Comparison of the effect of salinity on the D/H ratio of fatty acids of heterotrophic and photoautotrophic microorganisms

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One sentence summary: Metabolism is, compared to salinity, the main factor influencing the hydrogen isotopic composition of fatty acids.

Abstract

The core metabolism of microorganisms has a major influence on the hydrogen isotopic composition of their fatty acids. Heterotrophic microorganisms produce fatty acids with a deuterium to hydrogen (D/H) ratio either slightly depleted or enriched in D compared to the growth water, while photo- and chemoautotrophic microorganisms produce fatty acids which are heavily depleted in D. However, besides metabolism other biochemical and environmental factors (i.e. biosynthetic pathways, growth phase and temperature) have been shown to affect the D/H ratio of fatty acids and it is necessary to evaluate the magnitude of these effects compared to that of metabolism. Here we show that the effect of salinity on the D/H ratio of fatty acids depends on the core metabolism of the microorganism. While fatty acids of the photoautotroph *Isochrysis galbana* become more enriched in D with increasing salinity (enrichment of 30-40 ‰ over a range of 25 salinity units), no effect of salinity on the D/H ratio of fatty acids of the heterotrophic *Pseudomonas* str. LFY10 was observed ($\varepsilon_{lipid/water}$ of the C16:0 fatty acid of ~ 120 ‰ over a range of 10 salinity units). This can likely be explained by the relative contributions of different H and NADPH sources during fatty acid biosynthesis.

4.1. Introduction

Over the last 15 years the hydrogen isotope composition (δD) of different biomarker lipids has increasingly been used to understand and reconstruct diverse environmental factors such as salinity and the precipitation/evaporation balance (e.g. (Sauer et al., 2001; van der Meer et al., 2007; van der Meer et al., 2008; Berke et al., 2012; Garcin et al., 2012; Sachse et al., 2012; Coolen et al., 2013; van Soelen et al., 2013; Kasper et al., 2014). Several studies have also shown that the deuterium (D) to hydrogen ratio (D/H) of fatty acids reflects the core metabolism of microorganisms both in culture and in the environment (Zhang et al., 2009a; Osburn et al., 2011; Heinzelmann et al., 2015). Therefore, δD of lipids has attracted interest as a potential tool to study microbial activity *in situ* (Zhang et al., 2009a; Osburn et al., 2011). Microorganisms grown chemo- or photoautotrophically in culture produce fatty acids which are strongly depleted in D relative to the growth water, while heterotrophically grown organisms produce fatty acids enriched or only slightly depleted in D compared to the growth water. This also applies when the same microorganism is grown under different conditions expressing different core metabolisms (Zhang et al., 2009a), indicating that this effect is not species dependent but rather depends on metabolism.

The source of hydrogen is the most likely reason for this effect of metabolism on the hydrogen isotopic composition of fatty acids. The main source (~50 %) of hydrogen for fatty acid biosynthesis is nicotinamide adenine dinucleotide phosphate (NADPH) and thus the D/H ratio of fatty acids should reflect largely the D/H ratio of NADPH. Additional sources of hydrogen are the methyl group of acetyl-CoA (~25 %) and internal cell water (~25 %) (Saito et al., 1980; Robins et al., 2003; Schmidt et al., 2003). Zhang et al. (2009a) suggested that the isotopic composition of hydrogen provided by NADPH depends on the pathway by which $NADP^+$ is reduced to NADPH. The main source of hydrogen for the reduction of $NADP^+$ in a heterotrophic microorganism is the organic substrate which gets oxidized via the oxidative pentose phosphate (OPP) pathway and/or the tricarboxylic acid (TCA) cycle, with both pathways fractionating against the heavier isotope. Additionally, the NADH-NADPH converting transhydrogenase, an important enzyme which regulates the NADPH-pool in case of excess NADPH by reducing of nicotinamide adenine dinucleotide (NAD^+) to NADH and oxidation of NADPH (Kim and Gadd, 2008), is associated with a strong fractionation effect and

leaves the remaining NADPH-pool enriched in D. Oxygenic photoautotrophs on the other hand, mainly reduce $NADP^+$ during photosynthesis using hydrogen from water via ferredoxin-$NADP^+$ oxidoreductase, and the resulting NADPH is approximately 600 ‰ depleted in D compared to water ($\alpha = 0.4$) (Schmidt et al., 2003; Zhang et al., 2009a).

