

## B: Biomaterials and Membranes

**Molecular Dynamics and Metadynamics Insights of 1,4-Dioxane Induced Structural Changes of Biomembrane Models**

Zsófia Borbála Rózsa, Lukács József Németh, Balazs Jojart, Károly Nehéz, Béla Viskolcz, and Milán Sz#ri

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# Molecular Dynamics and Metadynamics Insights of 1,4-Dioxane Induced Structural Changes of Biomembrane Models

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**Abstract**

1,4-dioxane is a cytotoxic B2 type human carcinogen, a serious water pollutant produced solely by industrial activity. The effect of 1,4-dioxane on phospholipid membrane models composed by DPPC and its branched isomer (IPPC) was investigated using MD simulations. Clear and polluted membranes were compared by membrane parameters such as APL, VPL, compressibility modulus, membrane thickness and orderliness of lipid tails. While neat systems significantly differ from each other, the presence of the pollutant has the same effect on both types of lipid membranes: high density of dioxane appears at the vicinity of ester groups which pushes away lipid headgroups from each other, leading to an overall change in lipid structure: APL and VPL grows, while the orderliness of lipid tails, membrane thickness and compressibility modulus decreases. Orientational preferences of water and dioxane molecules were also investigated and different membrane regions have been specified according to the stance of water molecules. Free energy profile for 1,4-dioxane penetration mechanism into DPPC membranes was carried out using metadynamics for two different concentrations of the pollutant ( $c_1=7.51 \text{ g/dm}^3$ ,  $c_2=75.10 \text{ g/dm}^3$ ), which showed that the higher the concentration is, the lower the free energy of penetration gets. Only a small free energy barrier was found in the headgroup region and accumulation of dioxane is thermodynamically unfavored in the middle of the bilayer. The penetration mechanism has been described in detail based on the orientational preference of 1,4-dioxane molecules and the free energy profiles.

## Introduction

The passive membrane permeation of small molecules plays important role in their disposition and toxicity<sup>1</sup>. Passive diffusion is the main limitation of penetration of small molecules and thus the barrier for their infiltration into living cells (human, animal and bacteria). A key property of the passive transport of an investigated substance is its partitioning between water and membrane phases which is affected by the kinetics and thermodynamics of the specific permeation process<sup>2,3,4</sup>.

In most mammalian and bacterial cells phospholipids compose the base of the lipid bilayer with different types of head groups. In eukaryotic cells phosphatidylcholine (PC) is the most commonly found headgroup type for phospholipids (~50%), but others, like phosphatidylserine phosphatidylethanolamine, sphingomyelins and cholesterol can also be found as membrane components<sup>5</sup>. In the case of prokaryotic cells it is estimated that only 10% of bacteria produces phosphatidylcholines, while phosphatidylglycerols and phosphatidylethanolamines are the most common lipid types<sup>6</sup>. Thus both experimental and theoretical models usually utilize simple phospholipid bilayers to understand solute permeation processes<sup>2</sup>.

In terms of lipid chains several different types can be observed in cells, which can be characterized by their length, degree of unsaturation and branching. Branching fatty acids are widespread mostly among bacterial cell membranes. The most common ones contain one methyl group at the iso or anteiso position of the fatty acid<sup>7,8</sup>. In general both poly- and monomethyl-branching is associated with increasing the area per lipid, while decreasing the bilayer thickness and lowering chain ordering, thus enhancing the fluidity of the plasma membrane<sup>8,9</sup>. It has great importance in case of archaea and bacteria living in extreme conditions<sup>8</sup>. Iso- and midchain methyl-branching is associated with increasing the tolerance of the cell membrane to toxic compounds<sup>10</sup>. In the case of polymethyl-branching fatty acids - like phytanyl and phitanoyl - containing membranes it has been suggested, that they enhance the structural stability of cell membranes thus making it less permeable to water molecules, ions and small solutes compared to cell membranes composed of only straight chained lipids<sup>11</sup>, but through computational studies it has been proven that branching has no significant effect on the partition coefficients of neutral solutes<sup>12</sup>. These molecules have also been associated with the disturbance of the membrane interior's cavity distribution<sup>12</sup> which leads to a reduced amount of water molecules diffusing inside the membrane, while their mobility is also reduced.

Membrane/water interfaces are critical to many cellular functions. Water influences stability, fluidity and phase behavior of phospholipid membranes, and also has a crucial role in the electric properties of the bilayer<sup>13,14</sup>. Both experiments and molecular simulations have shown that water molecules lose their bulk-like nature near membrane surfaces<sup>15,16,17,18</sup>. Translational mobility and rotational entropy of individual water molecules decrease when their interactions with the lipid

1 headgroups become stronger <sup>19</sup> resulting in different interfacial water regions according to the  
2 membrane composition <sup>13,20</sup>. The total membrane dipole potential is a manifestation of the non-  
3 random orientation of the electric dipoles of headgroups, carbonyl groups and water <sup>14,15</sup>. The  
4 hydrating water molecules form an ordered, typically 1-2.5 nm thick stable layer (maximum 5 layers  
5 of structured water) at the membrane interface <sup>13</sup>, where adjacent water molecules are oriented by the  
6 headgroups as the dipoles of individual water molecules are aligned against the bilayer dipole <sup>14</sup>. The  
7 ester groups on the internal side of the headgroups can also establish hydrogen bonds closest to the  
8 bilayer center, which helps to create the dense, ordered state at the region of the acyl chains of the  
9 phospholipids, thus creating a barrier of penetration <sup>14</sup>. These layers are stable enough to present  
10 multiple energy barriers to approaching nanoscale objects <sup>16,21</sup>. Preferred molecular orientations can  
11 capture most of the structural features of these interfacial layers and they can be used for defining  
12 interfacial sublayers. The orientation of molecules relative to a membrane/water interface can be  
13 analyzed in terms of the orientation statistics of various vectors bound to the target molecule <sup>22,23,24</sup>.

24 Due to the complex nature and structure of biomembranes, its sublayers can be split into  
25 regions with different characteristics. There are several ways to split up the bilayer based on the  
26 investigated phenomena <sup>25,26</sup> from which we hereby adapted the four region model proposed by  
27 Marrink and Berendsen <sup>27</sup>:

31 **Region I:** The outer region of lipid bilayers, where the orientation of water molecules is already  
32 perturbed due to the headgroups and thus their diffusion constants, molecular orientations and  
33 solubilities differ from the aqueous bulk. Its border can be defined where lipid and water densities  
34 become comparable.

38 **Region II:** Highly structured region with a high mobile charge density of the head groups which can  
39 be a limiting factor for the penetration of polar and charged molecules. According to the original  
40 definition this region lasts until water density drops below 1%.

43 **Region III:** A region with high lipid chain density. It is considered as the main barrier for the  
44 permeation process of small molecules, as hydrophilic penetrants are not soluble in this region and  
45 water density is below 1% <sup>28</sup>.

48 **Region IV:** The middle of the bilayer where terminal methyl groups are found in the presence of a  
49 high fraction of free volume, therefore it is also called a low acyl chains density region.

52 Molecular Dynamics (MD) is a powerful tool to describe and understand the time evolution  
53 of a computational model of a complex chemical (biochemical) system. In the case of classical MD  
54 simulations the interatomic interactions are described by force fields – which are mathematical  
55 expressions describing the dependence of the systems potential energy on the atomic positions –, and  
56 atomic motions with the Newtonian equations of motion <sup>29</sup>. MD enables a thorough view of structure  
57 and dynamics of the simulated system and can provide relevant details which would be either  
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2 inaccessible or expensive and time consuming via experiments<sup>30</sup>. In the case of biological membranes  
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4 several studies have been carried out recently with the help of MD to understand - for example - the  
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6 structure and phase transition of membrane models of different lipid compositions<sup>31,32</sup>; the self-  
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8 assembly of bilayers<sup>33,34</sup>; the effect of ions<sup>35</sup>; interactions with proteins<sup>36,37</sup>; the permeation of small  
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10 molecules<sup>3,38,39,40,41</sup>.

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12 While the structural changes of such processes can be understood via the analysis of general  
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14 membrane parameters<sup>42,43</sup>, the energetic properties of the interactions remain hidden. The  
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16 partitioning and preferred position of an interacting molecule – like in the case of membrane  
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18 penetration – can be determined by a free energy profile along the bilayer normal where the change  
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20 in the free energy provides a clear view whether a process is thermodynamically favorable or not.  
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22 Thus free energy calculations are an efficient tool to estimate kinetic and dynamic characteristics of  
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24 chemical and biological processes, like rate constants, permeability coefficients or ionic conductance  
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26 <sup>44,45</sup>. To obtain the free energy profile of such process classical MD simulations are not well suited,  
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28 as high free energy barriers restrict the detailed sampling of the entire configuration space as the  
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30 system is likely to be found trapped in local free energy minima. Thus enhanced sampling algorithms  
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32 were developed which bias the system towards high energy regions which would be inaccessible  
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34 otherwise<sup>38,46</sup> such as umbrella sampling<sup>47</sup>, adaptive biasing<sup>48</sup>, thermodynamic integration<sup>49</sup>,  
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36 metadynamics<sup>50,51</sup> and its improved version, well-tempered metadynamics<sup>52</sup>.

