053	0.0872	0.3399	**3.30**
S18	18.07.2013	27.08.2013	23.4727	75.9096	0.0886	0.3456	**4.58**
S19	18.07.2013	29.08.2013	23.4686	2.3408	0.0112	0.0435	**4.12**
S20	18.07.2013	04.09.2013	23.4689	42.2671	0.1024	0.3995	**3.26**
Microcosm	**Freezing time**	**Extraction time**	**a15:0|RT**	**a15:0|Area**	**Amount in µg/g**	**Amount in nmol/g**	**% of Total (µg/g)**
S6	06.08.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S7	06.08.2013	21.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S8	06.08.2013	22.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S9	06.08.2013	27.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S10	06.08.2013	03.09.2013	0.0000	0.0000	0.0000	0.0000	0.00
S11	20.07.2013	03.09.2013	0.0000	0.0000	0.0000	0.0000	0.00
S12	20.07.2013	27.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S13	20.07.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S14	20.07.2013	03.09.2013	23.7422	23.3373	0.0528	0.2058	2.38
S15	20.07.2013	04.09.2013	23.7399	19.9270	0.0587	0.2289	1.91
S16	18.07.2013	04.09.2013	23.7378	9.9603	0.0492	0.1917	2.21
S17	18.07.2013	27.08.2013	23.7398	26.4000	0.0525	0.2049	1.99
S18	18.07.2013	27.08.2013	23.7422	43.5155	0.0508	0.1981	2.62
S19	18.07.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S20	18.07.2013	04.09.2013	23.7391	24.0983	0.0584	0.2278	1.86
Microcosm	**Freezing time**	**Extraction time**	**15:0|RT**	**15:0|Area**	**Amount in µg/g**	**Amount in nmol/g**	**% of Total (µg/g)**
S6	06.08.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	**0.00**
S7	06.08.2013	21.08.2013	0.0000	0.0000	0.0000	0.0000	**0.00**
S8	06.08.2013	22.08.2013	24.6522	5.4845	0.0070	0.0273	**6.39**
S9	06.08.2013	27.08.2013	24.6502	8.1523	0.0163	0.0636	**5.03**
S10	06.08.2013	03.09.2013	24.6476	2.9803	0.0098	0.0381	**6.04**
S11	20.07.2013	03.09.2013	0.0000	0.0000	0.0000	0.0000	**0.00**
S12	20.07.2013	27.08.2013	24.6521	1.4758	0.0052	0.0204	**5.73**
S13	20.07.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	**0.00**
S14	20.07.2013	03.09.2013	24.6491	14.1906	0.0321	0.1251	**5.65**
S15	20.07.2013	04.09.2013	24.6489	12.1676	0.0358	0.1398	**4.56**
S16	18.07.2013	04.09.2013	24.6475	5.6326	0.0278	0.1084	**4.88**
S17	18.07.2013	27.08.2013	24.6482	15.7638	0.0314	0.1223	**4.63**
S18	18.07.2013	27.08.2013	24.6480	28.0813	0.0328	0.1279	**6.60**
S19	18.07.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	**0.00**
S20	18.07.2013	04.09.2013	24.6470	15.8147	0.0383	0.1495	**4.75**
Microcosm	**Freezing time**	**Extraction time**	**14:0 3OH|RT**	**14:0 3OH|Area**	**Amount in µg/g**	**Amount in nmol/g**	**% of Total (µg/g)**
S6	06.08.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S7	06.08.2013	21.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S8	06.08.2013	22.08.2013	0.0000	0.0000	0.0000	0.0000	0.00

S9	06.08.2013	27.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S10	06.08.2013	03.09.2013	0.0000	0.0000	0.0000	0.0000	0.00
S11	20.07.2013	03.09.2013	0.0000	0.0000	0.0000	0.0000	0.00
S12	20.07.2013	27.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S13	20.07.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S14	20.07.2013	03.09.2013	26.2009	1.6130	0.0036	0.0000	0.16
S15	20.07.2013	04.09.2013	26.2032	1.7728	0.0052	0.0000	0.17
S16	18.07.2013	04.09.2013	0.0000	0.0000	0.0000	0.0000	0.00
S17	18.07.2013	27.08.2013	26.2013	2.9835	0.0059	0.0000	0.22
S18	18.07.2013	27.08.2013	26.2040	6.5944	0.0077	0.0000	0.40
S19	18.07.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S20	18.07.2013	04.09.2013	26.1948	1.7999	0.0044	0.0000	0.14
Microcosm	**Freezing time**	**Extraction time**	**i16:0|RT**	**i16:0|Area**	**Amount in µg/g**	**Amount in nmol/g**	**% of Total (µg/g)**
S6	06.08.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S7	06.08.2013	21.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S8	06.08.2013	22.08.2013	26.7222	5.0706	0.0065	0.0240	1.52
S9	06.08.2013	27.08.2013	26.7263	9.0847	0.0182	0.0672	1.44
S10	06.08.2013	03.09.2013	26.7264	3.0691	0.0101	0.0372	1.59
S11	20.07.2013	03.09.2013	26.7259	2.1660	0.0073	0.0269	1.69
S12	20.07.2013	27.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S13	20.07.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S14	20.07.2013	03.09.2013	26.7273	10.3790	0.0235	0.0868	1.06
S15	20.07.2013	04.09.2013	26.7253	9.3114	0.0274	0.1014	0.89
S16	18.07.2013	04.09.2013	26.7252	4.8092	0.0237	0.0878	1.07
S17	18.07.2013	27.08.2013	26.7290	13.2873	0.0264	0.0978	1.00
S18	18.07.2013	27.08.2013	26.7327	22.6688	0.0265	0.0979	1.37
S19	18.07.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S20	18.07.2013	04.09.2013	26.7290	10.5253	0.0255	0.0943	0.81
Microcosm	**Freezing time**	**Extraction time**	**16:1w7|RT**	**16:1w7|Area**	**Amount in µg/g**	**Amount in nmol/g**	**% of Total (µg/g)**
S6	06.08.2013	29.08.2013	27.2411	2.6941	0.0083	0.0309	**11.34**
S7	06.08.2013	21.08.2013	27.2463	63.6910	0.0782	0.2912	**11.03**
S8	06.08.2013	22.08.2013	27.2430	30.7681	0.0393	0.1466	**9.20**
S9	06.08.2013	27.08.2013	27.2464	50.0158	0.1000	0.3726	**7.92**
S10	06.08.2013	03.09.2013	27.2401	15.7934	0.0517	0.1928	**8.21**
S11	20.07.2013	03.09.2013	27.2349	13.6180	0.0457	0.1702	**10.63**
S12	20.07.2013	27.08.2013	27.2365	10.7172	0.0380	0.1416	**10.67**
S13	20.07.2013	29.08.2013	27.2364	7.5828	0.0254	0.0945	**13.00**
S14	20.07.2013	03.09.2013	27.2528	146.6884	0.3317	1.2356	**14.98**
S15	20.07.2013	04.09.2013	27.3412	312.5805	0.9208	3.4303	**30.01**
S16	18.07.2013	04.09.2013	27.3185	123.4768	0.6095	2.2707	**27.43**
S17	18.07.2013	27.08.2013	27.3432	394.7045	0.7854	2.9259	**29.75**
S18	18.07.2013	27.08.2013	27.2968	16.8377	0.0197	0.0732	**1.02**
S19	18.07.2013	29.08.2013	27.3133	19.9838	0.0952	0.3547	**35.18**
S20	18.07.2013	04.09.2013	27.3453	394.7902	0.9568	3.5645	**30.41**
Microcosm	**Freezing time**	**Extraction time**	**16:1w6|RT**	**16:1w6|Area**	**Amount in µg/g**	**Amount in nmol/g**	**% of Total (µg/g)**
S6	06.08.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	**0.00**
S7	06.08.2013	21.08.2013	27.4501	40.8573	0.0501	0.1868	**7.08**
S8	06.08.2013	22.08.2013	27.4450	17.0035	0.0217	0.0810	**5.08**
S9	06.08.2013	27.08.2013	27.4467	31.2272	0.0624	0.2326	**4.95**
S10	06.08.2013	03.09.2013	27.4427	8.7493	0.0287	0.1068	**4.55**
S11	20.07.2013	03.09.2013	27.4496	7.5657	0.0254	0.0946	**5.91**
S12	20.07.2013	27.08.2013	27.4439	4.7760	0.0169	0.0631	**4.76**

