

Effects of fatty acid from deep-sea microorganisms on lipid bilayer membrane fluidity under high pressure: comparison of branched-chain and polyunsaturated fatty acid

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Abstract. In this study, the effects of unsaturated and saturated branched-chain fatty acids in biomembranes of microorganisms living under high temperature and high pressure on the fluidity of biomembranes were investigated by time-resolved fluorescence anisotropy measurements. First, the relationship between the order parameter S and the rotational diffusion coefficient D_w , which can be calculated from the fluorescence anisotropy measurements, and the motion of lipid molecules was investigated using lipids with three different structures, and it was found that the former was related to the spacing of lipid molecules and the latter to the motion of lipid molecules. Next, we investigated the S and D_w values of lipid bilayer membrane containing the saturated branched-chain fatty acid 12-methyltridecanoic acid (12-MTA) and the polyunsaturated fatty acid (PUFA) *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA). The results showed that 12-MTA increased the S value and decreased the D_w value. On the other hand, DHA tended to reduce the S value and increase the D_w value, albeit slightly. These results mean that 12-MTA narrows the molecular spacing of lipids and inhibits lipid molecular movement, while DHA tends to widen the molecular spacing of lipids and promote lipid molecular movement.

1 Introduction

An extreme environment, such as the deep sea or hydrothermal vents, is called an extreme environment, and the organisms that live in it are called extreme environment organisms. Extreme environment organisms adapt their cellular components to survive in such harsh environments. Among the cell members, the biological membrane is the one that is exposed to changes in the surrounding physical environment. The expression of proteins in the biomembrane is essential for maintaining the organism's life. The movement of lipid molecules around the proteins, i.e., the fluidity of the biomembrane, is necessary to make this possible. For example, organisms contain a large amount of polyunsaturated fatty acids

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(PUFA) in their biomembrane to prevent lipid molecules' movement from deteriorating under low temperature and high-pressure conditions such as in the deep sea. In this way, organisms are said to adjust the fatty acid composition of their biomembrane according to the surrounding environment to maintain the fluidity of the biomembrane, which is important for life support. Such a mechanism is called "homeoviscous adaptation" [1].

In addition to these reports on the temperature of Homeoviscous adaptation [2, 3], adaptation to high-pressure environments has also been actively studied [4-7]. Recently, many organisms living under high pressure, such as in the deep sea, have been isolated. Their biological membranes have been shown to contain PUFAs and branched-chain fatty acids [8, 9]. Although unsaturated fatty acids such as PUFA are said to increase biomembrane fluidity under low temperature, and high pressure, the effect of branched-chain fatty acids on biomembrane fluidity has not been investigated in detail. On the other hand, Gram-positive bacteria, such as *Bacillus subtilis*, are known to be rich in branched-chain fatty acids [10, 11]. Since the tolerance of these bacteria at growth temperatures is wider on the high-temperature side, branched-chain fatty acids are thought to play some role in high-temperature environments. Although environmental conditions differ between deep-sea and high-temperature environments, branched-chain fatty acids are essential components of biological membranes for microorganisms living in these environments. Therefore, clarifying the role of branched-chain fatty acids in biological membranes may play a role in elucidating the survival strategies of organisms.

So far, research on the biological membrane fluidity of living organisms has been investigated by measuring light transmittance and differential scanning calorimetry [12, 13]. However, such a measuring method regards the fluidity of the biological membrane as a change in a uniform phase state. It is difficult to observe it as the movement of lipid molecules. A technique for studying the properties of lipids at the molecular level is fluorescence anisotropy measurement. This is done by using fluorescent molecules to understand the movement of surrounding molecules. Using this technique, Usui et al. have investigated in detail the biomembrane properties of *Shewanella violacea* at an optimum pressure of 30 MPa [14]. As a result, they reported that EPA also has a role in preventing hyperfluidization of biomembranes, which is a new finding that differs from the general concept based on previous studies on the properties of biomembranes.

This study used time-resolved fluorescence anisotropy to investigate the detailed role of branched-chain fatty acids on biomembrane properties in a high-pressure environment. The results were compared with PUFA, which are known to enhance biomembrane fluidity in high-pressure environments.