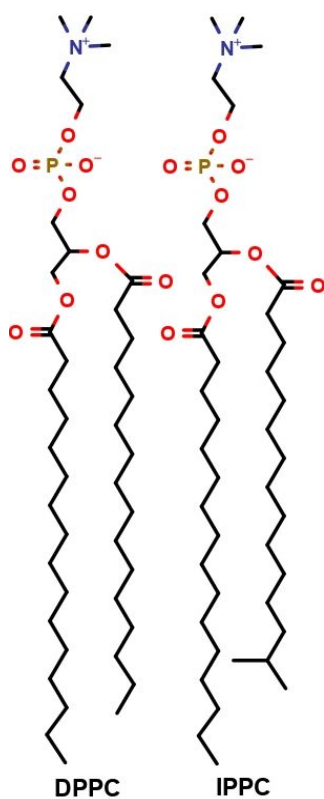
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38 1,4-dioxane (C<sub>4</sub>H<sub>8</sub>O<sub>2</sub>, M<sub>DIOX</sub>=88.11 g/mol) is a pleasant odour synthetic industrial chemical  
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40 with no known natural sources. Before the Montreal Convention it has been used as a stabilizing  
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42 agent for chlorinated solvents, primarily during the production of 1,1,1-trichloroethane (TCA).  
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44 Nowadays, it is a widely used industrial solvent during the production of organic compounds,  
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46 disinfectants, cooling liquids and hygienic products. It also appears as a side product of several  
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48 industrial processes, like PET manufacturing<sup>53,54</sup>. 1,4-dioxane pollution has been detected all over  
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50 the world in different areas, from surface water, groundwater to landfills, mostly coming from  
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52 industrial wastewater, industrial waste and illegal dumping<sup>54,55,56</sup>.

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54 At the same time 1,4-dioxane has several health effects. Inside the body it is instantly adsorbed  
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56 through the lungs and the gastrointestinal tract, and distributes quickly and uniformly between the  
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58 lungs, liver, kidney, spleen, colon and skeletal muscle tissues. Animal studies proved<sup>54</sup> that it causes  
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60 kidney and liver cancer, thus it is considered as a B2 type ‘Probable human carcinogen’<sup>57</sup>. 1,4-  
dioxane appears to be a typical cytotoxic carcinogen, but it requires a high level of exposure for its  
metabolism to happen<sup>58</sup>. As the substance has nonlinear toxicokinetics it can accumulate in the body,  
mostly in the liver. In rats it has been shown that its half-life increases with the dose of 1,4-dioxane  
from 1h to 14h<sup>59</sup>. 1,4-dioxane eliminates the body after its metabolism into 2-(2-  
hydroxyethoxy)acetic acid (HEAA) via urination, of which the amount depends on the consumed

1  
2 dose <sup>60</sup>.

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4 The physico-chemical properties of 1,4-dioxane make it a dangerous and persistent  
5 groundwater pollutant, as it is almost completely miscible with water <sup>61</sup>, its density is roughly the  
6 same as water (1.0329 g/mL) <sup>62</sup> and its low octanol-water partition coefficient ( $\log K_{OW} = -0.27$ ) <sup>63, 64</sup>  
7 indicating that it is also compatible with lipophilic matrices.  
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10 Despite the amphiphilic nature of 1,4-dioxane and its serious health risk, to the best of our  
11 knowledge, neither its passive membrane permeation nor its effect on the membrane structure was  
12 not investigated yet. Therefore, the aim of our study was to get detailed information about structural  
13 changes in biomembranes caused by 1,4-dioxane using DPPC (dipalmitoyl-phosphatidylcholine) and  
14 IPPC (isodipalmitoyl-phosphatidylcholine, 1-14-methylpentadecanoyl-2-palmitoyl-  
15 phosphatidylcholine, 14-MpPPC, **Figure 1**) model membrane systems. DPPC membranes are one of  
16 the most extensively investigated model bilayer systems <sup>65, 28</sup> and IPPC only differs in a branching  
17 methyl group at the C14 position of one of the hexadecanoates <sup>9</sup>. Changes induced by 1,4-dioxane  
18 are analyzed through the inflicted alterations in general membrane parameters, density profiles, area  
19 per lipid (APL), volume per lipid (VPL), isothermal compressibility ( $K_A$ ), deuterium order parameter  
20 ( $S_{CD}$ ) and membrane thickness ( $D_{HH}$ ). To thoroughly describe the penetration mechanism orientations  
21 of water and the pollutant molecules in the membrane/water interfacial regions were described using  
22 vectors bound to the molecules. Furthermore, we also determined the concentration dependence of  
23 the height of the free energy barrier of 1,4-dioxane penetration in order to estimate how easily it can  
24 cross biomembranes to exert its carcinogenic effects.  
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**Figure 1** 2D structure of DPPC and IPPC molecules



## Methods

### *System Preparation*

Total four different systems were used to understand the effects of 1,4-dioxane: DPPC and IPPC model membranes, and DPPC and IPPC membranes (**Figure 1**) in the presence of the pollutant molecule. Throughout this work pure systems will be referred to as 'clear', and 1,4-dioxane containing systems as 'polluted' systems. Initial membrane configurations have been generated using automated online membrane builder, CHARMM-GUI<sup>66,67</sup>. The simulated systems consisted of 128 ( $n_{lipid} = 128$ , 64-64 lipid molecules each membrane leaflet, which size have been proven to be sufficiently large to reproduce membrane structural parameters<sup>3,38,41,68,69</sup>) either DPPC or IPPC molecules, with 50 water molecules per headgroup ( $n_{water} = 6400$  in total), which is well above the value of the water molecules needed for total hydration of bilayer<sup>70</sup>. In the case of polluted systems an additional 100 1,4-dioxane molecules have been inserted into the simulation box in random positions. Such high concentration (75.102 g/dm<sup>3</sup>) had been chosen to exaggerate studied effects to distinguish it from statistical noise as suggested by Oh and Klein<sup>71</sup>. Lipid molecules were modelled by CHARMM36<sup>72</sup>, water molecules by CHARMM TIP3P<sup>73</sup> and CGenFF<sup>74</sup> force field parameters were applied for 1,4-dioxane molecules in a cubic simulation box using periodic boundary conditions. All dynamics calculations were carried out using GROMACS 5.1.2<sup>75</sup>, while VMD<sup>76</sup> was used for molecular visualization.

### *Parameters of MD simulations*

Production runs have been set up with an equilibration protocol where the number of optimization steps has been maximized at 5000. This was followed by a 25 ps long simulation using NVT ensemble where at first the P atoms were restrained to their positions with a 1000 kJ/(mol·nm<sup>2</sup>) force constant along axis  $z$  to prevent decomposition of the bilayer<sup>72</sup>. The next equilibration step was 800 ps long in an  $N_{p_{semi-isotropic}}T$  ensemble. The temperature ( $T = 330$  K, above the phase transition temperature, which is 313.35-313.45K experimentally<sup>77,78,79</sup>) and pressure ( $p = 1$  atm) was set using Berendsen temperature and pressure control<sup>80</sup>, where the value of coupling constants were chosen as  $\tau_T = 1.0$  ps and  $\tau_p = 5.0$  ps. Non-bonded interactions have been calculated using 1.2 nm cut-off value<sup>72</sup>, while the long distance electrostatic interactions have been accounted using smooth Particle Mesh Ewald summation method<sup>81</sup>. During calculations the heavy-atom-hydrogen distances have been fixed by the means of P-LINCS algorithm<sup>82</sup> with the exception of water molecules which have been kept rigid by using SETTLE<sup>83</sup>. The equations of motion were integrated in 2 fs time steps and structures have been saved to a trajectory file every 10 ps. Further simulations have been carried out in  $N_{p_{semi-isotropic}}T$  ensemble using Nosé-Hoover temperature<sup>84,85</sup> and Parrinello-Rahman pressure<sup>86</sup> control for 125 ns. Temperature and pressure values were the same as during previous  $N_{p_{semi-isotropic}}T$  equilibration to maintain a biologically relevant liquid-crystalline ( $L_\alpha$ ) phase. Out of these simulations

the first 25 ns were considered as part of the equilibration protocol, as the box sizes were still drastically changing in the case of 1,4-dioxane polluted systems, thus it was left out from the analysis, and the remaining 100 ns was considered as the production run. For each system 5 parallel production runs were conducted, resulting in 500 ns of total simulation for clear and polluted systems.

### ***Analysis of structural membrane parameters***

The analysis of membrane parameters was carried out using the equations listed in **Table 1**. Membrane thickness was calculated as the distance between the two peaks of the electron density profiles of the systems <sup>87</sup>.