Although compound-specific δD of fatty acids is a promising tool to characterize the metabolic activity of microbial communities in the environment, the possible impact of factors other than metabolism on the D/H ratio of fatty acids needs to be studied in more detail. Previous studies have shown that the fatty acid synthesis pathway influences the D/H ratio. For example, in the piezophilic, heterotrophic bacterium *Moritella japonica* DSK 1 long-chain polyunsaturated fatty acids, synthesized by the polykektide (PKS) pathway, were 120–210 ‰ more depleted in D than short-chain fatty acids, synthesized by the fatty acid synthase (FAS) pathway (Fang et al., 2014). Furthermore, temperature was also shown to influence the D/H ratio of some fatty acids leading either to depletion or enrichment in D by up to 30–40 ‰ and 20 ‰, respectively (Zhang et al., 2009b; Dirghangi and Pagani, 2013b).

One of the best studied environmental parameter affecting the hydrogen isotopic composition of aquatic microorganisms so far is salinity. It has been shown to affect the D/H ratio of long chain alkenones of haptophyte algae (Schouten et al., 2006; Chivall et al., 2014; M'Boule et al., 2014) and of algal steroids such as dinosterol and brassicasterol (Sachse and Sachs, 2008; Sachs and Schwab, 2011; Nelson and Sachs, 2014). The D/H ratio of isoprenoid lipids derived from the heterotrophic euryarchaeote *Haloarcula marismortui* were also seemingly affected by salinity, although the salinity effect might have been indirect, with the original cause likely being growth rate rather than salinity itself (Dirghangi and Pagani, 2013a). Interestingly, the impact of salinity on the D/H ratio of fatty acids of microorganisms has not been studied so far and it is unknown how large this effect is compared to that imposed by metabolism. Therefore, the goal of this study is to evaluate the possible impact of salinity on the D/H ratio of fatty acids and compare it to the effect of metabolism. To this end, we studied the D/H ratios of fatty acids produced by a heterotrophic bacterium *Pseudomonas* str. LFY10 grown at three different salinities and compare them to changes in the D/H ratios of fatty acids produced by the photoautotrophic alga *Isochrysis galbana*, grown at six different salinities and harvested at three different growth phases (exponential, stationary and death phase). The salinity ranges from brackish to marine covering the range encountered in coastal and marine environments.

4.2. Material and Methods

4.2.1. Cultures

A freshwater *Pseudomonas* str. LFY10 was grown at 25 ºC on an ammonium-glucose medium at salinities of 6.4, 10.9 and 16.9 (Heinzelmann et al., 2015). The salinities were obtained by addition of different amounts of NaCl to the medium. Biomass samples and water samples for hydrogen analysis were collected during the stationary phase by filtration over a 0.7 µm GF/F filter. The photoautotrophic eukaryote *Isochrysis galbana* (strain CCMP 1323) was cultured previously at salinities of 10.3, 15.3, 20.2, 25.1, 30.2 and 35.5 in f/2 medium (Guillard, 1975) by M'Boule *et al*. (2014). The cultures were incubated at 15 °C and a light intensity of ~50 µE m^{-2} s^{-1} of a cool white fluorescent light (16 h light, 8 h dark). Biomass and water samples were collected during the exponential, stationary and death phase as previously described by Chivall et al. (2014) and Heinzelmann et al. (2015).

4.2.2. Fatty acid and hydrogen isotope analysis

Fatty acids were extracted by saponification of the freeze dried filters as described by Heinzelmann et al. (2015). Briefly, the filters were refluxed for 1 h in 1 N KOH in methanol (MeOH), after which the pH was adjusted to pH = 4 using 2 N HCl/MeOH (1/1, v/v). The solid phase was washed with MeOH/H_2O (1/1, v/v), MeOH and dichloromethane (DCM), the liquid phases were combined and washed with DCM. Fatty acids were derivatized by methylation with 13–15 % boron trifluoride in-methanol (BF_3-MeOH) after which a fraction containing the methylated fatty acids was obtained by eluting with DCM over an aluminium oxide column. In order to identify the position of double bonds in unsaturated fatty acids, an aliquot of the methylated fatty acids was derivatized with dimethyldisulfide (DMDS) (Nichols et al., 1986) and analysed by gas chromatography (GC) and mass spectrometry (MS).