S13	20.07.2013	29.08.2013	27.4425	2.8361	0.0095	0.0353	**4.86**
S14	20.07.2013	03.09.2013	27.4573	54.2305	0.1226	0.4568	**5.54**
S15	20.07.2013	04.09.2013	27.4520	46.2213	0.1362	0.5072	**4.44**
S16	18.07.2013	04.09.2013	27.4467	23.2902	0.1150	0.4283	**5.17**
S17	18.07.2013	27.08.2013	27.4558	65.3288	0.1300	0.4843	**4.92**
S18	18.07.2013	27.08.2013	27.4707	119.2590	0.1392	0.5187	**7.19**
S19	18.07.2013	29.08.2013	27.4428	2.7000	0.0129	0.0479	**4.75**
S20	18.07.2013	04.09.2013	27.4570	68.6764	0.1664	0.6201	**5.29**
Microcosm	**Freezing time**	**Extraction time**	**16:1w5|RT**	**16:1w5|Area**	**Amount in µg/g**	**Amount in nmol/g**	**% of Total (µg/g)**
S6	06.08.2013	29.08.2013	27.6179	3.6024	0.0111	0.0413	**15.16**
S7	06.08.2013	21.08.2013	27.6215	71.2815	0.0875	0.3259	**12.35**
S8	06.08.2013	22.08.2013	27.6183	42.5778	0.0544	0.2028	**12.73**
S9	06.08.2013	27.08.2013	27.6189	64.7355	0.1294	0.4822	**10.25**
S10	06.08.2013	03.09.2013	27.6127	21.0836	0.0691	0.2573	**10.95**
S11	20.07.2013	03.09.2013	27.6125	16.3023	0.0547	0.2038	**12.73**
S12	20.07.2013	27.08.2013	27.6122	13.0975	0.0465	0.1731	**13.04**
S13	20.07.2013	29.08.2013	27.6136	8.7234	0.0292	0.1087	**14.96**
S14	20.07.2013	03.09.2013	27.6224	122.9465	0.2780	1.0356	**12.56**
S15	20.07.2013	04.09.2013	27.6232	105.7087	0.3114	1.1601	**10.15**
S16	18.07.2013	04.09.2013	27.6136	39.8732	0.1968	0.7333	**8.86**
S17	18.07.2013	27.08.2013	27.6239	132.4816	0.2636	0.9821	**9.99**
S18	18.07.2013	27.08.2013	27.6389	249.9227	0.2918	1.0870	**15.07**
S19	18.07.2013	29.08.2013	27.6119	6.7755	0.0323	0.1203	**11.93**
S20	18.07.2013	04.09.2013	27.6252	143.6819	0.3482	1.2973	**11.07**
Microcosm	**Freezing time**	**Extraction time**	**16:0|RT**	**16:0|Area**	**Amount in µg/g**	**Amount in nmol/g**	**% of Total (µg/g)**
S6	06.08.2013	29.08.2013	27.9587	7.3643	0.0227	0.0802	**31.00**
S7	06.08.2013	21.08.2013	27.9666	148.2654	0.1819	0.6441	**25.68**
S8	06.08.2013	22.08.2013	27.9593	73.6232	0.0941	0.3333	**22.00**
S9	06.08.2013	27.08.2013	27.9657	126.4520	0.2528	0.8951	**20.02**
S10	06.08.2013	03.09.2013	27.9538	39.3586	0.1289	0.4565	**20.45**
S11	20.07.2013	03.09.2013	27.9538	28.8684	0.0969	0.3430	**22.54**
S12	20.07.2013	27.08.2013	27.9532	20.3553	0.0722	0.2556	**20.27**
S13	20.07.2013	29.08.2013	27.9570	13.0654	0.0437	0.1547	**22.40**
S14	20.07.2013	03.09.2013	27.9681	161.3944	0.3649	1.2919	**16.49**
S15	20.07.2013	04.09.2013	27.9673	137.4790	0.4050	1.4338	**13.20**
S16	18.07.2013	04.09.2013	27.9596	65.4928	0.3233	1.1446	**14.55**
S17	18.07.2013	27.08.2013	27.9699	184.3348	0.3668	1.2986	**13.89**
S18	18.07.2013	27.08.2013	27.9854	325.8294	0.3804	1.3468	**19.65**
S19	18.07.2013	29.08.2013	27.9558	9.8048	0.0467	0.1654	**17.26**
S20	18.07.2013	04.09.2013	27.9662	172.2485	0.4175	1.4780	**13.27**
Microcosm	**Freezing time**	**Extraction time**	**i17:1w8|RT**	**i17:1w8|Area**	**Amount in µg/g**	**Amount in nmol/g**	**% of Total (µg/g)**
S6	06.08.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S7	06.08.2013	21.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S8	06.08.2013	22.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S9	06.08.2013	27.08.2013	29.3138	7.2429	0.0145	0.0513	1.15
S10	06.08.2013	03.09.2013	29.3107	2.6110	0.0086	0.0303	1.36
S11	20.07.2013	03.09.2013	0.0000	0.0000	0.0000	0.0000	0.00
S12	20.07.2013	27.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S13	20.07.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S14	20.07.2013	03.09.2013	29.3142	10.0646	0.0228	0.0806	1.03
S15	20.07.2013	04.09.2013	29.3144	8.8825	0.0262	0.0926	0.85
S16	18.07.2013	04.09.2013	29.3149	3.6337	0.0179	0.0635	0.81