**Table 1** Definition of the used membrane parameters.

<b>Membrane parameter</b>	<b>Definition</b>	<b>Formula</b>	<b>Abbreviation</b>
<b>APL</b>	Area per lipid <sup>88</sup>	$\frac{2 \times L_x \times L_y}{n_{lipid}}$	$L$ : average x and y dimensions of the simulation box
<b>VPL</b>	Volume per lipid <sup>88</sup>	$\frac{V_{box} - n_{water}V_{water} - n_{dioxane}V_{dioxane}}{n_{lipid}}$	$V_{box}$ : average volume of the simulation box $V_{water}$ : the volume of a TIP3P water molecule (0.03053 nm <sup>3</sup> ) <sup>87</sup> $V_{dioxane}$ : derived from the density of neat 1,4 dioxane (1.03 g/cm <sup>3</sup> <sup>54</sup> - 0.146 nm <sup>3</sup> )
<b>K<sub>A</sub></b>	Isothermal compressibility modulus, a dynamic parameter to measure stress resistance of the membrane <sup>89</sup>	$\frac{2k_B T \langle APL \rangle}{n_{lipid} \sigma_{APL}^2}$	$k_B$ : Boltzmann's constant $T$ : temperature $\sigma_{APL}^2$ : the variance of APL
<b>S<sub>CD</sub></b>	Deuterium order parameter, which measures the orientation of C-H bonds with respect to the bilayer normal <sup>87</sup>	$\frac{1}{2} \langle 3 \cos^2 \theta - 1 \rangle$	$\theta$ : the angle between bilayer normal (Z) and the vector between C <sub>i</sub> -H <sub>i</sub>

### ***Definition of vectors bound to water and 1,4-dioxane molecules***

To be able to describe the orientational preference of water and 1,4-dioxane molecules in the membrane/water interface vectors were defined bound to each investigated species. Three local vectors have been defined to describe the orientation of water molecules (upper panel of **Figure 2**): the dipole vector ( $z$ ), the normal vector ( $x$ ) and the vector joining the two hydrogen atoms (H-H vector,  $y$ ) in order to obtain their relative orientation to the membrane surface normal ( $Z$ ). Their orientation is characterized by their angles denoted  $\alpha$ ,  $\beta$  and  $\gamma$  respectively, formed with the interface normal. In

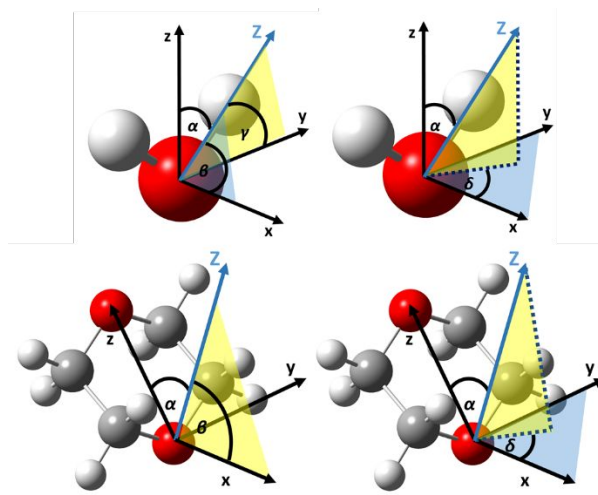
the case of 1,4-dioxane two vectors and the corresponding angles ( $\alpha$ ,  $\beta$  in the bottom panel of **Figure 2**) have been chosen, the vector joining the two oxygen atoms (O-O vector,  $z$ ), and the normal of this vector ( $x$ ). It should be noted that due to the symmetry of the molecules the molecular normal, and H-H vectors can be directed in two opposite directions, and there is no physical way to distinguish them. Therefore, their corresponding angles ( $\beta$  and  $\gamma$ ) can only be scattered between  $0^\circ$  and  $90^\circ$ , and thus their cosines between 0 and 1, while  $\alpha$  angles defined by the dipole moment vector of water or O-O vector of dioxane (as a direction is defined by selecting the O-O vector) can fall into the entire angular range of  $0^\circ$  to  $180^\circ$ , and cosine range of -1 and 1. To make interpretation easy for their ensemble averages, uniformly distributed orientation belongs to value 0, which is the case for the expected value ( $\langle \cos(\alpha) \rangle$ ), of  $\cos(\alpha)$ , while  $\cos(\beta)$  and  $\cos(\gamma)$  must be shifted downwards by 0.5. To characterize the variations of the average alignment of water and dioxane molecules along the interface normal axis,  $Z$  has been divided into 1 Å slices, where  $\Phi_\alpha(Z)$ ,  $\Phi_\beta(Z)$  and  $\Phi_\gamma(Z)$  – orientational function – profiles have been calculated, defined as:

$$\Phi_\alpha(Z) = \rho(Z) \langle \cos(\alpha) \rangle(Z) \quad \text{Eq. 1}$$

$$\Phi_\beta(Z) = \rho(Z) \langle \cos(\beta) - 0.5 \rangle(Z) \quad \text{Eq. 2}$$

$$\Phi_\gamma(Z) = \rho(Z) \langle \cos(\gamma) - 0.5 \rangle(Z) \quad \text{Eq. 3}$$

where  $\rho(Z)$  is the number density of the molecule (water or dioxane) in the corresponding slice of the simulation box. It is important to note that while any two of these angles can describe the orientation of a single molecule relative to the interface, these angles are not independent of each other. To create bivariate angle distribution maps of the water and 1,4-dioxane molecules two independent angles have been chosen as angular polar coordinates of the interface normal vector (**Figure 2**):  $\alpha$  and  $\delta$ , which are the angle of the molecular dipole vector enclosing with the bilayer normal, and the angle formed by the molecular normal vector and the projection of the interface normal ( $Z$ ) to the plane perpendicular to the molecular dipole vector ( $xy$  plane, shown in blue in **Figure 2**), respectively. For local Cartesian coordinates were fixed to each investigated molecule, where  $x$ -axis corresponds to the molecular normal,  $y$  to the H-H and  $z$  to the molecular dipole vector (**Figure 2**). It should be noted, that angle  $\delta$  can always be chosen in a way to fall in the range of  $0^\circ \leq \delta \leq 90^\circ$  because the normal can point towards two opposite directions.



**Figure 2** Definition of the local vectors ( $x, y, z$ ) bound to water and 1,4-dioxane molecules. The orientation of the molecules relative to the membrane normal ( $Z$ ) are defined by  $\alpha, \beta, \gamma, \delta$ .

### Free energy calculations

In metadynamics<sup>50,52</sup>, a history-dependent bias potential ( $P(s, t)$ ) is calculated along a collective variable ( $s$ ), which itself is a function of the positions of the particles throughout the simulation time ( $t$ ). The bias potential appears during the simulations as a repulsive Gaussian, centered on the explored points of a collective variable space ( $CV(s)$ ), leading the system towards previously unexplored configurations. These Gaussians have a preassigned width ( $\sigma$ ) and height ( $\omega$ ) and are deposited at every time step ( $t'$ ) as the simulation proceeds, where the collective variable has a value of  $s(t')$ . Then the bias potential can be calculated the following way:

$$P(s, t) = \omega_0 \sum_{t' < t} e^{-\frac{(s - s(t'))^2}{2\sigma^2}} \quad \text{Eq. 4}$$

By adding these Gaussians the system is discouraged of the exploration of already visited states of  $CV(s)$ , and their sum provides an immediate estimate of the free-energy surface ( $F(s)$ )<sup>50</sup>.

While metadynamics has been used successfully in a large variety of membrane penetration studies of different small molecules<sup>90,91,92,93,94</sup> it still has problems arising from that the free energy not converges to an exact value but fluctuates around the correct result as similar sized Gaussians are deposited along the CV<sup>52,38</sup>. Thus well-tempered metadynamics (WT-MD)<sup>52</sup> was proposed, where the height of the hills decreases as passing by previously mapped regions of the free energy profile which enables the convergence of the profile to an exact value<sup>95,46</sup>. The rescaling of the height of the Gaussians ( $\omega_{t'}$ ) goes the following:

$$\omega_{t'} = \omega_0 e^{-\frac{P(s, t')}{k_B \Delta T}}, \quad \text{Eq. 5}$$

where  $\Delta T$  is an input parameter in units of temperature and  $k_B$  is the Boltzmann constant. Then if we

replace  $\omega_0$  to  $\omega_i$  in Eq 4, the bias potential on a long time limit converges to:

$$P(s, t \rightarrow \infty) = -\frac{T + \Delta T}{\Delta T} F(s) + C, \quad \text{Eq. 6}$$

where  $C$  is an arbitrary constant and  $\frac{T + \Delta T}{\Delta T}$  is the bias factor in which  $T$  is the simulation temperature.