The fatty acid fractions were analysed and identified by gas chromatography (GC) and GC/mass spectrometry (GC/MS) (as described by Heinzelmann et al. (2015). Hydrogen isotope analysis of the fatty acid fraction was performed by GC-thermal conversion-isotope ratio monitoring MS (GC/TC/irMS) using an Agilent 7890 GC connected via Thermo GC Isolink and Conflo IV interfaces to a Thermo Delta V MS according to Chivall, *et al.,* 2014. The H_3^+ correction factor was determined daily and was constant at 5.3±0.2. An internal standard, squalane (δD = -170±4 ‰), was co-injected with each fatty acid sample in order to monitor the accuracy of the measurements. The mean measured δD of the internal standard was -165±3 ‰.

The δD of the individual fatty acids was measured in duplicate and corrected for the addition of methyl hydrogen (δD_{ME} = -171±1 ‰; Heinzelmann et al. (2015).

The δD of culture medium and the non-exchangeable hydrogen of glucose, after derivatization with acetic anhydride, were determined by elemental analysis/TC/irMS (EA/TC/irMS) according to Chivall et al. (2014) and Heinzelmann et al. (2015), respectively.

4.3. Results

Pseudomonas str. LFY10 was grown heterotrophically with glucose as the substrate at three different salinities and was harvested during the stationary phase. It produced C16:1ω7, C16:0, C18:1ω7 fatty acids and traces of C17:cyc and C18:0 fatty acids at all three salinities. The hydrogen isotopic compositions of the most abundant fatty acids was determined and $\varepsilon_{lipid/water}$ and $\varepsilon_{lipid/glucose}$ values are summarised in Table 1, with ε expressing the hydrogen isotopic fractionation between lipid and water/substrate. All fatty acid did not show any difference in $\varepsilon_{lipid/water}$ with increasing salinity and no significant correlation was observed between $\varepsilon_{lipid/water}$ and salinity for the different fatty acids ($R^2 < 0.25$; p values > 0.7).

Batch cultures of *Isochrysis galbana* were grown at six different salinities (M'Boule et al., 2014) and harvested at three different growth phases (Chivall et al., 2014). *I. galbana* produced C14:0, C16:0 and C18:1ω9 fatty acids at all salinities and growth phases (Table 2). Low amounts of the C18:0 and other, unsaturated, fatty acids were also detected. Hydrogen isotope ratios were determined for the C14:0, C16:0 and C18:1 fatty acids. The $\varepsilon_{lipid/water}$ values of fatty acids produced by *I. galbana* at the highest salinity (~35) has been reported previously (Heinzelmann et al., 2015). During all three growth phases $\varepsilon_{lipid/water}$ for individual fatty acids became less negative with increasing salinity (Table 2). The $\varepsilon_{lipid/water}$ values for all three fatty acids showed a strong correlation with salinity at every growth phase studied (Figure 1).

4.4. Discussion

Fatty acids synthesized by the heterotrophically grown *Pseudomonas* str. LFY10 are substantially enriched in D compared to the growth water, while fatty acids produced by *I.*

galbana are depleted in D. This pattern is consistent with that previously observed for other heterotrophs and photoautotrophs (Sessions et al., 1999; Chikaraishi et al., 2004; Zhang and Sachs, 2007; Zhang et al., 2009a; Heinzelmann et al., 2015), which is related to their metabolism. In the following, we discuss the effect of salinity.