S17	18.07.2013	27.08.2013	29.3140	10.6621	0.0212	0.0751	0.80
S18	18.07.2013	27.08.2013	29.3175	18.8109	0.0220	0.0778	1.13
S19	18.07.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S20	18.07.2013	04.09.2013	29.3105	9.9913	0.0242	0.0857	0.77
Microcosm	**Freezing time**	**Extraction time**	**cy17:0|RT**	**cy17:0|Area**	**Amount in µg/g**	**Amount in nmol/g**	**% of Total (µg/g)**
S6	06.08.2013	29.08.2013	30.9010	1.7610	0.0054	0.0202	**7.41**
S7	06.08.2013	21.08.2013	30.8998	31.3659	0.0385	0.1434	**5.43**
S8	06.08.2013	22.08.2013	30.8995	19.1511	0.0245	0.0912	**5.72**
S9	06.08.2013	27.08.2013	30.8992	34.4853	0.0690	0.2569	**5.46**
S10	06.08.2013	03.09.2013	30.8969	10.9230	0.0358	0.1333	**5.68**
S11	20.07.2013	03.09.2013	30.8983	8.7063	0.0292	0.1088	**6.80**
S12	20.07.2013	27.08.2013	30.8960	5.8378	0.0207	0.0771	**5.81**
S13	20.07.2013	29.08.2013	30.8989	3.2415	0.0108	0.0404	**5.56**
S14	20.07.2013	03.09.2013	30.9013	49.5319	0.1120	0.4172	**5.06**
S15	20.07.2013	04.09.2013	30.8981	45.9547	0.1354	0.5043	**4.41**
S16	18.07.2013	04.09.2013	30.8964	20.7270	0.1023	0.3812	**4.60**
S17	18.07.2013	27.08.2013	30.9000	60.4364	0.1203	0.4480	**4.56**
S18	18.07.2013	27.08.2013	30.9078	106.8058	0.1247	0.4645	**6.44**
S19	18.07.2013	29.08.2013	30.8960	2.7996	0.0133	0.0497	**4.93**
S20	18.07.2013	04.09.2013	30.8984	51.6579	0.1252	0.4664	**3.98**
Microcosm	**Freezing time**	**Extraction time**	**10Me16:0|RT**	**10Me16:0|Area**	**Amount in µg/g**	**Amount in nmol/g**	**% of Total (µg/g)**
S6	06.08.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S7	06.08.2013	21.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S8	06.08.2013	22.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S9	06.08.2013	27.08.2013	29.4241	6.3732	0.0127	0.0000	1.01
S10	06.08.2013	03.09.2013	29.4223	2.0702	0.0068	0.0000	1.08
S11	20.07.2013	03.09.2013	0.0000	0.0000	0.0000	0.0000	0.00
S12	20.07.2013	27.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S13	20.07.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S14	20.07.2013	03.09.2013	29.4190	6.7795	0.0153	0.0000	0.69
S15	20.07.2013	04.09.2013	29.4229	6.2303	0.0184	0.0000	0.60
S16	18.07.2013	04.09.2013	29.4175	3.4473	0.0170	0.0000	0.77
S17	18.07.2013	27.08.2013	29.4194	8.0995	0.0161	0.0000	0.61
S18	18.07.2013	27.08.2013	29.4231	6.3513	0.0074	0.0000	0.38
S19	18.07.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S20	18.07.2013	04.09.2013	29.4172	7.3356	0.0178	0.0000	0.57
Microcosm	**Freezing time**	**Extraction time**	**i17:0|RT**	**i17:0|Area**	**Amount in µg/g**	**Amount in nmol/g**	**% of Total (µg/g)**
S6	06.08.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S7	06.08.2013	21.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S8	06.08.2013	22.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S9	06.08.2013	27.08.2013	30.0852	6.7778	0.0136	0.0476	1.07
S10	06.08.2013	03.09.2013	30.0842	2.1368	0.0070	0.0246	1.11
S11	20.07.2013	03.09.2013	0.0000	0.0000	0.0000	0.0000	0.00
S12	20.07.2013	27.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S13	20.07.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S14	20.07.2013	03.09.2013	30.0778	6.7951	0.0154	0.0540	0.69
S15	20.07.2013	04.09.2013	30.0830	6.1113	0.0180	0.0633	0.59
S16	18.07.2013	04.09.2013	30.0817	3.4585	0.0171	0.0600	0.77
S17	18.07.2013	27.08.2013	30.0835	9.0430	0.0180	0.0633	0.68
S18	18.07.2013	27.08.2013	30.0815	15.3057	0.0179	0.0628	0.92
S19	18.07.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S20	18.07.2013	04.09.2013	30.0795	7.3645	0.0178	0.0627	0.57

Microcosm	Freezing time	Extraction time	a17:0|RT	a17:0|Area	Amount in µg/g	Amount in nmol/g	% of Total (µg/g)
S6	06.08.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S7	06.08.2013	21.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S8	06.08.2013	22.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S9	06.08.2013	27.08.2013	30.3816	3.7569	0.0075	0.0264	0.59
S10	06.08.2013	03.09.2013	0.0000	0.0000	0.0000	0.0000	0.00
S11	20.07.2013	03.09.2013	0.0000	0.0000	0.0000	0.0000	0.00
S12	20.07.2013	27.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S13	20.07.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S14	20.07.2013	03.09.2013	30.3800	4.2568	0.0096	0.0338	0.43
S15	20.07.2013	04.09.2013	30.3803	3.7245	0.0110	0.0386	0.36
S16	18.07.2013	04.09.2013	30.3777	1.7167	0.0085	0.0298	0.38
S17	18.07.2013	27.08.2013	30.3845	5.6162	0.0112	0.0393	0.42
S18	18.07.2013	27.08.2013	30.3846	9.0901	0.0106	0.0373	0.55
S19	18.07.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S20	18.07.2013	04.09.2013	30.3817	4.4640	0.0108	0.0380	0.34
Microcosm	**Freezing time**	**Extraction time**	**17:1w8|RT**	**17:1w8|Area**	**Amount in µg/g**	**Amount in nmol/g**	**% of Total (µg/g)**
S6	06.08.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S7	06.08.2013	21.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S8	06.08.2013	22.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S9	06.08.2013	27.08.2013	30.5850	8.5198	0.0170	0.0603	1.35
S10	06.08.2013	03.09.2013	30.5829	3.1809	0.0104	0.0369	1.65
S11	20.07.2013	03.09.2013	0.0000	0.0000	0.0000	0.0000	0.00
S12	20.07.2013	27.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S13	20.07.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S14	20.07.2013	03.09.2013	30.5842	14.8419	0.0336	0.1188	1.52
S15	20.07.2013	04.09.2013	30.5813	13.7182	0.0404	0.1431	1.32
S16	18.07.2013	04.09.2013	30.5819	6.0113	0.0297	0.1051	1.34
S17	18.07.2013	27.08.2013	30.5819	17.7151	0.0353	0.1248	1.34
S18	18.07.2013	27.08.2013	30.5855	31.3797	0.0366	0.1297	1.89
S19	18.07.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S20	18.07.2013	04.09.2013	30.5813	17.1775	0.0416	0.1474	1.32
Microcosm	**Freezing time**	**Extraction time**	**18:2w6.9|RT**	**18:2w6.9|Area**	**Amount in µg/g**	**Amount in nmol/g**	**% of Total (µg/g)**
S6	06.08.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S7	06.08.2013	21.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S8	06.08.2013	22.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S9	06.08.2013	27.08.2013	33.6924	6.9088	0.0138	0.0469	1.09
S10	06.08.2013	03.09.2013	33.6880	1.8841	0.0062	0.0210	0.98
S11	20.07.2013	03.09.2013	0.0000	0.0000	0.0000	0.0000	0.00
S12	20.07.2013	27.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S13	20.07.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S14	20.07.2013	03.09.2013	33.6903	7.5879	0.0172	0.0583	0.78
S15	20.07.2013	04.09.2013	33.6916	4.4802	0.0132	0.0448	0.43
S16	18.07.2013	04.09.2013	33.6906	2.3222	0.0115	0.0389	0.52
S17	18.07.2013	27.08.2013	33.6921	7.9291	0.0158	0.0536	0.60
S18	18.07.2013	27.08.2013	33.6926	14.3177	0.0167	0.0568	0.86
S19	18.07.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S20	18.07.2013	04.09.2013	33.6926	6.7728	0.0164	0.0557	0.52
Microcosm	**Freezing time**	**Extraction time**	**18.1w9c|RT**	**18.1w9c|Area**	**Amount in µg/g**	**Amount in nmol/g**	**% of Total (µg/g)**
S6	06.08.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S7	06.08.2013	21.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S8	06.08.2013	22.08.2013	33.8704	11.2160	0.0143	0.0000	3.35