2 Materials and methods

2.1. Materials and sample preparation

The lipids 1, 2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). The fatty acids were *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA), a polyunsaturated fatty acid, and 12-Methyltridecanoic acid (12-MTA), a branched-chain fatty acid, purchased from Sigma-Aldrich Corp. (St. Luis, MO, USA). Fluorescent probes, 1-[4-(trimethylamine) phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH) was obtained from Marker Gene Technologies, Inc. (Eugene, OR, USA). Fig. 1 shows the structures of the lipids and fatty acids used.

The samples were prepared by the Bangham method [15]. This study's detailed samples and preparation conditions were as follows: the fluorescent probe was added to the lipid-

chloroform solution at a molar ratio of lipid to fluorescent probe = 500:1. The lipid film was prepared by vacuum drying under reduced pressure for 90 minutes. Then, ultrapure water was added and sonicated at 60-70 °C to prepare liposome solution with a lipid concentration of 1 mmol kg⁻¹.

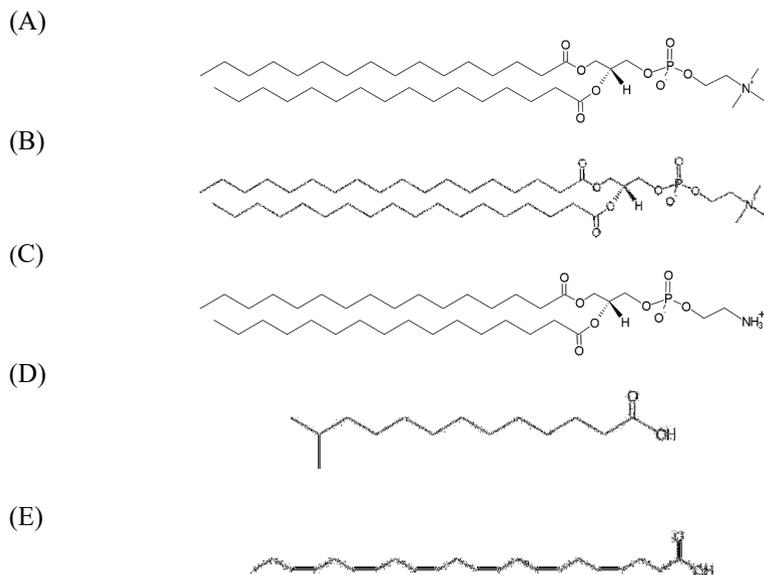


Fig. 1. Structures of the lipids and fatty acids.: (A) 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), (B) 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), (C) 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPE), (D) 12-Methyltridecanoic acid (12-MTA), (E) *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA).

2.2 Time-resolved fluorescence anisotropy measurements

PCI-200-PH cell holder (Shin Corporation, Kyoto) was installed on a FluoroMax-4 Spectrofluorometer (HORIBA, Ltd., Kyoto) for measurement. The measurement was performed at an excitation wavelength of $\lambda_{ex} = 370$ nm and a fluorescence wavelength of $\lambda_{em} = 430$ nm under a constant 70 °C and a pressure range of 0.1-15 MPa or a continuous 50 °C and a pressure range of 0.1-150 MPa. The temperature and pressure of the experimental conditions in this study were determined from the pressure-temperature phase diagram [16] of each lipid in the range that includes the liquid crystalline phase with high fluidity.

2.3 Principle of time-resolved fluorescence anisotropy measurement [17]

Light is an electromagnetic wave that has the property of a transverse wave and oscillates in a plane perpendicular to the traveling direction. On the other hand, when a fluorescent probe is excited from the ground state S_0 to the first excited state S_1 , the transition moment has an intrinsic directionality determined by the molecular structure. When this transition moment and the incident light match, the probability of being excited becomes maximum, and if orthogonal, it becomes 0. This is called light selection. In addition, the emitted fluorescence also has a unique direction. If the fluorescence polarization is strong, the polarization component of the fluorescence parallel to the incident light becomes strong. The parameter representing the degree of this polarization is the fluorescence anisotropy value r , which is expressed by the following equation.

$$r = \frac{I_{VV} - G \cdot I_{VH}}{I_{VV} + 2G \cdot I_{VH}} \quad G = \frac{I_{HV}}{I_{HH}} \quad (1)$$

Where I is the fluorescence intensity, its first and second subscripts represent the fluorescence component for the excitation polarization, V is the vertical direction, and H is the horizontal direction. G is the correction factor of the diffraction grating. In terms of the fluidity of the lipid membrane, the smaller the value of this anisotropy, the higher the fluidity, and the larger the value of the anisotropy, the lower the fluidity.