To characterize the energetic nature of the penetration mechanism of 1,4-dioxane, metadynamics simulation protocols have been carried out using PLUMED 2.3.0<sup>96,97</sup> combined with GROMACS 5.1.4<sup>75</sup>. Here, Gaussians were deposited at every 2500 ps, with a height of 0.1 kJ/mol and width of 0.5 Å. Walls for the pulling were set 37 Å from the center of the membrane bilayer. The 1000 ns simulation time was analyzed while other parameters of the metadynamics were identical to the production runs. The free energy profile of the penetration can depend on the concentration of the dioxane, therefore an additional 500 ns long metadynamics simulation had also been carried out for a system having an order of magnitude less dioxane in the simulation box (10 dioxane molecules).

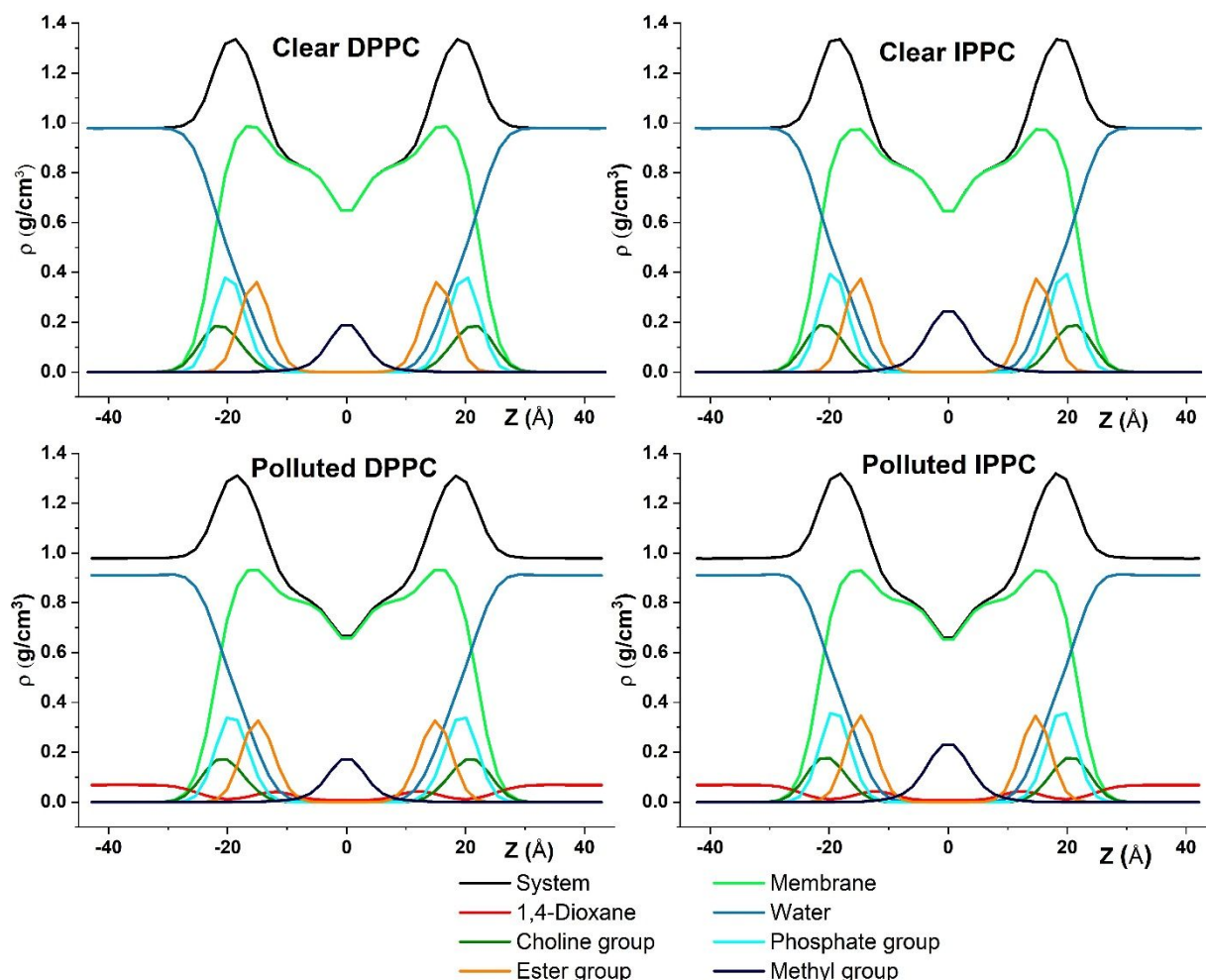
As mentioned before, metadynamics simulations can suffer from convergence issues, thus an additional 600 ns well-tempered metadynamics simulation has been run for the higher pollutant concentration, to have a guess whether the penetration process has a method dependence. During the WT-MD calculations Gaussians were deposited at every 3000 ps, with a height of 2.2 kJ/mol and width of 0.1 Å, while the bias potential was set to be 6.0. Other parameters of the WT-MD were identical to metadynamics calculations.

## Results and Discussion

### *Density profiles*

The symmetrized and centered mass density profiles along the membrane normal are shown in **Figure 3** for the four studied systems. Besides the total mass density, the spatial contributions of water molecules and 1,4-dioxane as well as that of choline, phosphate, ester and methyl groups of DPPC/IPPC are presented. The obtained electron density profiles of clear DPPC bilayer agrees reasonably well with experimental data<sup>70</sup> and the comparison can be found in the **SFigure 1 of Supplementary Information**. The clear DPPC and IPPC density profiles differ only in the density for the terminal methyl groups in the middle of the bilayer (at  $Z \approx 0$  Å) due to the branching of the isopalmitic-acid in IPPC membrane. Presence of 1,4-dioxane as a pollutant affects both membranes similarly. The mass density profile of the pollutant indicates a local maximum at the inner side of the ester groups (at  $|Z| \approx 12$  Å, which is the border of Region III and IV). Here, dioxane density ( $\rho_{\text{DIOX, ester}} = 0.042$  g/cm<sup>3</sup>) is more than half of its aqueous phase density ( $\rho_{\text{DIOX, bulk}} = 0.068$  g/cm<sup>3</sup>), while it is significantly smaller for the remaining part of the membrane. In the inner regions of the membrane a small accumulation can be observed around 5 Å distance of the penetrating 1,4-dioxane molecules radial distribution function which can be found in the **SFigure 2 of Supplementary Information**.

Furthermore, as an effect of dioxane pollution a slight growth in water density can also be observed at  $|Z| \approx 17 \text{ \AA}$  which means more water molecules can access the high density of acyl chains region (Region III) of the membranes.

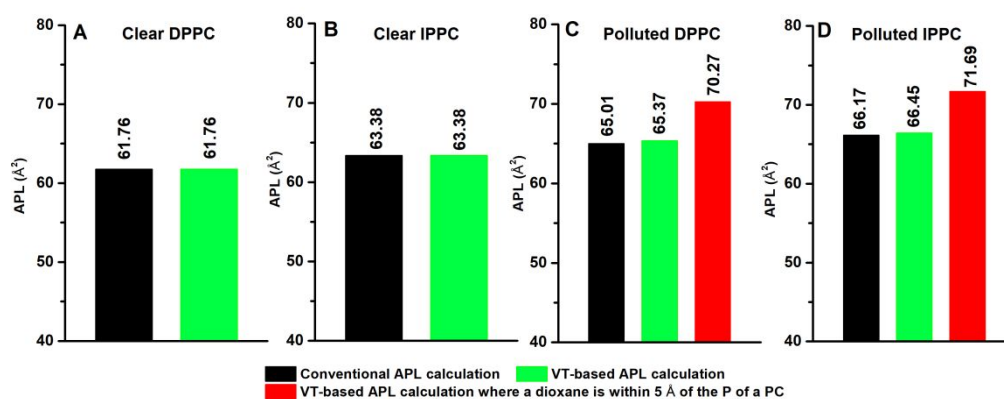


**Figure 3** Mass density profiles of the membrane systems along the membrane normal ( $Z$ ). The overall mass densities are decomposed into subsections to highlight the inner structure of the biomembranes. Black – complete system, green – DPPC/IPPC, blue – water, dark green – cholines, cyan – phosphates, orange – esters, dark green – terminal methyl groups, red – 1,4-dioxane.