S9	06.08.2013	27.08.2013	33.8770	23.7131	0.0474	0.0000	3.76
S10	06.08.2013	03.09.2013	33.8702	6.7321	0.0221	0.0000	3.50
S11	20.07.2013	03.09.2013	33.8789	2.3705	0.0080	0.0000	1.85
S12	20.07.2013	27.08.2013	33.8807	2.9869	0.0106	0.0000	2.97
S13	20.07.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S14	20.07.2013	03.09.2013	33.8809	20.9777	0.0474	0.0000	2.14
S15	20.07.2013	04.09.2013	33.8779	18.3596	0.0541	0.0000	1.76
S16	18.07.2013	04.09.2013	33.8779	5.4770	0.0270	0.0000	1.22
S17	18.07.2013	27.08.2013	33.8780	16.1884	0.0322	0.0000	1.22
S18	18.07.2013	27.08.2013	33.8840	40.7475	0.0476	0.0000	2.46
S19	18.07.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S20	18.07.2013	04.09.2013	33.8788	20.2236	0.0490	0.0000	1.56
Microcosm	**Freezing time**	**Extraction time**	**18:1w8c?|RT**	**18:1w8c?|Area**	**Amount in µg/g**	**Amount in nmol/g**	**% of Total (µg/g)**
S6	06.08.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S7	06.08.2013	21.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S8	06.08.2013	22.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S9	06.08.2013	27.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S10	06.08.2013	03.09.2013	0.0000	0.0000	0.0000	0.0000	0.00
S11	20.07.2013	03.09.2013	0.0000	0.0000	0.0000	0.0000	0.00
S12	20.07.2013	27.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S13	20.07.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S14	20.07.2013	03.09.2013	33.9468	6.0787	0.0137	0.0000	0.62
S15	20.07.2013	04.09.2013	33.9481	5.4042	0.0159	0.0000	0.52
S16	18.07.2013	04.09.2013	0.0000	0.0000	0.0000	0.0000	0.00
S17	18.07.2013	27.08.2013	34.0790	112.5497	0.2240	0.0000	**8.48**
S18	18.07.2013	27.08.2013	33.9496	11.9268	0.0139	0.0000	0.72
S19	18.07.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S20	18.07.2013	04.09.2013	33.9534	6.8139	0.0165	0.0000	0.52
Microcosm	**Freezing time**	**Extraction time**	**18:1w7c/9t|RT**	**18:1w7c/9t|Area**	**Amount in µg/g**	**Amount in nmol/g**	**% of Total (µg/g)**
S6	06.08.2013	29.08.2013	34.0643	8.3343	0.0256	0.0865	**35.08**
S7	06.08.2013	21.08.2013	34.0761	141.8965	0.1741	0.5873	**24.58**
S8	06.08.2013	22.08.2013	34.0693	74.6124	0.0954	0.3218	**22.30**
S9	06.08.2013	27.08.2013	34.0739	144.7235	0.2894	0.9760	**22.92**
S10	06.08.2013	03.09.2013	34.0639	42.3247	0.1387	0.4677	**21.99**
S11	20.07.2013	03.09.2013	34.0674	32.3918	0.1087	0.3666	**25.29**
S12	20.07.2013	27.08.2013	34.0646	25.1491	0.0892	0.3009	**25.04**
S13	20.07.2013	29.08.2013	34.0629	14.7352	0.0493	0.1662	**25.27**
S14	20.07.2013	03.09.2013	34.0762	179.7098	0.4063	1.3705	**18.36**
S15	20.07.2013	04.09.2013	34.0771	162.3765	0.4783	1.6133	**15.59**
S16	18.07.2013	04.09.2013	34.0680	70.8740	0.3499	1.1800	**15.74**
S17	18.07.2013	27.08.2013	34.0838	85.6087	0.1703	0.5746	**6.45**
S18	18.07.2013	27.08.2013	34.0948	349.7455	0.4083	1.3773	**21.09**
S19	18.07.2013	29.08.2013	34.0626	10.3948	0.0495	0.1670	**18.30**
S20	18.07.2013	04.09.2013	34.0761	187.5232	0.4545	1.5329	**14.44**
Microcosm	**Freezing time**	**Extraction time**	**18:1w5c?|RT**	**18:1w5c?|Area**	**Amount in µg/g**	**% of Total (µg/g)**	**% of Total**
S6	06.08.2013	29.08.2013	0.0000	0.0000	0.0000	0.00	0.0000
S7	06.08.2013	21.08.2013	0.0000	0.0000	0.0000	0.00	0.0000
S8	06.08.2013	22.08.2013	0.0000	0.0000	0.0000	0.00	0.0000
S9	06.08.2013	27.08.2013	34.2128	4.7127	0.0094	0.75	0.0000
S10	06.08.2013	03.09.2013	0.0000	0.0000	0.0000	0.00	0.0000
S11	20.07.2013	03.09.2013	0.0000	0.0000	0.0000	0.00	0.0000
S12	20.07.2013	27.08.2013	0.0000	0.0000	0.0000	0.00	0.0000