Fatty acids of *I. galbana* are produced via the FAS pathway (Lengeler et al., 1999; Huerlimann and Heimann, 2013) and get enriched in D with increasing salinity. There is a clear correlation between their $\varepsilon_{lipid/water}$ values and salinity with an increase of ~1.5 ‰ per salinity unit for all growth phases (Figure 1). A similar salinity effect has been previously observed for alkenones produced by various haptophyte algae (Schouten et al., 2006; Chivall et al., 2014; M'Boule et al., 2014). Furthermore, environmental data also seems to suggest that photoautotrophic organisms, including cyanobacteria, decrease hydrogen isotopic fractionation at increasing salinities (Sachse and Sachs, 2008), suggesting it may be a common phenomenon among photoautotrophs. The salinity effect was hypothesized to be caused by a reduction of water transport across the cell membrane with increasing salinity, which would lead to a D enrichment of the internal cell water relative to the medium and, therefore, a D enrichment in lipids (Sachse and Sachs, 2008; Sachs and Schwab, 2011). It has been estimated, that the main sources of hydrogen during biosynthesis of fatty acids in general are NADPH (~50 %), the methyl group of acetyl-CoA (~25 %) and internal cell water (~25 %) (Saito et al., 1980; Robins et al., 2003; Schmidt et al., 2003). In photoautotrophic organisms both NADPH and acetyl-CoA ultimately gain their H from internal cell water during photosynthesis. Therefore, if a D enrichment of the internal cell water is indeed the cause of the D enrichment in alkenones with increasing salinity, then fatty acids should also be enriched in D with salinity, which we indeed observe. Our results, therefore, seem to confirm the hypothesis that the D enrichment of lipids with salinity in photoautotrophs is caused by a D enrichment of internal cell water. The fact that all the hydrogen in fatty acids derives ultimately from internal cell water, which gets enriched with increasing salinity, finally leads to enrichment in D of ~15 ‰ of fatty acids over a range of 10 salinity units in *I. galbana*.

None of the fatty acids produced by *Pseudomonas* str. LFY10 showed significant change of $\varepsilon_{lipid/water}$ over a range of 10 salinity units (Table 1). The lack of change agrees with the observations of Dirghangi and Pagani (2013a), who concluded that changes in the δD of archaeol in the heterotrophically grown euryarchaeote *H. marismortui* is mainly due to growth rate changes and not directly to changes in salinity. There are several possible explanations for this lack of change in $\varepsilon_{lipid/water}$ values with increasing salinity as discussed below.

Differences in hydrogen isotopic fractionation during biosynthesis leading to major differences in the $\varepsilon_{lipid/water}$ values of specific fatty acids have been attributed to different lipid biosynthetic pathways (Fang et al., 2014). However, similar to *I. galbana*, *Pseudomonas* str. LFY10 likely also expresses the FAS pathway, since this pathway has been described to operate in other *Pseudomonas* species (Hoang and Schweizer, 1997). Therefore, differences in the salinity effect on the $\varepsilon_{lipid/water}$ values of fatty acids in both microorganisms are most likely not due to differences in the pathway of fatty acid biosynthesis.

Salt stress can lead to changes in enzyme activity in biochemical pathways. Danevčič and Stopar (2011) and Fu *et al*. (2014) observed that salt stress can lead to an up regulation of the glucose-6-phosphate dehydrogenase activity in the OPP pathway. This would lead to an increased contribution of NADPH produced in the OPP pathway, which is D-depleted with an $\varepsilon_{lipid/water}$ value of ~-250 ‰ compared to the general NADPH pool (Schmidt et al., 2003). Increased production of D-depleted NADPH by the OPP pathway at higher salinities could counter act the hypothetical increase in D of the internal cell water as a result of increasing salinity, potentially resulting in the absence of a salinity effect on hydrogen isotopic fractionation in fatty acids produced by *Pseudomonas* str. LFY10.

As previously mentioned, the $\varepsilon_{lipid/water}$ values of fatty acids from *I. galbana* increase by ~15 ‰ over a range of 10 salinity units with all hydrogen of the fatty acids originating from internal cell water. In *Pseudomonas* str. LFY10 on the other hand the majority of H originates from the substrate (~75 %), since NADPH (~50 %) gains its hydrogen from substrate and acetyl-CoA (~25 %), which is also derived from the substrate, and only ~25 % from internal cell water (Zhang et al., 2009a). The δD of glucose (-8±11 ‰) was the same at all salinities and therefore should not result in changes in the D/H ratio of fatty acids. Since approximately one quarter of the H should come from the internal cell water we would expect an D enrichment in fatty acids of one quarter of the magnitude observed in *I. galbana* assuming that both organisms modulate their internal cell water similarly. This should lead to an enrichment of ~3.8 ‰ in fatty acids over a range of 10 salinity units in *Pseudomonas* str. LFY10. Differences of ~3.8 ‰ are, however, close to the external precision of the measurement of 3 ‰ (as suggested by the precision in the measurement of the isotopic composition of our internal standard $\delta D_{Squalene}$ -165±3 ‰). Therefore, it is possible that there is an effect of salinity on the $\varepsilon_{lipid/water}$ values of fatty acids of *Pseudomonas* str. LFY10 but that it is too small to be detected. Alternatively, *Pseudomonas* str. LFY10 might not adapt its internal cell water as it is hypothesized for *I. galbana*.