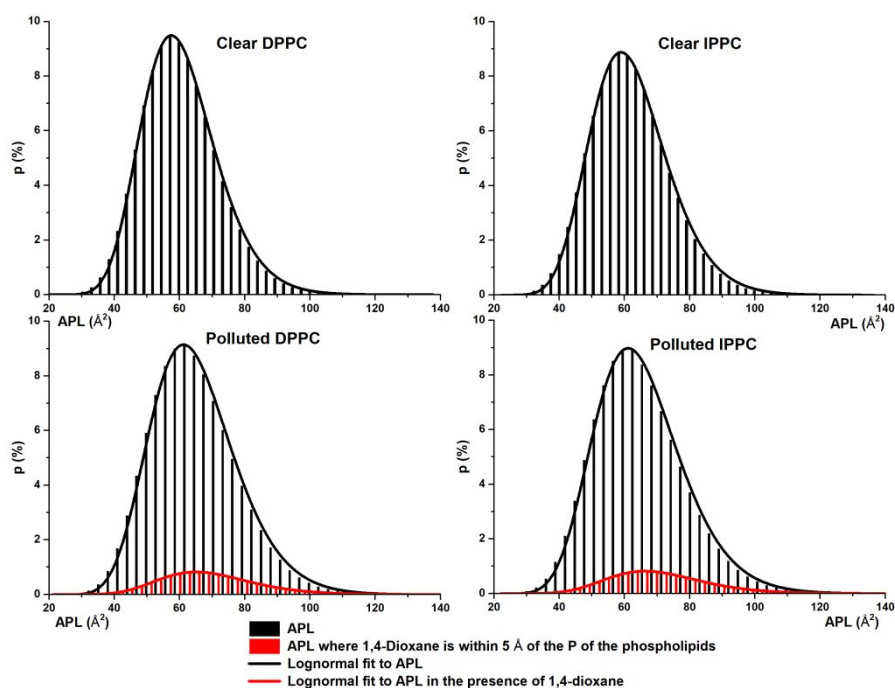
### Area per Lipid

Calculation of area per lipid (APL) is one of the most generally applied test for membrane simulations<sup>98</sup> since APL is accessible experimentally *via* NMR<sup>99</sup>, neutron diffraction<sup>100</sup> or X-ray<sup>101</sup> measurements. It is worth mentioning that literature APL values are scattered in a large range depending on its indirect measurement and state of the membrane phase<sup>98</sup>. Based on molecular simulations, the average APL can also be derived from different methods. Conventionally, it is calculated from the lateral dimensions of the simulation box divided by the number of lipids in a membrane leaflet (**Table 1**), but in this case the APL distribution remains unknown. Besides the

conventional APL calculation, the average APL can also be obtained *via* a Voronoi tessellation (VT) based technique for simple and multicomponent lipid bilayers<sup>102,103</sup> as well as complex protein-membrane<sup>104,105</sup> systems. The great advantage of VT technique is that it also provides not only APL distribution beyond the average but also local structural information of the bilayer surface<sup>105</sup>. For instance, local disturbances caused by the direct permeation of small molecules can be identified by VT-based APL distribution<sup>106,107,102</sup>. To find such local disturbances, the NpT trajectories had been analyzed by a VT-based in-house Python script (powered by Python 2.7<sup>108</sup> and MDAnalysis 0.18.1<sup>109</sup>), in which the phosphorous atom was projected to the macroscopic *XY* surface of the membrane<sup>110</sup>, while the periodic boundary conditions were also taken into account. The area belonging to each phospholipid molecule has been calculated as the area of a Voronoi polygon by using Delaunay triangulation<sup>111</sup>. The VT-based APL calculation has been carried out for all 5 runs of the individual systems to obtain statistically relevant results. The comparison of the different types of average APL calculations and the distributions of the VT-based calculations are shown in **Figure 4** and **Figure 5**, respectively.



**Figure 4** Average area per lipid (APL) calculated by conventional and Voronoi tessellation-based approaches for DPPC and IPPC membranes without (the first two diagrams) and with the presence of 1,4-dioxane (the last two diagrams). Average APL obtained from all phosphorus atoms using conventional (black) and Voronoi tessellation approach (green). APL is also calculated from all phosphorus atoms which have 1,4-dioxane molecules within its 5 Å radius (red).



**Figure 5** Probability distributions of area per lipid (APL) obtained by Voronoi tessellation approach. The total APL distribution is shown in black while the local APL distribution of phosphorus atoms which have 1,4-dioxane molecules within its 5 Å radius are in red. Lognormal distribution fits are also indicated in the diagrams.

In the case of the clear systems, the average APL values obtained from conventional and VT-based approaches match perfectly (see the first two panels of **Figure 4**) and their relative deviation from experiment is only 2% in the case of DPPC ( $APL_{\text{calc}} = 61.76 \pm 1.44 \text{ \AA}^2$ ,  $APL_{\text{exp}} = 62.9 \pm 1.3 \text{ \AA}^2$ <sup>112</sup>), basically within its uncertainty. However, no experimental data was found for IPPC membranes for which the APL value is larger by 1.6 Å<sup>2</sup> than that of DPPC membrane due to the branching lipid tail of isopalmitic-acid.

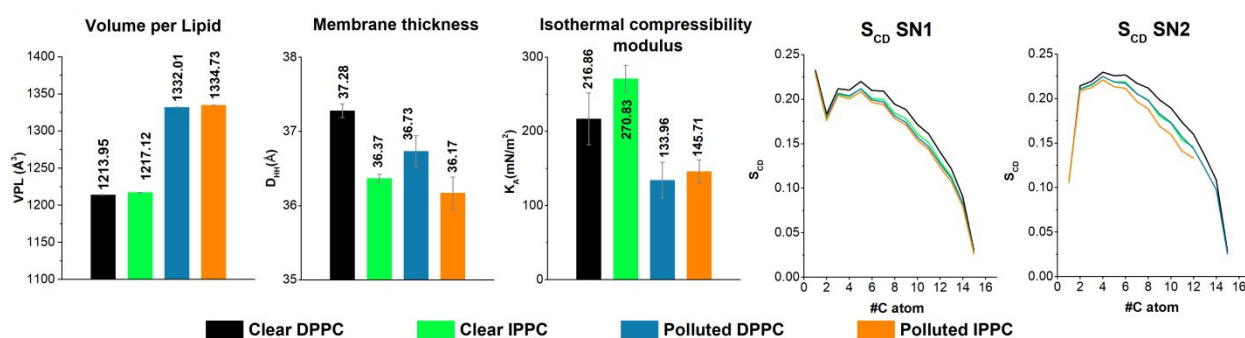
In the presence of dioxane, the average APL values increased by more than 3 Å<sup>2</sup> for both DPPC and IPPC membranes. To understand the local disturbances caused by 1,4-dioxane molecules, phosphorus atoms which are within 5 Å of dioxane molecules are selected from the VT-based APL calculation (see red columns in **Figure 4** and **Figure 5**), where the probability distributions of the total and local APL values are calculated and shown in **Figure 5**. The average of such local APL values found to be significantly larger than that of the conventional APL (more than 8% growth), namely 70.27 Å<sup>2</sup> for DPPC and 71.69 Å<sup>2</sup> for IPPC membrane which means that the lipid heads are pushed away due to the vicinity of dioxane. It is worth mentioning that the total APL distributions of clear systems are in the range of 30 and 110 Å<sup>2</sup> while in the case of polluted systems 30 and 120 Å<sup>2</sup> and have an upper tail (shown in black in **Figure 5**) which can be well described using lognormal distribution. However, assumption of normal distribution and arithmetic mean as expected value results within 2 Å<sup>2</sup> of the expected values of APL obtained from the proper lognormal fit (**Figure 5**) making a reasonable trade-off of APL widely used in the literature<sup>102,113,30</sup>.



Further analyzing the probability distribution functions of **Figure 5**, the APL value with the highest probability (APL mode) is  $57.48 \text{ \AA}^2$  and  $58.60 \text{ \AA}^2$  for the clear DPPC and IPPC systems, respectively, while they shifted towards larger APL values in the case of polluted system ( $62.31 \text{ \AA}^2$  for DPPC and  $63.24 \text{ \AA}^2$  for IPPC).

### *Volume per lipid, membrane thickness, isothermal compressibility modulus and deuterium order parameter*

Volume per lipid (VPL), membrane thickness ( $D_{HH}$ ), isothermal compressibility ( $K_A$ ) and deuterium order parameter ( $S_{CD}$ ) have also been calculated based on the equations collected in **Table 1**. Calculated membrane parameters are shown in **Figure 6** for the simulated membrane systems to further understand the effect of 1,4-dioxane pollution. To the best of our knowledge, experimental data is available only for DPPC composed membranes where the calculated results are in excellent agreement with literature values. For example, the relative deviation of VPL and  $D_{HH}$  values are less than 2% and 3%, respectively. ( $VPL_{calc} = 1214 \pm 0,23 \text{ \AA}^3$ ,  $VPL_{exp} = 1232 \text{ \AA}^3$ <sup>114</sup>,  $D_{HH,calc} = 37.2 \pm 4.5 \text{ \AA}$ ,  $D_{HH,exp} = 38.3 \text{ \AA}$ <sup>114</sup>).



**Figure 6** Volume per lipid (VPL in  $\text{\AA}^3$ ), Membrane thickness ( $D_{HH}$  in  $\text{\AA}$ ), Isothermal Compressibility ( $K_A$  in  $\text{mN/m}^2$ ) and Deuterium Order Parameter ( $S_{CD}$ ) of the investigated PC membrane - 1,4-dioxane systems. Clear DPPC and IPPC membrane parameters are indicated with black and green respectively, while polluted DPPC-s are IPPC membranes are indicated with blue and orange, respectively.