S13	20.07.2013	29.08.2013	0.0000	0.0000	0.0000	0.00	0.0000
S14	20.07.2013	03.09.2013	34.2087	2.1980	0.0050	0.22	0.0000
S15	20.07.2013	04.09.2013	34.2137	2.4091	0.0071	0.23	0.0000
S16	18.07.2013	04.09.2013	34.2017	2.9173	0.0144	0.65	0.0000
S17	18.07.2013	27.08.2013	34.2168	2.6534	0.0053	0.20	0.0000
S18	18.07.2013	27.08.2013	34.2847	0.2762	0.0003	0.02	0.0000
S19	18.07.2013	29.08.2013	0.0000	0.0000	0.0000	0.00	0.0000
S20	18.07.2013	04.09.2013	34.2097	2.4827	0.0060	0.19	0.0000
Microcosm	**Freezing time**	**Extraction time**	**18:0|RT**	**18:0|Area**	**Amount in µg/g**	**Amount in nmol/g**	**% of Total (µg/g)**
S6	06.08.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S7	06.08.2013	21.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S8	06.08.2013	22.08.2013	34.7003	6.5251	0.0083	0.0279	1.95
S9	06.08.2013	27.08.2013	34.9591	10.8903	0.0218	0.0729	1.72
S10	06.08.2013	03.09.2013	34.9543	3.3636	0.0110	0.0369	1.75
S11	20.07.2013	03.09.2013	34.9602	2.4728	0.0083	0.0278	1.93
S12	20.07.2013	27.08.2013	34.9589	1.8049	0.0064	0.0214	1.80
S13	20.07.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S14	20.07.2013	03.09.2013	34.6991	11.3630	0.0257	0.0861	1.16
S15	20.07.2013	04.09.2013	34.7009	10.0284	0.0295	0.0990	0.96
S16	18.07.2013	04.09.2013	34.7007	6.5201	0.0322	0.1078	1.45
S17	18.07.2013	27.08.2013	34.7021	15.3462	0.0305	0.1023	1.16
S18	18.07.2013	27.08.2013	34.7047	12.1506	0.0142	0.0475	0.73
S19	18.07.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S20	18.07.2013	04.09.2013	34.6975	13.8776	0.0336	0.1127	1.07
Microcosm	**Freezing time**	**Extraction time**	**10Me18:0?|RT**	**10Me18:0?|Area**	**Amount in µg/g**	**Amount in nmol/g**	**% of Total (µg/g)**
S6	06.08.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S7	06.08.2013	21.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S8	06.08.2013	22.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S9	06.08.2013	27.08.2013	36.0094	3.4265	0.0069	0.0219	0.54
S10	06.08.2013	03.09.2013	0.0000	0.0000	0.0000	0.0000	0.00
S11	20.07.2013	03.09.2013	0.0000	0.0000	0.0000	0.0000	0.00
S12	20.07.2013	27.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S13	20.07.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S14	20.07.2013	03.09.2013	36.0101	6.1164	0.0138	0.0442	0.62
S15	20.07.2013	04.09.2013	36.0124	5.4290	0.0160	0.0512	0.52
S16	18.07.2013	04.09.2013	36.0091	2.7097	0.0134	0.0428	0.60
S17	18.07.2013	27.08.2013	36.0115	9.2681	0.0184	0.0590	0.70
S18	18.07.2013	27.08.2013	36.0130	13.9273	0.0163	0.0520	0.84
S19	18.07.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S20	18.07.2013	04.09.2013	36.0112	5.9242	0.0144	0.0459	0.46
Microcosm	**Freezing time**	**Extraction time**	**12Me18:0|RT**	**12Me18:0|Area**	**Amount in µg/g**	**Amount in nmol/g**	**% of Total (µg/g)**
S6	06.08.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S7	06.08.2013	21.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S8	06.08.2013	22.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S9	06.08.2013	27.08.2013	36.1365	7.2511	0.0145	0.0000	1.15
S10	06.08.2013	03.09.2013	36.1331	1.9941	0.0065	0.0000	1.04
S11	20.07.2013	03.09.2013	36.1371	1.3992	0.0047	0.0000	1.09
S12	20.07.2013	27.08.2013	36.1305	1.2765	0.0045	0.0000	1.27
S13	20.07.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S14	20.07.2013	03.09.2013	36.1332	8.2845	0.0187	0.0000	0.85
S15	20.07.2013	04.09.2013	36.1338	7.2593	0.0214	0.0000	0.70
S16	18.07.2013	04.09.2013	36.1329	4.0753	0.0201	0.0000	0.91

S17	18.07.2013	27.08.2013	36.1316	13.0206	0.0259	0.0000	0.98
S18	18.07.2013	27.08.2013	36.1370	18.5429	0.0216	0.0000	1.12
S19	18.07.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	0.00
S20	18.07.2013	04.09.2013	36.1308	8.2810	0.0201	0.0000	0.64
Microcosm	**Freezing time**	**Extraction time**	**19:1|RT**	**19:1|Area**	**Amount in µg/g**	**Amount in nmol/g**	**% of Total (µg/g)**
S6	06.08.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	**0.00**
S7	06.08.2013	21.08.2013	37.6493	17.0612	0.0209	0.0674	**2.96**
S8	06.08.2013	22.08.2013	37.6490	10.7892	0.0138	0.0444	**3.22**
S9	06.08.2013	27.08.2013	37.6494	22.7933	0.0456	0.1468	**3.61**
S10	06.08.2013	03.09.2013	37.6468	8.1867	0.0268	0.0864	**4.25**
S11	20.07.2013	03.09.2013	37.6464	6.8460	0.0230	0.0740	**5.35**
S12	20.07.2013	27.08.2013	37.6504	4.4376	0.0157	0.0507	**4.42**
S13	20.07.2013	29.08.2013	37.6484	2.1940	0.0073	0.0236	**3.76**
S14	20.07.2013	03.09.2013	37.6459	25.1235	0.0568	0.1829	**2.57**
S15	20.07.2013	04.09.2013	37.6451	23.4485	0.0691	0.2225	**2.25**
S16	18.07.2013	04.09.2013	37.6458	13.9028	0.0686	0.2210	**3.09**
S17	18.07.2013	27.08.2013	37.6486	30.6147	0.0609	0.1962	**2.31**
S18	18.07.2013	27.08.2013	37.6502	47.3210	0.0552	0.1779	**2.85**
S19	18.07.2013	29.08.2013	0.0000	0.0000	0.0000	0.0000	**0.00**
S20	18.07.2013	04.09.2013	37.6469	26.1790	0.0634	0.2043	**2.02**
Microcosm	**Freezing time**	**Extraction time**	**19:0|RT**	**19:0|Area**	**Amount in µg/g (Control)**		
S6	06.08.2013	29.08.2013	38.0447	157.3788	0.4842		
S7	06.08.2013	21.08.2013	38.0653	394.5714	0.4842		
S8	06.08.2013	22.08.2013	38.0626	378.6972	0.4842		
S9	06.08.2013	27.08.2013	38.0520	242.1579	0.4842		
S10	06.08.2013	03.09.2013	38.0423	147.7988	0.4842		
S11	20.07.2013	03.09.2013	38.0426	144.2937	0.4842		
S12	20.07.2013	27.08.2013	38.0426	136.4988	0.4842		
S13	20.07.2013	29.08.2013	38.0401	144.7825	0.4842		
S14	20.07.2013	03.09.2013	38.0502	214.1516	0.4842		
S15	20.07.2013	04.09.2013	38.0421	164.3707	0.4842		
S16	18.07.2013	04.09.2013	38.0424	98.0895	0.4842		
S17	18.07.2013	27.08.2013	38.0546	243.3367	0.4842		
S18	18.07.2013	27.08.2013	38.0645	414.7268	0.4842		
S19	18.07.2013	29.08.2013	38.0396	101.6266	0.4842		
S20	18.07.2013	04.09.2013	38.0463	199.7845	0.4842		