Although the exact mechanism remains uncertain, we observe that the relationship between salinity and $\varepsilon_{lipid/water}$ values of fatty acids and the magnitude of $\varepsilon_{lipid/water}$ may depend on the metabolism of the source organism. Importantly, it shows that salinity has a relatively small impact on hydrogen isotopic fractionation compared to metabolism. It will be necessary to extend this type of research to a wider range of microorganisms, including chemoautotrophic microorganisms and microorganisms which express a wide metabolic range. Additionally, it will be necessary to study how microorganisms adapt to changes in salinity i.e. changes in metabolic activity and water transport. This will increase our understanding of how environmental and biochemical parameters affect hydrogen isotopic fractionation in lipids and improve the application of the D/H ratio of fatty acids as an indicator for the general metabolism of microbial communities.

4.5. Conclusion

The D/H ratio of fatty acids produced by the heterotrophic microorganism *Pseudomonas* str. LFY10 showed no significant change in $\varepsilon_{lipid/water}$ values with increasing salinity, whereas fatty acids produced by the photoautotrophic algae *Isochrysis galbana* became increasingly enriched in D with increasing salinity. This suggests that the effect of salinity on the D/H ratio of fatty acids depends on the metabolism of the organism. This difference can likely be explained by the fact that hydrogen in fatty acids in heterotrophs are only for a small part derived from internal cell water whereas *I. galbana* gains all its hydrogen from internal cell water. Overall, our results shows that salinity has a relatively small to minor effect on hydrogen isotopic fractionation compared to metabolism.

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Figure and tables legends

Figure 1

Hydrogen isotopic fractionation ($\varepsilon_{lipid/water}$ values) of the C16:0 fatty acid produced by the heterotrophic *Pseudomonas* str. LFY10 and the photoautotrophic *Isochrysis galbana* against salinity.

Table 1

ε values of fatty acids produced by *Pseudomonas* str. LFY10 at three different salinities.

Salinity	δD_{water} (‰)	$\delta D_{glucose}$ (‰)	$\varepsilon_{lipid/water}$ (‰)			$\varepsilon_{lipid/glucose}$ (‰)		
			C16:1*	C16:0	C18:1ϕ	C16:1*	C16:0	C18:1ϕ
6.4	-58±1	-8±11	96±5	122±2	121±1	35±5	60±2	59±1
10.9	-56±3	-8±11	92±1	116±2	116±3	34±1	57±2	56±3
16.9	-57±2	-8±11	97±2	118±4	118±2	38±2	58±4	57±2

C16:1*: double bond is at the ω7 position; C18:1ϕ: double bond is at the ω7 position.