Furthermore, a good agreement can be found also for calculated  $S_{CD}$  values (for details see **SFigure 3 in Supplementary Information**), as their average relative deviations from experimental data measured in liquid-crystalline phase<sup>115</sup> are around 7%. The largest relative deviation was obtained in the case of compressibility modulus ( $K_{A,calc} = 216.9 \pm 34.8 \text{ mN/m}^2$ ,  $K_{A,exp} = 231 \pm 20 \text{ mN/m}^2$ <sup>114</sup>), which is close to 10%, but uncertainties of  $K_A$  in experiment and in calculation are still in overlap, therefore our computed results are in acceptable range.

As seen from **Figure 6**, the computed APL, VPL and compressibility modulus are significantly higher in the case of IPPC membranes than those for DPPC, while the membrane thickness of IPPC membrane is significantly lower, along with the orderliness of the lipid tails

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2 measured by  $S_{CD}$ . Since the branched lipids need larger space in the middle of the bilayer leading to  
3 larger disorder, resulting in the reduction of the relative length of such fatty acids. As the relative  
4 length of such lipids shortens, their spatial space requirement grows, increasing the APL and VPL of  
5 the IPPC composed membranes. The hereby obtained results also agree well with the previous  
6 simulations of IPPC and DPPC <sup>9</sup> (IPPC was noted as 14-MpPPC there).  
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10 The presence of the pollutant affects both membranes in a similar way, although the degree  
11 of the change is different. 1,4-dioxane pollution led to a significant rise in both APL and VPL while  
12 the thickness of the membrane, the compressibility modulus and the orderliness of the lipid chains  
13 decreased. As clear DPPC was found to be in liquid-crystalline phase and the  $S_{CD}$  values of clear  
14 IPPC membranes is slightly lower than DPPC, we can assume that it can also be found in  $L_{\alpha}$  phase.  
15 As an effect of the pollution the lipid tails can be found in an even more disordered structure, thus we  
16 can be certain that the pollution does not induce phase transition. As mentioned before in the case of  
17 mass density profiles, 1,4-dioxane penetrates inside the membrane as deep as the ester groups (Region  
18 III) and the pollutant pushes away the membrane headgroups and lipid tails from each other leading  
19 to the swelling of the membrane which was already mentioned in the case of APL diagrams (**Figure**  
20 **4** and **Figure 5**). This swelling leads to a larger available space for the lipid tails, thus turning into a  
21 more disordered state with shorter relative length, affecting the membrane thickness. As IPPCs were  
22 found in an initially more disordered state, its change in the orderliness of the lipid chains and  
23 membrane thickness is less remarkable compared to the change for DPPC.  
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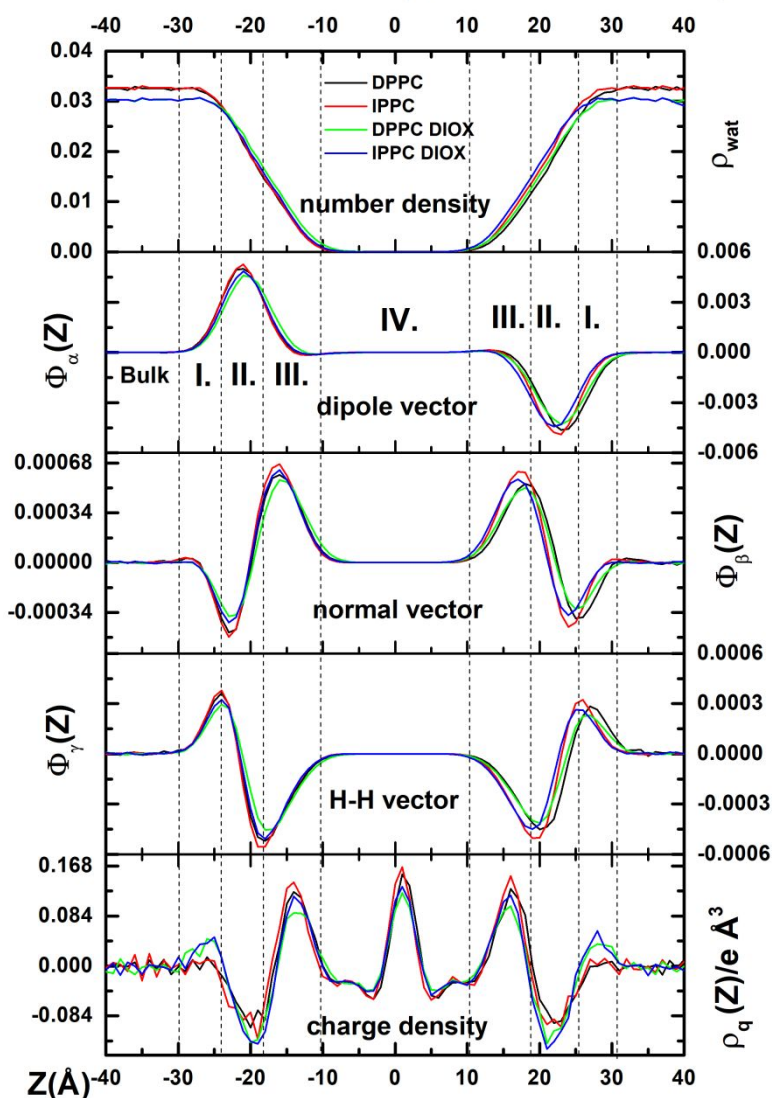
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27 The most drastic change was found in the case of compressibility modulus. While the clear  
28 IPPC membrane has significantly higher  $K_A$  compared to the clear DPPC membrane ( $K_{A,DPPC\ clear} =$   
29  $216.9 \pm 34.9$  mN/m,  $K_{A,IPPC\ clear} = 270.8 \pm 18.14$  mN/m), these values dropped for both membranes to  
30 similar value ( $K_{A,DPPC\ polluted} = 174.8 \pm 19.1$  mN/m,  $K_{A,IPPC\ polluted} = 179.7 \pm 17.0$  mN/m) due to the  
31 1,4-dioxane pollution. This is an interesting finding in the light of the definition of  $K_A$ , since  
32 compressibility modulus is a quantity for the response of the membrane area to tension, which may  
33 come from environmental stress, like perturbations, or the addition of molecules to one of the  
34 membrane leaflets <sup>116</sup>. Therefore, in this case, smaller  $K_A$  values mean smaller resistance towards  
35 environmental stress. This is in contradiction with the previous literature about branching fatty acids,  
36 that iso-branching would increase the tolerance of the cell membranes to toxic compounds <sup>10</sup> and it  
37 shows no sign of enhancing the structural stability of the membrane-like phytanyl and phitanoyl <sup>11</sup>.  
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### 55 **Water orientational profiles and the determination of the interfacial regions**

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57 Previous studies proved that based on the orientational order of the water molecules, four  
58 different regions can be separated in the membrane interface <sup>23,22,24</sup>. By means of orientational  
59 functions, charge densities and water density profiles, we have defined the bulk phase and these four  
60

different regions in the interfacial area, which are in line with the Marrink and Berendsen model<sup>27</sup>. Data will only be discussed regarding the negative side of the membrane normal  $Z$ . As the interface normal is fixed to point from the negative side to the positive, and  $\alpha$  can be in the range of  $0$  and  $180^\circ$ , opposite orientations can be observed on the two sides of the membrane in the case of dipole orientation distribution. If the molecules do not have preferential alignment, then  $\Phi_\alpha(Z)$ ,  $\Phi_\beta(Z)$  and  $\Phi_\gamma(Z)$  are fluctuating around  $0$ . If  $\Phi_\beta(Z)$  is lower than  $0$ , it indicates a more perpendicular, and if  $\Phi_\beta(Z)$  larger than  $0$ , it indicates a more parallel alignment of the normal vector with the membrane surface. For  $\Phi_\gamma(Z)$  the opposite is true, thus if  $\Phi_\gamma(Z)$  is lower than  $0$ , it indicates that the H-H vector can be found in a more parallel alignment with the membrane surface, and if its larger than  $0$  it is in a more perpendicular alignment with the membrane surface.

### Water orientational density profiles across bilayers



**Figure 7** Profiles of number density and orientational functions of water and total charge density profile across the membrane/water interfaces. The  $Z=0$  corresponds to the center of the membrane. The dashed vertical lines indicate the borders between the regions of the interface. The results for clear DPPC and IPPC membranes are shown as black and red solid lines, respectively. The results for the DPPC and IPPC membrane in the presence of 1,4-dioxane are depicted by green and blue solid lines.

The number density, the orientational function of the dipole, normal and H-H vector as well

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2 as the charge density profile of water molecules are shown in **Figure 7**. In the bulk phase, all these  
3 orientational functions are practically zero which in this case means there is no preferred direction  
4 for water molecules. Since the phosphatidylcholine headgroups contain negatively charged phosphate  
5 and positively charged tetramethylammonium groups, they determine mainly the orientation of the  
6 dipole moments of water molecules as they preferentially turn towards the negatively charged groups.  
7 Dipole, normal and H-H vectors are all sensitive to the presence of charged headgroups, therefore  
8 their orientational functions can be used for determining the border of the bulk phase (**Figure 7**) thus  
9 defining the outer border of Region I. Since the orientational function of H-H vector,  $\Phi_\gamma(Z)$ , is the  
10 most responsive to the structural changes, the borders of the Region II are defined by its stationary  
11 points, which coincidence with the inflexion points of the orientational function of the dipole vector  
12  $\Phi_\alpha(Z)$ . Finally, the border between Region III and IV can be given by the coordinate where  $\Phi_\alpha(Z)$   
13 becomes zero again.