The fluorescence lifetime of a fluorescent probe from the time it is exciting to the time it emits fluorescence is generally several tens of seconds to several nanoseconds. In time-resolved fluorescence anisotropy measurement, the fluorescence anisotropy value is measured in nanoseconds, and the time variation of the anisotropy value shown in Eq.(2) can be obtained.

$$r(t) = \frac{I_{VV}(t) \cdot G \cdot I_{VH}(t)}{I_{VV}(t) + 2G \cdot I_{VH}(t)} \quad (2)$$

In aqueous solution, the movement of the fluorescent probe is not restricted. However, in lipid bilayers such as the one used in this study, the rotational motion of the fluorescent probe is restricted, and the value of the fluorescence anisotropy is expressed in the one-component approximation of equation (3).

$$r(t) = (r_0 - r_\infty) \cdot \exp\left(-\frac{t}{\theta}\right) + r_\infty \quad (3)$$

Where r_0 is the anisotropy at $t=0$, r_∞ is the anisotropy when completely depolarized, and θ is the rotational correlation time of the fluorescent probe. The values of r_0 , r_∞ , and θ can be calculated from the results of time-resolved fluorescence anisotropy measurements. The order parameter S and the rotational diffusion coefficient D_w can be calculated using these values as shown in the following equation.

$$S = \sqrt{\frac{r_\infty}{r_0}} \quad (4)$$

$$D_w = \frac{r_0 - r_\infty}{6\theta r_0} \quad (5)$$

The S value represents the tilt of the fluorescent probe, and the D_w value represents the rotational motion of the fluorescent probe; a smaller S value indicates a more significant tilt of the fluorescent probe, and a larger D_w value indicates a larger rotational movement of the fluorescent probe.

3 Results

3.1 Evaluation of order parameter S and rotational diffusion coefficient D_w for pressure change

It is known that a single lipid bilayer shows a clear phase transition, and at high pressure, the phase state becomes low fluid, like a gel phase. Since biomembrane generally remains in a highly fluid condition. We investigated the relationship between order parameter S and rotational diffusion coefficient D_w and lipid molecules under the liquid state. The

fluorescence anisotropy, r , DPPC, DSPC, and DPPE with three different structures were measured at 0.1-15 MPa, and S and D_w were calculated. In this pressure range, the lipid bilayers exhibit a liquid crystalline phase.

Fig. 2 shows the pressure dependence of S of lipid bilayer membrane labeled TMA-DPH. The smaller the S value, the larger the slope of the fluorescent probe. The value of DPPE showed a higher than DPPC and DSPC. This indicates that the hill of fluorescent probes in lipid bilayers prepared with DPPE is smaller than fluorescent probes in lipid bilayers designed with the other two lipids. Furthermore, the S values of DPPC and DSPC were almost the same. This means that the slope of the fluorescent probe is the same for both lipids.

Fig. 3 shows the pressure dependence of D_w of lipid bilayer membrane labeled TMA-DPH. The D_w value indicates the speed of rotation of the fluorescent probe, and the larger the value, the faster the process of the fluorescent probe. The D_w value of DSPC is more significant than that of DPPC and DPPE. In addition, the D_w values of DPPC and DPPE were almost the same.