PLFA GC-C-IRMS measurements

MK	Date	Component	Rt(s)	Area All(Vs)	delta $_{13}$C/$_{12}$C (per mil)
S6	After 18 days	C_{16} label peak	1011.4	2.911	94.004
S6	After 18 days	13:0 Std	1057.5	27.344	-30.166
S6	After 18 days	16:1ω7	1561.9	7.336	-49.713
S6	After 18 days	16:1ω5	1578.7	1.462	-59.624
S6	After 18 days	16:0	1597.4	2.772	-38.183
S6	After 18 days	cy17:0	1771.2	0.863	-32.663
S6	After 18 days	18:1ω7c/9t	1956.1	3.263	-39.364

S6	After 18 days	19:0 Std	2193.3	54.11	-30.152
S7	After 18 days	C_{16} label peak	1011.7	3.248	92.139
S7	After 18 days	13:0 Std	1057.6	25.846	-30.155
S7	After 18 days	i14:0	1158.8	0.765	31.877
S7	After 18 days	14:0	1222.6	2.739	-30.75
S7	After 18 days	i15:0	1336.4	3.553	-24.456
S7	After 18 days	a15:0	1352.4	2.141	-13.578
S7	After 18 days	15:0	1404.9	0.903	-33.168
S7	After 18 days	i16:0	1525.9	0.934	-19.11
S7	After 18 days	16:1ω7	1566.2	45.145	4.577
S7	After 18 days	16:1ω6	1571.8	4.907	-19.249
S7	After 18 days	16:1ω5	1580.9	8.579	-32.238
S7	After 18 days	16:0	1600.1	16.47	13.728
S7	After 18 days	17:1ω6	1752.4	1.065	-30.942
S7	After 18 days	cy17:0	1771.7	3.351	-33.305
S7	After 18 days	18:2ω6.9	1935.8	0.924	31.879
S7	After 18 days	18:1ω9c	1945.2	1.935	8.131
S7	After 18 days	18:1ω7c/9t	1958.4	15.996	6.692
S7	After 18 days	18:0	2006.8	0.982	-29.421
S7	After 18 days	19:1	2166.7	1.561	-33.079
S7	After 18 days	19:0 Std	2192.5	43.658	-31.114
S8	After 18 days	C_{16} label peak	1011.4	1.772	70.268
S8	After 18 days	C13:0	1058.7	43.478	-30.377
S8	After 18 days	14:0	1222.4	2.204	-48.424
S8	After 18 days	i15:0	1336.4	3.541	-38.019
S8	After 18 days	a15:0	1352.3	2.116	-29.119
S8	After 18 days	15:0	1404.8	0.817	-39.975
S8	After 18 days	i16:0	1525.7	0.956	-32.738
S8	After 18 days	16:1ω7	1565.1	33.356	-20.841
S8	After 18 days	16:1ω6	1570.9	3.324	-39.817
S8	After 18 days	16:1ω5	1580.4	7.563	-45.356
S8	After 18 days	16:0	1599.3	12.49	-16.999
S8	After 18 days	cy17:0	1771.6	3.413	-35.454
S8	After 18 days	18:1ω9c	1944.9	1.785	-19.371
S8	After 18 days	18:1ω7c/9t	1957.8	13.932	-11.088
S8	After 18 days	?	1991.4	0.916	-33.281
S8	After 18 days	19:1	2166.7	1.798	-36.496
S8	After 18 days	C19:0	2194.1	63.446	-30.533
S9	After 18 days	C_{16} label peak	1011.1	1.572	82.995
S9	After 18 days	13:0 Std	1057	24.373	-30.06
S9	After 18 days	i14:0	1158.4	1.018	-1.4
S9	After 18 days	14:0	1222.2	4.657	-45.875
S9	After 18 days	i15:0	1336.5	7.654	-33.758
S9	After 18 days	a15:0	1352.2	4.148	-26.418

S9	After 18 days	15:0	1404.4	1.746	-37.678
S9	After 18 days	i16:0	1525.8	2.54	-28.216
S9	After 18 days	16:1ω7 + 16:1ω6	1567.8	77.777	-10.905
S9	After 18 days	16:1ω5	1581.8	15.783	-42.711
S9	After 18 days	16:0	1601.3	28.695	-6.514
S9	After 18 days	?	1682.3	2.927	-27.146
S9	After 18 days	?	1720.6	1.33	-33.61
S9	After 18 days	17:1ω6	1752.2	2.259	-31.142
S9	After 18 days	cy17:0	1771.9	7.464	-33.417
S9	After 18 days	?	1793.6	0.864	-31.046
S9	After 18 days	18:2ω6.9	1935.5	1.482	5.586
S9	After 18 days	18:1ω9c	1945.1	3.423	-13.351
S9	After 18 days	18:1ω7c/9t	1959.8	32.761	-3.847
S9	After 18 days	18:0	2006.5	1.99	-31.701
S9	After 18 days	?	2074.5	1.519	-28.987
S9	After 18 days	19:1	2166.5	4.404	-36.725
S9	After 18 days	19:0 Std	2192.4	50.926	-30.815
S10	After 18 days	C_{16} label peak	1012.4	15.153	100.523
S10	After 18 days	13:0 Std	1058	37.816	-30.312
S10	After 18 days	14:0	1222	3.14	-47.458
S10	After 18 days	i15:0	1336	5.222	-39.826
S10	After 18 days	a15:0	1351.9	3.096	-31.464
S10	After 18 days	15:0	1404.3	1.391	-37.129
S10	After 18 days	i16:0	1525.3	1.449	-33.218
S10	After 18 days	16:1ω7 + 16:1ω6	1565.4	49.803	-27.535
S10	After 18 days	16:1ω5	1580.4	9.889	-48.75
S10	After 18 days	16:0	1599.4	17.554	-22.182
S10	After 18 days	?	1681.8	0.928	-45.357
S10	After 18 days	?	1720.2	0.829	-36.236
S10	After 18 days	17:1ω6	1751.7	1.774	-34.378
S10	After 18 days	17:1ω6	1771.3	5.334	-34.749
S10	After 18 days	18:2ω6.9	1934.9	1.28	-16.161
S10	After 18 days	18:1ω9c	1944.5	2.592	-18.372
S10	After 18 days	18:1ω7c/9t	1957.9	20.647	-18.323
S10	After 18 days	?	1990.7	1.384	-32.255
S10	After 18 days	18:0	2006	1.147	-41.403
S10	After 18 days	19:1	2166	3.437	-37.457
S10	After 18 days	19:0 Std	2193	63.648	-30.482
S11	After 2 days	C_{16} label peak	1012.2	12.475	101.042
S11	After 2 days	13:0 Std	1057.3	27.15	-30.471
S11	After 2 days	14:0	1222	1.767	-58.74
S11	After 2 days	i15:0	1335.7	1.97	-40.593
S11	After 2 days	a15:0	1351.7	1.516	-29.612
S11	After 2 days	15:0	1404.3	0.754	-43.72
S11	After 2 days	16:1ω7 + 16:1ω6	1563.5	23.018	-48.239