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15 In the first interfacial region (noted as Region I), water density started to decrease slowly as  
16 seen in the top panel of **Figure 7**.  $\Phi_\beta(Z)$  almost reached a minimum here, while  $\Phi_\alpha(Z)$  showed quick  
17 growth, and  $\Phi_\gamma(Z)$  reached a maximum, which was chosen as a border of this region. These changes  
18 manifested as the plane of water molecules are more likely to be found in a perpendicular orientation  
19 relative to the bilayer. The increasement of  $\Phi_\alpha(Z)$  indicates that the dipole moments of water  
20 molecules start to point towards the phosphate groups. For the polluted systems, Region I is more  
21 positively charged while the clear system showed no charge accumulation here.

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23 In the second region,  $\Phi_\alpha(Z)$  reached a maximum, the orientational function of the normal  
24 vector linearly increased while  $\Phi_\gamma(Z)$  decreased as getting closer to the middle of the membrane. This  
25 region is negatively charged for each system while this effect is more pronounced for the polluted  
26 systems resulting a more polarized outer layer for these membranes. Here, the dipole moment vectors  
27 enclose around  $\sim 0^\circ$  with the surface normal, which means that the dipoles are pointing towards the  
28 membrane surface.

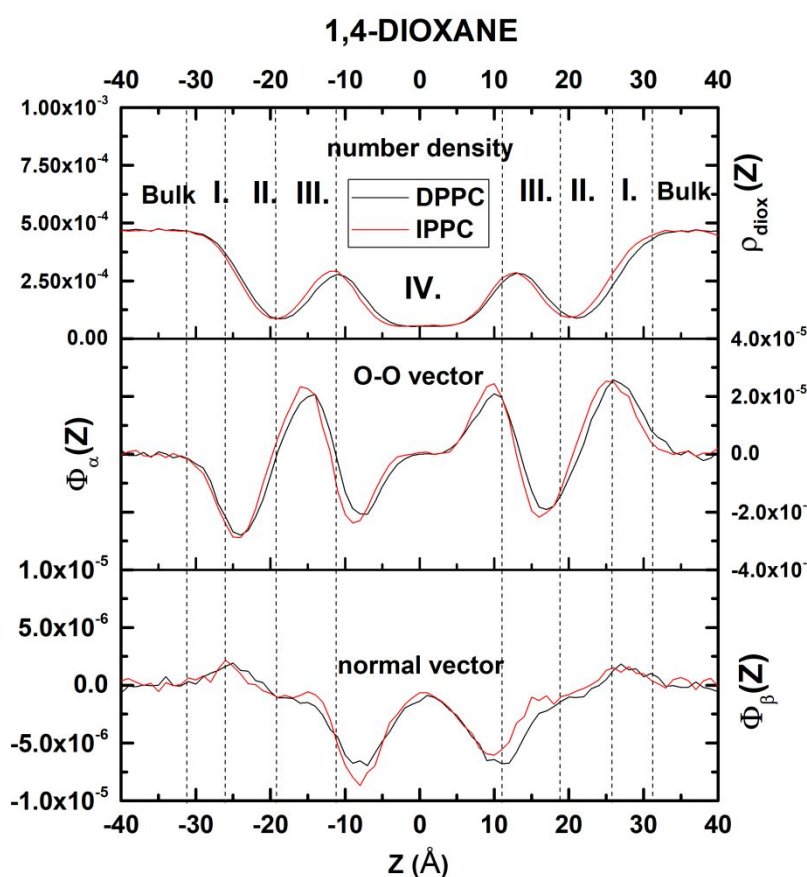
29  
30 In the third region, water density vanished slowly and the decrease of  $\Phi_\alpha(Z)$ , as well as the  
31 increase of  $\Phi_\gamma(Z)$  to zero, indicate that water molecules are slowly losing their orientational preference.  
32 Before the vanish of the orientational preference occurring at the inner part of Region III, the presence  
33 of the peak of  $\Phi_\beta(Z)$  indicates that water molecules are taking a more planar alignment due to the  
34 presence of the ester groups.

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36 In the central region (Region IV) no preferred orientations for water molecules can be seen  
37 based on the orientational profiles most probably due to the low statistical sampling of water  
38 molecules.

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*1,4-dioxane orientational profiles along the membrane normal*

Similar to water, the orientational preference of 1,4-dioxane had been quantitatively described by orientational functions of the O-O ( $\Phi_\alpha(Z)$ ) and normal vector ( $\Phi_\beta(Z)$ ) as a function of the distance from the center of mass of the membrane (**Figure 8**). When  $\Phi_\beta$  is lower than 0, it indicates perpendicular, if larger, parallel alignment of the normal vector of the pollutant molecule with the membrane surface. DPPC and IPPC membranes have almost identical features for the number density profile of dioxane, as well as for the orientational profiles of the O-O and normal vectors. Orientational function of the O-O vector is centrosymmetric having minima at the border of Region I and II and at about  $Z = -5 \text{ \AA}$  (in Region IV) as well as maximum at  $Z = -15 \text{ \AA}$  (in Region III), while the orientational function of the normal vector is symmetric with a minimum at around  $|Z|=8 \text{ \AA}$ .

A structural manifestation of these profiles is that random orientations are preferred in the bulk phase, then the molecular planes are in a planar position with the bilayer surface in the first two interfacial regions. The turning of the O-O vector throughout Region III and IV indicates, that while 1,4-dioxanes approach the membrane, the molecules turn around their  $y$  axis, then turn back into a planar position. This turning can also be observed from the direct examination of the trajectories. In the inner side of the ester groups of the bilayer, 1,4-dioxanes can be found more likely in a parallel position with the membrane interface. Between the two types of membranes, no significant differences can be inspected.



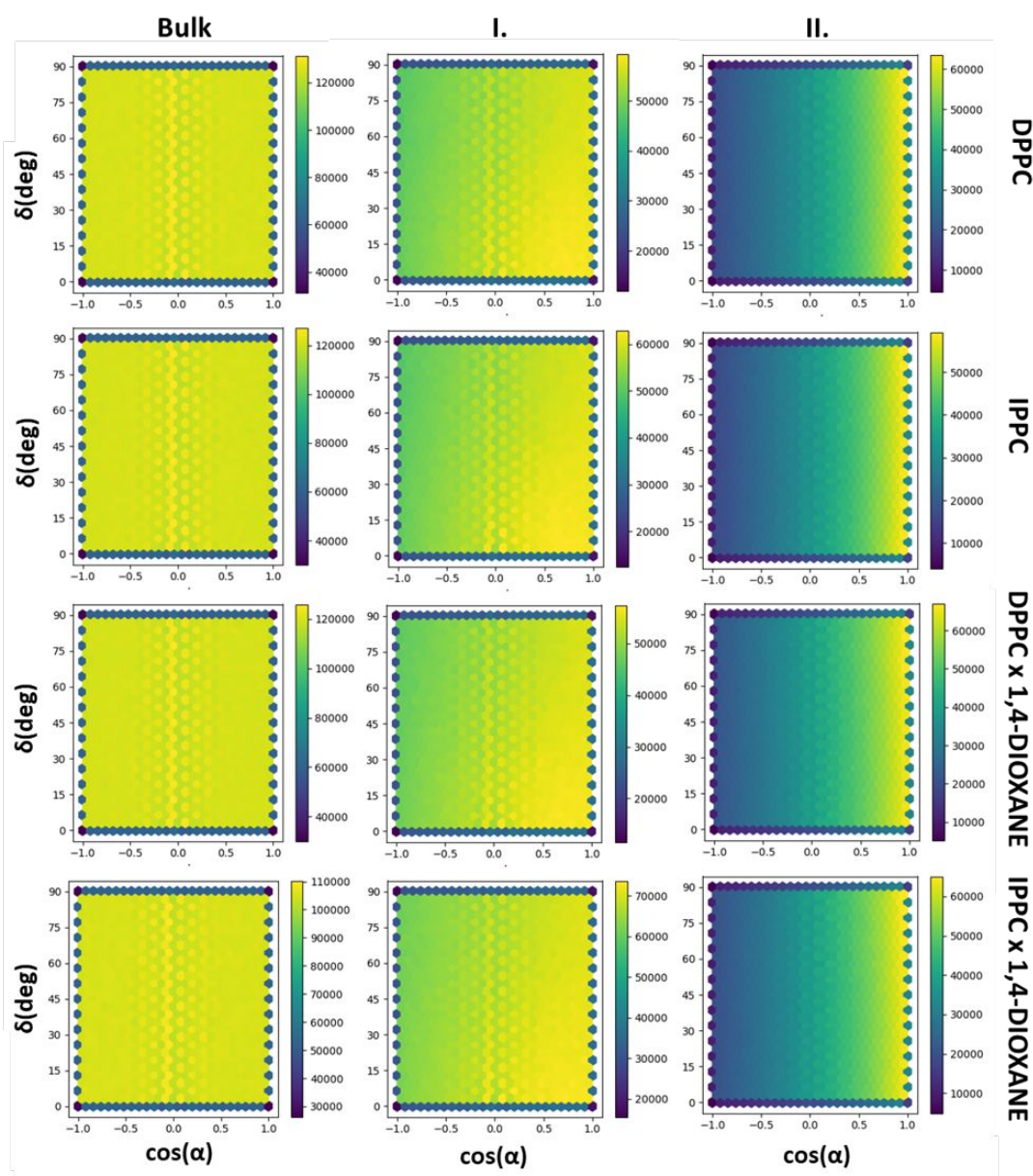
**Figure 8** Orientational profiles of dioxane molecules across the membrane/water interfaces. The density of dioxane