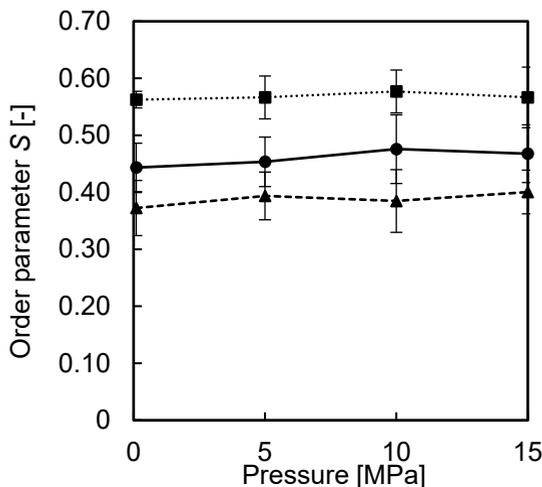


Fig. 2. Pressure dependence of order parameter S of each lipid (TMA-DPH): (●) DPPC, (■) DPPE, (▲) DSPC.

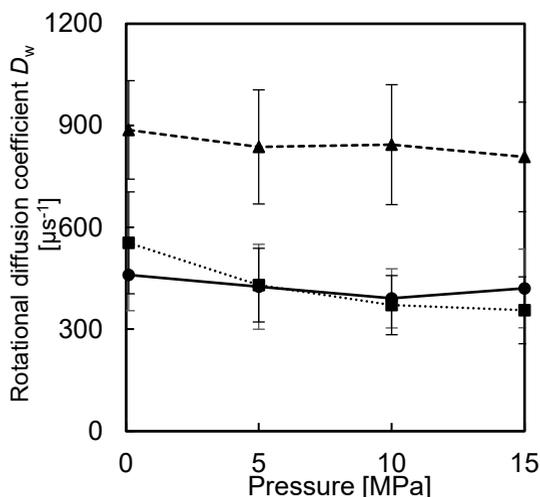


Fig. 3. Pressure dependence of rotational diffusion coefficient D_w of each lipid (TMA-DPH): (●) DPPC, (■) DPPE, (▲) DSPC.

3.2 Effect of branched-chain fatty acid and PUFA to fluorescence anisotropy r under high-pressure

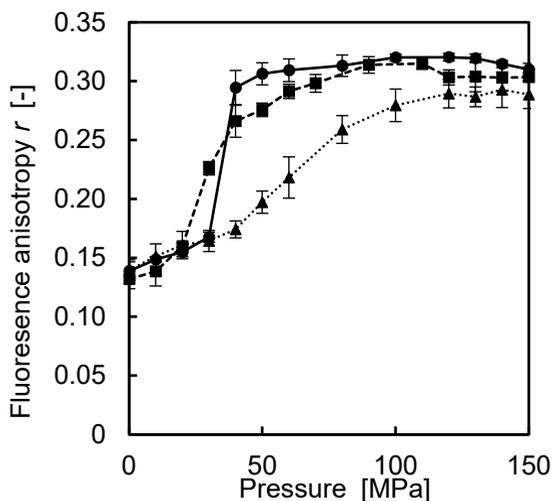


Fig. 4. Pressure dependence of fluorescence anisotropy r of DPPC bilayers labeled with TMA-DPH: (●) 0 mol kg⁻¹ fatty acid, (■) 300 mol kg⁻¹ 12-MTA, (▲) 300 mol kg⁻¹ DHA 300 mol kg⁻¹.

Fig. 4 shows the fluorescence anisotropy value r in the absence and presence of DHA and 12-MTA in DPPC bilayer membrane. The r -value correlates with the fluidity of the biological membrane, and the larger the r -value, the lower the fluidity. At 50 °C, which is the measurement temperature in this study, it is known that the DPPC bilayer membrane pure system changes from a liquid crystal phase with high fluidity to a gel phase with low fluidity at a pressure of about 37 MPa. This phase change from the liquid crystal phase to the gel phase is called the primary transition, and the pressure at which the major transition occurs is called the primary transition pressure. In this study's pure system of DPPC bilayers membrane, a significant change in r value was observed at about 37 MPa, confirming the phase transition. When 12-MTA, a branched-chain fatty acid, was added to the DPPC bilayer membrane, the primary transition pressure shifted to the lower pressure side. On the other hand, when DHA, a polyunsaturated fatty acid, was added to the DPPC bilayer membrane, the primary transition pressure shifted to the high-pressure side. This indicates that 12-MTA stabilizes the gel phase, and DHA stabilizes the liquid crystal phase.