S11	After 2 days	16:1ω5	1579.2	5.409	-56.898
S11	After 2 days	16:0	1598.1	8.964	-38.948
S11	After 2 days	17:1ω6	1751.8	1.053	-36.062
S11	After 2 days	cy17:0	1771.1	3.076	-34.292
S11	After 2 days	?	1911.2	0.976	-35.025
S11	After 2 days	18:1ω7c/9t	1956.9	11.715	-39.81
S11	After 2 days	?	1991	0.913	-38.169
S11	After 2 days	19:1	2166.3	2.212	-35.505
S11	After 2 days	19:0 Std	2191.9	44.536	-30.329
S12	After 2 days	C_{16} label peak	1011	2.146	95.174
S12	After 2 days	13:0 Std	1056.5	18.524	-30.154
S12	After 2 days	i14:0	1158.3	0.194	-40.959
S12	After 2 days	14:0	1221.9	1.302	-59.847
S12	After 2 days	i15:0	1335.6	1.781	-41.715
S12	After 2 days	a15:0	1351.7	0.946	-36.626
S12	After 2 days	15:0	1404.3	0.495	-47.409
S12	After 2 days	i16:0	1525.1	0.43	-53.182
S12	After 2 days	16:1ω7 + 16:1ω6	1563.3	21.417	-50.909
S12	After 2 days	16:1ω5	1579.1	4.432	-61.198
S12	After 2 days	16:0	1597.8	6.694	-39.945
S12	After 2 days	?	1677.4	0.392	-41.668
S12	After 2 days	?	1682	0.373	-41.76
S12	After 2 days	?	1720.4	0.332	-43.41
S12	After 2 days	17:1ω6	1751.9	0.658	-34.373
S12	After 2 days	cy17:0	1771.1	2.316	-33.624
S12	After 2 days	18:2ω6.9	1935.3	0.298	-42.56
S12	After 2 days	18:1ω9c	1944.6	0.9	-32.1
S12	After 2 days	18:1ω7c/9t	1956.7	8.831	-38.514
S12	After 2 days	18:0	2006.3	0.49	-30.741
S12	After 2 days	?	2074.5	0.422	-16.788
S12	After 2 days	19:1	2166.1	1.417	-36.599
S12	After 2 days	19:0 Std	2191.9	43.53	-30.266
	After 2 days				
S13	After 2 days	C_{16} label peak	1011.2	0.864	77.954
S13	After 2 days	13:0 Std.	1057.4	26.749	-30.195
S13	After 2 days	14:0	1222.2	0.979	-59.8
S13	After 2 days	i15:0	1335.9	1.191	-42.112
S13	After 2 days	a15:0	1352	0.668	-35.781
S13	After 2 days	15:0	1404.7	0.364	-44.611
S13	After 2 days	i16:0	1525.2	0.267	-51.818
S13	After 2 days	16:1ω7 + 16:1ω6	1562.9	14.619	-50.652
S13	After 2 days	16:1ω5	1579	2.994	-61.942
S13	After 2 days	16:0	1597.7	4.318	-39.657

S13	After 2 days	?	1677.6	0.18	-54.376
S13	After 2 days	?	1682.2	0.183	-51.075
S13	After 2 days	?	1720.8	0.137	-60.649
S13	After 2 days	17:1ω6	1752.2	0.416	-36.303
S13	After 2 days	cy17:0	1771.3	1.398	-34.578
S13	After 2 days	18:2ω6.9	1935.5	0.173	-46.448
S13	After 2 days	18:1ω9c	1944.8	0.574	-36.153
S13	After 2 days	18:1ω7c/9t	1956.5	5.402	-39.289
S13	After 2 days	18:0	2006.7	0.312	-33.544
S13	After 2 days	?	2074.9	0.285	-21.906
S13	After 2 days	19:1	2166.5	0.814	-36.518
S13	After 2 days	19:0 Std	2192.9	49.135	-30.132
S14	After 2 days	C_{16} label peak	1011.3	1.278	65.29
S14	After 2 days	13:0 Std	1057.9	33.851	-30.397
S14	After 2 days	i14:0	1158.6	1.278	-42.017
S14	After 2 days	14:0	1223.1	9.971	-61.014
S14	After 2 days	i15:0	1337.2	11.45	-42.622
S14	After 2 days	a15:0	1352.8	6.514	-37.565
S14	After 2 days	15:0	1405	3.747	-46.017
S14	After 2 days	i16:0	1526.7	3.656	-39.527
S14	After 2 days	16:1ω7	1571.1	153.915	-50.543
S14	After 2 days	16:1ω5	1584.8	32.855	-61.956
S14	After 2 days	16:0	1603.4	41.08	-39.688
S14	After 2 days	?	1678.3	3.826	-40.096
S14	After 2 days	?	1682.8	1.772	-44.965
S14	After 2 days	?	1721	1.65	-38.832
S14	After 2 days	17:1ω6	1752.9	4.189	-34.108
S14	After 2 days	cy17:0	1773.1	13.435	-33.597
S14	After 2 days	18:2ω6.9	1935.7	1.884	-48.767
S14	After 2 days	18:1ω9c	1945.8	4.738	-43.701
S14	After 2 days	18:1ω7c/9t	1961.3	44.784	-40.121
S14	After 2 days	18:0	2006.8	2.585	-38.775
S14	After 2 days	?	2074.6	2.379	-27.796
S14	After 2 days	19:1	2166.6	5.631	-39.511
S14	After 2 days	19:0 Std	2192.3	47.93	-31.638
S15	After 2 days	C_{16} label peak	1015.6	7.677	96.909
S15	After 2 days	13:0 Std	1061.4	28.748	-30.255
S15	After 2 days	i14:0	1162.6	1.193	-40.803
S15	After 2 days	14:0	1227.2	9.105	-60.813
S15	After 2 days	i15:0	1341.2	9.664	-42.286
S15	After 2 days	a15:0	1357	5.586	-36.753
S15	After 2 days	15:0	1409.3	3.247	-45.442