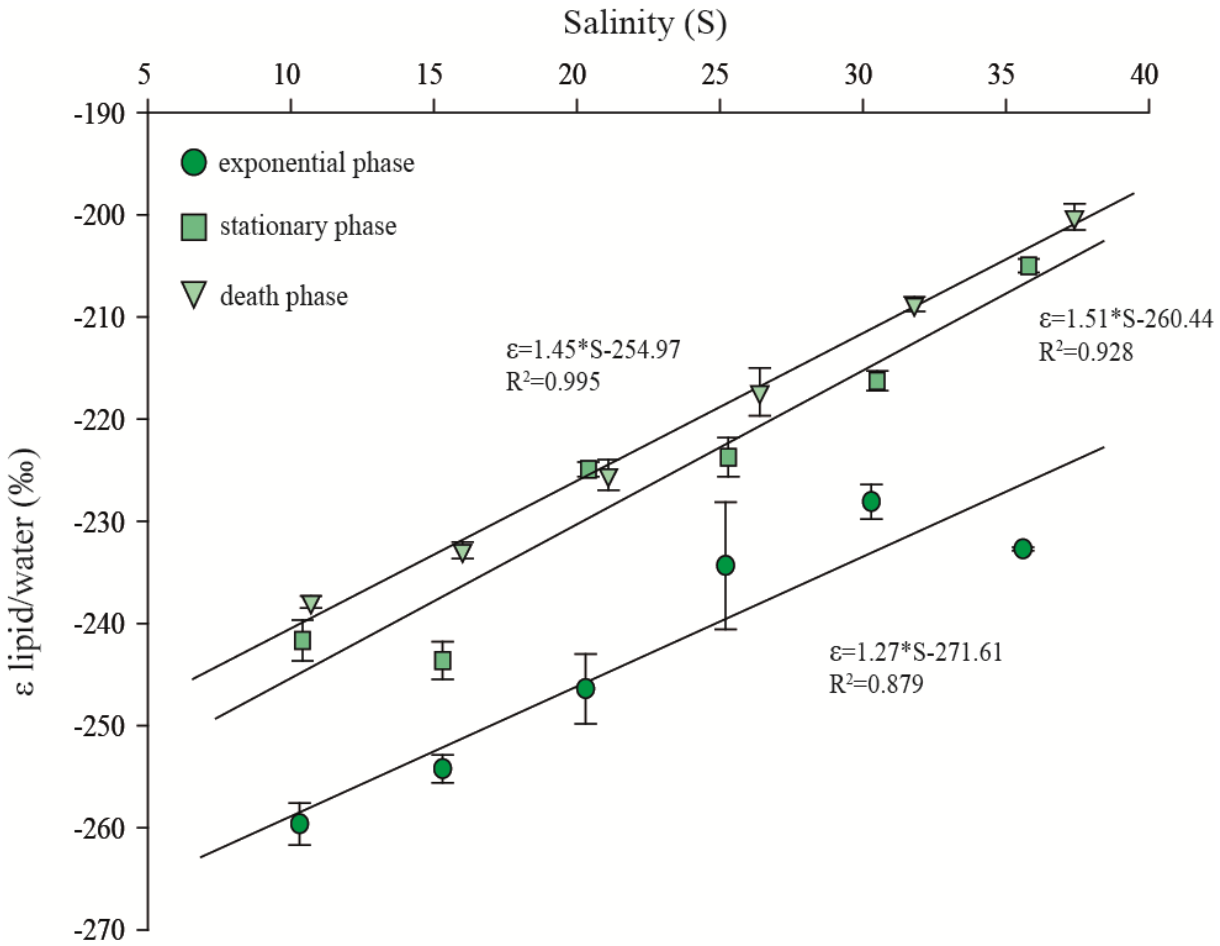
Table 2

ε values of fatty acids produced by *Isochrysis galbana* at different salinities and during different growth phases. δD_{water} and salinity values are the mean of measurements taken before inoculation and at the time of sampling (Chivall et al., 2014). $\varepsilon_{lipid/water}$ values of fatty acids produced at salinity ~35 were taken from Heinzelmann *et al.* 2015.

Growth Phase	Salinity	δD_{water} (‰)	$\varepsilon_{lipid/water}$ (‰)		
			C14:0	C16:0	C18:1$^{\phi}$
exponential	10.3	-29±1	-269±0	-260±2	-192±1
	15.3	-22±1	-268±1	-254±1	-186±1
	20.3	-17±2	-260±1	-246±3	-170±2
	25.2	-9±2	-247±5	-234±6	-162±6
	30.3	-3±2	-239±0	-228±2	-161±1
	35.6	+4±2*	-237±1*	-233±0*	-149±4*
stationary	10.4	-27±1	-248±1	-242±2	-207±2
	15.3	-21±1	-251±1	-244±2	-203±0
	20.4	-15±1	-220±6	-225±1	-205±1
	25.3	-10±1	-229±2	-224±2	-196±2
	30.5	-2±1	-223±0	-216±1	-186±1
	35.8	+5±1*	-215±1*	-205±1*	-179±1*
death	10.7	-24±1	-242±1	-238±1	-195±1
	16	-19±2	-230±2	-233±1	-198±0
	21.1	-14±2	-228±0	-225±2	-196±1
	26.4	-5±2	-220±3	-217±2	-192±1
	31.8	+2±2	-216±2	-209±1	-182±3
	37.4	+9±1*	-201±1*	-200±1*	-184±1*

C18:1$^{\phi}$: double bond is at the ω9 position. * data previously reported by Heinzelmann *et al.* 2015

12



13 Figure 1