3.3 Effect of branched-chain fatty acid and PUFA to order parameter S and rotational diffusion coefficient D_w under high pressure

Fig. 5 and Fig. 6 show the S and D_w values of the liquid crystalline phase of the DPPC bilayer membrane calculated from the r values. In the DPPC bilayer membrane in 12-MTA, the S value increased and the D_w value decreased. This means that the insertion of 12-MTA into the lipid bilayer membrane reduced the slope of the fluorescent probe and decreased the motion.

On the other hand, the S and D_w values of DPPC bilayer membrane in the presence of DHA were shown to be almost the same as those of the pure DPPC system, but the S values tended to be slightly higher, and the D_w values marginally lower. In other words, the presence of DHA in the DPPC bilayer slightly increased the slope of the fluorescent probe, although the hill was somewhat more significant, and the motion was also slightly larger.

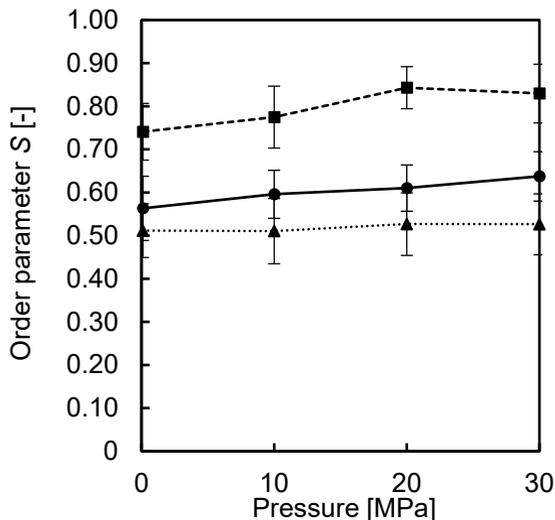


Fig. 5. Pressure dependence of order parameter S of DPPC bilayers labeled with TMA-DPH: (●) 0 mol kg⁻¹ fatty acid, (■) 300 mol kg⁻¹ 12-MTA, (▲) 300 mol kg⁻¹ DHA 300 mol kg⁻¹.

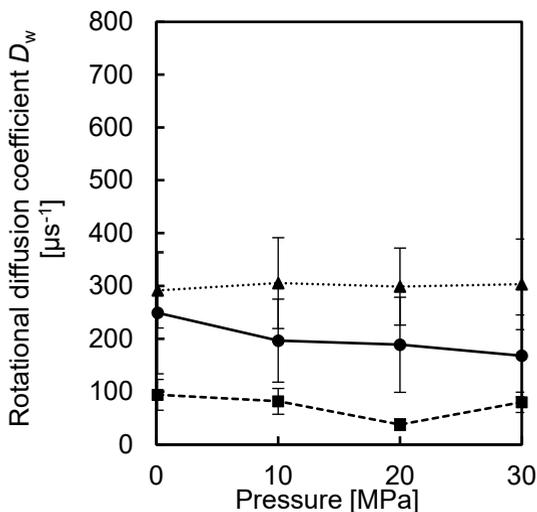


Fig. 6. Pressure dependence of rotational diffusion coefficient D_w of DPPC bilayers labeled with TMA-DPH: (●) 0 μmol kg⁻¹ fatty acid, (■) 300 μmol kg⁻¹ 12-MTA, (▲) 300 μmol kg⁻¹ DHA 300 μmol kg⁻¹.

4 Discussion

4.1 Relationship between the motion of lipid molecules under high pressure and the order parameter S and rotational diffusion coefficient D_w

The differences in the S value results at high pressures studied for lipids with three different structures mean differences in the fluorescent probes' slopes. The slope of the fluorescent probe changes depending on the packing of the surrounding lipid molecules. The S value of

DPPE was higher than that of the other two lipid molecules, and the slope of the fluorescent probe was smaller. This may be attributed to the fact that DPPE, which has a relatively small ethanolamine group in the hydrophilic group of the lipid, has a denser orientation and narrower spacing between lipid molecules than the other two lipids with choline groups. DPPC and DSPC have the same hydrophilic group. The choline group and the hydrophobic group differ by two carbons. Still, this difference is not considered to be enough to change the distance between lipid molecules. Therefore, there was no clear difference in the slope of the fluorescent probes, and the S values were considered to be almost the same. These results suggest that the S value is related to the distance between lipid molecules.