S15	After 2 days	i16:0	1531.1	3.09	-38.414
S15	After 2 days	16:1ω7 + 16:1ω6	1574.5	128.973	-50.421
S15	After 2 days	16:1ω5	1588.6	27.477	-60.888
S15	After 2 days	16:0	1607.2	34.67	-39.667
S15	After 2 days	?	1682.9	3.377	-38.341
S15	After 2 days	?	1687.4	1.525	-35.905
S15	After 2 days	?	1725.8	1.319	-39.636
S15	After 2 days	17:1ω6	1757.7	3.852	-33.236
S15	After 2 days	cy17:0	1777.7	11.104	-33.195
S15	After 2 days	18:2ω6.9	1940.6	1.634	-49.865
S15	After 2 days	18:1ω9c	1950.6	3.898	-40.056
S15	After 2 days	18:1ω7c/9t	1965.7	43.905	-40.071
S15	After 2 days	18:0	2011.9	2.457	-39.041
S15	After 2 days	?	2079.8	1.736	-28.535
S15	After 2 days	19:1	2171.9	4.832	-37.044
S15	After 2 days	19:0 Std	2196.3	35.593	-30.875
S16	After 0 days	13:0 Std	1060.8	21.933	-30.282
S16	After 0 days	14:0	1226.4	2.88	-59.049
S16	After 0 days	i15:0	1340.4	2.91	-40.489
S16	After 0 days	a15:0	1356.5	2.167	-33.756
S16	After 0 days	15:0	1409.2	1.105	-42.875
S16	After 0 days	i16:0	1530.2	1.157	-35.851
S16	After 0 days	16:1ω7 + 16:1ω6	1569.5	39.318	-49.264
S16	After 0 days	16:1ω5	1585	8.278	-58.777
S16	After 0 days	16:0	1603.9	13.03	-38.598
S16	After 0 days	?	1682.5	1.279	-38.469
S16	After 0 days	?	1687.1	0.632	-38.552
S16	After 0 days	?	1725.5	0.669	-40.253
S16	After 0 days	17:1ω6	1757.1	1.567	-32.281
S16	After 0 days	cy17:0	1776.6	4.377	-32.883
S16	After 0 days	18:2ω6.9	1940.3	0.867	-46.577
S16	After 0 days	18:1ω9c	1950.1	1.541	-35.436
S16	After 0 days	18:1ω7c/9t	1962.9	15.184	-39.175
S16	After 0 days	18:0	2011.6	0.872	-35.526
S16	After 0 days	?	2079.8	0.678	-29.196
S16	After 0 days	19:1	2171.8	2.378	-36.412
S16	After 0 days	19:0 Std	2196.1	32.477	-30.419
S17	After 0 days	13:0 Std	1056.8	20.486	-30.269
S17	After 0 days	i14:0	1158.4	1.059	-42.478
S17	After 0 days	14:0	1222.9	8.778	-61.7
S17	After 0 days	i15:0	1336.9	10.19	-41.832
S17	After 0 days	a15:0	1352.7	6.362	-36.826
S17	After 0 days	15:0	1404.8	3.685	-45.772

S17	After 0 days	i16:0	1526.6	3.651	-38.528
S17	After 0 days	16:1ω7 + 16:1ω6	1570.4	127.234	-49.797
S17	After 0 days	16:1ω5	1584.1	29.662	-62.989
S17	After 0 days	16:0	1603.2	40.352	-41.403
S17	After 0 days	?	1678.1	3.591	-37.497
S17	After 0 days	?	1682.7	1.775	-47.055
S17	After 0 days	?	1721	1.681	-40.441
S17	After 0 days	17:1ω6	1752.8	4.266	-34.148
S17	After 0 days	cy17:0	1773	13.028	-33.542
S17	After 0 days	18:2ω6.9	1935.6	1.581	-50.832
S17	After 0 days	18:1ω9c	1945.7	4.645	-43.524
S17	After 0 days	18:1ω7c/9t	1961	42.951	-40.531
S17	After 0 days	18:0	2006.8	2.638	-39.154
S17	After 0 days	?	2074.7	2.863	-32.455
S17	After 0 days	19:1	2166.6	5.85	-39.122
S17	After 0 days	19:0 Std	2192	47.351	-31.935
S18	After 0 days	13:0 Std	1057	21.221	-30.27
S18	After 0 days	i14:0	1158.6	1.008	-41.09
S18	After 0 days	14:0	1223	8.607	-61.542
S18	After 0 days	i15:0	1337	9.703	-41.897
S18	After 0 days	a15:0	1352.8	5.859	-37.018
S18	After 0 days	15:0	1404.9	3.628	-46.22
S18	After 0 days	i16:0	1526.7	3.461	-38.825
S18	After 0 days	16:1ω7 + 16:1ω6	1570.6	147.565	-51.452
S18	After 0 days	16:1ω5	1584.6	31.104	-59.694
S18	After 0 days	16:0	1603.4	39.58	-41.177
S18	After 0 days	?	1678.3	3.434	-37.695
S18	After 0 days	?	1682.8	1.619	-38.837
S18	After 0 days	?	1721.1	1.601	-39.652
S18	After 0 days	17:1ω6	1753	4.227	-32.513
S18	After 0 days	cy17:0	1773.2	12.991	-32.828
S18	After 0 days	18:2ω6.9	1935.8	1.587	-48.397
S18	After 0 days	18:1ω9c	1945.8	4.338	-38.294
S18	After 0 days	18:1ω7c/9t	1961.2	48.865	-40.629
S18	After 0 days	18:0	2006.9	2.787	-38.847
S18	After 0 days	?	2074.8	2.312	-27.561
S18	After 0 days	19:1	2166.7	5.128	-37.097
S18	After 0 days	19:0 Std	2192.4	47.776	-31.381
S19	After 0 days	C13:0	1056.7	16.889	-30.125
S19	After 0 days	14:0	1222.1	0.757	-60.273
S19	After 0 days	i15:0	1335.8	0.869	-40.158
S19	After 0 days	16:1ω7	1562.5	10.726	-51.145

S19	After 0 days	16:1ω6	1569.6	1.29	-67.48
S19	After 0 days	16:1ω5	1578.8	2.52	-63.637
S19	After 0 days	16:0	1597.6	3.487	-41.353
S19	After 0 days	17:1ω6	1752.2	0.407	-34.013
S19	After 0 days	cy17:0	1771.2	1.159	-33.622
S19	After 0 days	18:2ω6.9	1935.6	0.158	-58.244
S19	After 0 days	18:1ω9c	1944.8	0.46	-41.725
S19	After 0 days	18:1ω7c/9t	1956.2	4.041	-39.098
S19	After 0 days	19:0 Std	2191.8	36.945	-30.123
S20	After 0 days	13:0 Std	1061.1	23.752	-30.571
S20	After 0 days	i14:0	1162.6	0.876	-41.084
S20	After 0 days	14:0	1226.9	7.356	-61.694
S20	After 0 days	i15:0	1340.9	6.933	-42.32
S20	After 0 days	a15:0	1356.7	4.222	-37.426
S20	After 0 days	15:0	1409.2	2.625	-46.502
S20	After 0 days	i16:0	1530.9	2.155	-40.333
S20	After 0 days	16:1ω7 + 16:1ω6	1573.4	104.384	-51.791
S20	After 0 days	16:1ω5	1587.7	23.092	-60.897
S20	After 0 days	16:0	1606.3	27.141	-41.268
S20	After 0 days	?	1682.7	2.392	-37.592
S20	After 0 days	?	1687.2	1.038	-45.601
S20	After 0 days	?	1725.6	1.162	-39.84
S20	After 0 days	17:1ω6	1757.4	3.132	-33.3
S20	After 0 days	cy17:0	1777.3	8.781	-33.199
S20	After 0 days	18:2ω6.9	1940.6	1.288	-51.776
S20	After 0 days	18:1ω9c	1950.3	2.894	-44.056
S20	After 0 days	18:1ω7c/9t	1964.8	34.051	-40.75
S20	After 0 days	18:0	2011.7	1.964	-40.136
S20	After 0 days	?	2079.7	1.184	-24.105
S20	After 0 days	19:;1	2171.8	3.702	-36.724
S20	After 0 days	C19:0 Std	2195.8	29.935	-30.639