The difference in the D_w values of the three lipids can be attributed to the difference in the structure of the hydrophobic groups of the lipids: DPPC and DPPE have 16 carbons of hydrophobic groups, whereas DSPC has two more carbons. The rotation of the fluorescent probe is affected by the movement of the lipid molecules. Therefore, it can be said that the action of lipid molecules is more significant for DSPC with an enormous D_w value than for other lipids. It is considered that the more substantial number of carbons in the hydrophobic group means that the intramolecular carbon-carbon movement is more significant and the effect on the surrounding lipid molecules is greater. These results suggest that the D_w value is related to the mobility of lipid molecules.

4.2 Role of branched-chain fatty acids and PUFAs on biomembrane fluidity under high pressure

The effect of 12-MTA and DHA on the biomembrane fluidity under high pressure was investigated from the S and D_w values. 12-MTA increased the S value and decreased the D_w value. This means that 12-MTA narrows the lipid molecular spacing and decreases the lipid molecular motion. On the other hand, DHA tended to increase the lipid spacing and lipid movement, albeit slightly. These results can be attributed to the structural differences between 12-MTA and DHA. Although 12-MTA has a branched methyl group at the second carbon from the end of the hydrocarbon chain, the hydrocarbon chain structure is much more compact than DHA. Therefore, lipid molecules in lipid bilayers containing 12-MTA tend to become denser under pressure. Still, the branched methyl group of 12-MTA creates a slight bulk at the end of the hydrocarbon chain, which may inhibit the movement of the lipid molecules. The structure of DHA contains many unsaturated bonds in the hydrocarbon chain, and when it exists in a lipid bilayer membrane, it exists in a spiral form [18]. Therefore, even if lipid molecules are tightly packed due to pressure, the bulkiness of the DHA structure is thought to create some gaps between the hydrocarbon chains of the lipid bilayer, facilitating lipid molecular movement.

The presence of each fatty acid in the DPPC bilayer membrane shifted the phase transition pressure of 12-MTA to the lower pressure side and that of DHA to the higher-pressure side compared to the pure DPPC bilayer membrane system. This indicates that 12-MTA stabilizes the gel phase with low biomembrane fluidity, and DHA stabilizes the liquid crystal phase with high biomembrane fluidity. Considering that biomembrane generally needs to maintain a high degree of fluidity, the present results do not indicate that saturated branched-chain fatty acids maintain or improve the fluidity of biomembrane under pressure. In contrast, DHA tended to increase biomembrane fluidity under pressure. Regarding the relationship between pressure and branched-chain fatty acids in the biomembrane of bacteria isolated from pressure-tolerant and barophilic bacteria, it has been reported that saturated branched-chain fatty acids decrease with pressure and that increasing branched-chain fatty acids contain unsaturated bonds [8]. It has also been reported that PUFA such as DHA in the biomembranes of bacteria in the deep sea increase with pressure [5], and the results of the present study are consistent with these reports. Changes in the fatty acid composition of biomembranes with

changes in temperature have been investigated not only for PUFA but also for unsaturated branched-chain fatty acids, the content of which increases as the fluidity of biomembranes decreases at low temperatures [19]. Therefore, unsaturated fatty acids may be necessary for increasing the fluidity of biomembranes, which is reduced under high pressure or low-temperature conditions.

5 Conclusion

This study suggests that saturated branched-chain fatty acids may be involved in the reduction of biomembrane fluidity. In other words, saturated branched-chain fatty acids do not play a role in increasing the fluidity of biomembrane in high-pressure environments. However, when biomembrane fluidity increases in environments, such as at high temperatures, they may play a role in suppressing excessive increases in biomembrane fluidity and maintaining biomembrane fluidity that is just right for preserving life.

On the other hand, unsaturated fatty acids can increase the fluidity of biological membranes under high pressure. This result indicates that unsaturated fatty acids are involved in maintaining biomembrane fluidity sufficient for life support in a high-pressure environment.

6 Acknowledgement

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