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2 molecules is shown as a reference. The dashed vertical lines indicate the borders between the different regions.  
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5 *Orientalional maps of water and 1,4-dioxane molecules in the membrane interfacial regions*  
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7 The two independent vectors ( $\alpha$  and  $\delta$ ) of water and 1,4-dioxane molecules can be represented  
8 as maps which gives us a clearer view of their orientational preference in the different regions of the  
9 membrane/water interface. In the bulk phase and the first two regions of the water orientation maps  
10 significant differences cannot be observed between the two membranes (**Figure 9**), as expected from  
11 the presence of similar headgroups. In the bulk phase, no preferred orientation can be found. In the  
12 first region a slight orientational preference can be seen as the water molecules are more likely to be  
13 found in the  $0 \leq \cos(\alpha) \leq 1$  region, while  $\delta$  has no effect on the orientations, which means that the  
14 dipole moment vectors are starting to point towards the negatively charged headgroups of lipids,  
15 while the orientation of the plane of water molecules has no preference. This reference is more  
16 pronounced in the second region, but here the dipole orientations ( $0.5 \leq \cos(\alpha) \leq 1$ ) obviously show  
17 that the water molecules are in a perpendicular position with the membrane surface still without any  
18  $\delta$  preference.  
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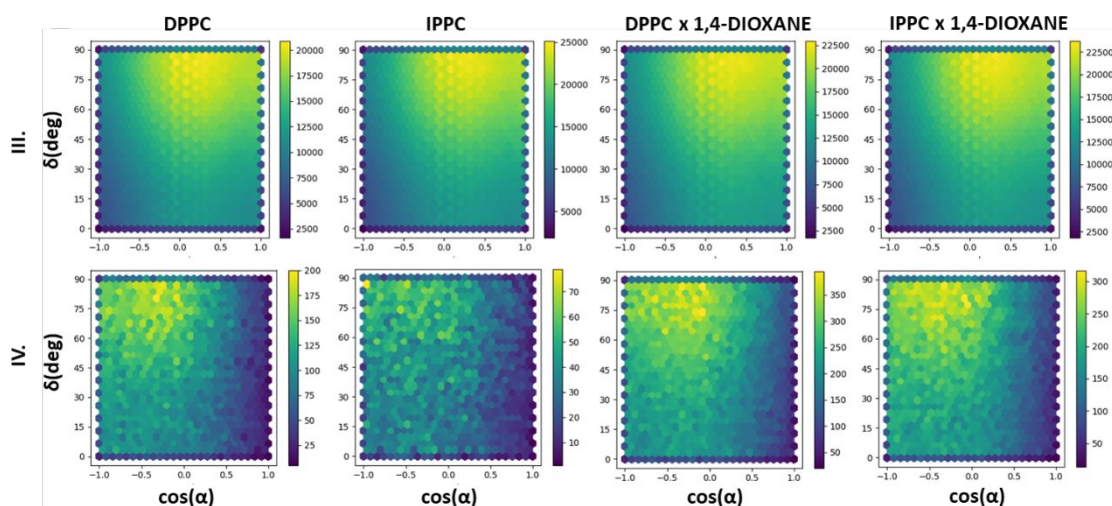


**Figure 9** Orientational distribution maps of water molecules in the first three regions (Bulk and Region I and II) of the DPPC and IPPC membrane/water interfaces. While  $\cos(\alpha)$  can be varied from -1 to 1,  $\delta$  is from  $0^\circ$  to  $90^\circ$  due to the symmetry of the water molecule. The color bars indicate the number of water molecules appearing in each region over 500 ns total simulation.

Angular distributions of water molecules found in the interior regions (Region III and IV) of DPPC and IPPC membranes are shown in **Figure 10**. In the third membrane region, the orientational peak is at the same place for both membrane types where the likeliness of perpendicular orientation of the water plane ( $60^\circ \leq \delta \leq 90^\circ$ ) is the highest, while the dipole moment vector is parallel with the bilayer surface ( $-0.5 \leq \cos(\alpha) \leq 0.5$ ). At the same time, more water molecules can be found in this region in the case of clear IPPC membranes compared to clear DPPC membranes.

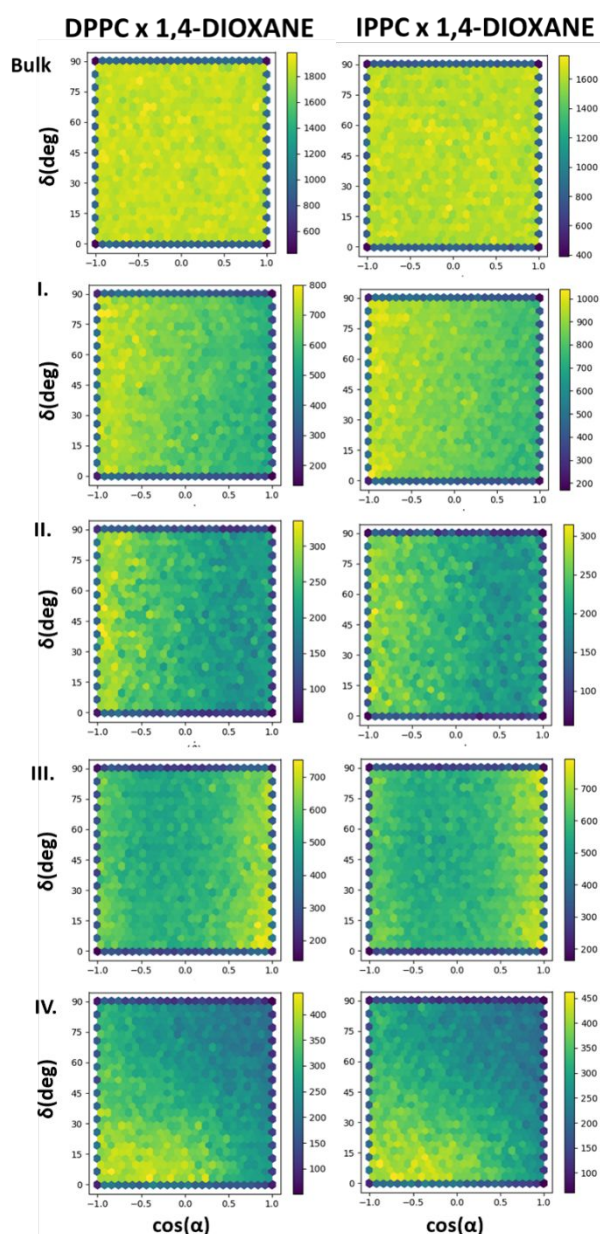
In the central region (Region IV) a distinct peak can be observed in the orientation of water molecules at  $60^\circ \leq \delta \leq 90^\circ$  and  $0.0 \leq \cos(\alpha) \leq -1$ , which indicates a quite similar orientation as in the

fourth region, but the dipole vector turns around as the molecules approach the middle of the bilayer. At the same time the amount of water molecules is only third as much in this region in the case of branching fatty acids compared to the DPPC membranes in clean system. As an effect of 1,4-dioxane pollution, this difference disappears: almost the same amount of water molecules appear both in the third region and in the middle of the bilayer of both membranes.



**Figure 10** Orientational distribution maps of water molecules in the inner membrane regions (Region III and IV) of the DPPC and IPPC membrane/water interfaces. While  $\cos(\alpha)$  can be varied from -1 to 1,  $\delta$  is from  $0^\circ$  to  $90^\circ$  due to the symmetry of the water molecules. The color bars indicate the number of water molecules appearing in each region over 500 ns total simulation.





**Figure 11** Orientational distribution maps of 1,4-dioxane molecules in the different region of the DPPC and IPPC membrane/water interfaces. While  $\cos(\alpha)$  can be varied from -1 to 1,  $\delta$  is from  $0^\circ$  to  $90^\circ$  due to the symmetry of the dioxane molecule. The color bars indicate the number of dioxane molecules appearing in each region over 500 ns total simulation.

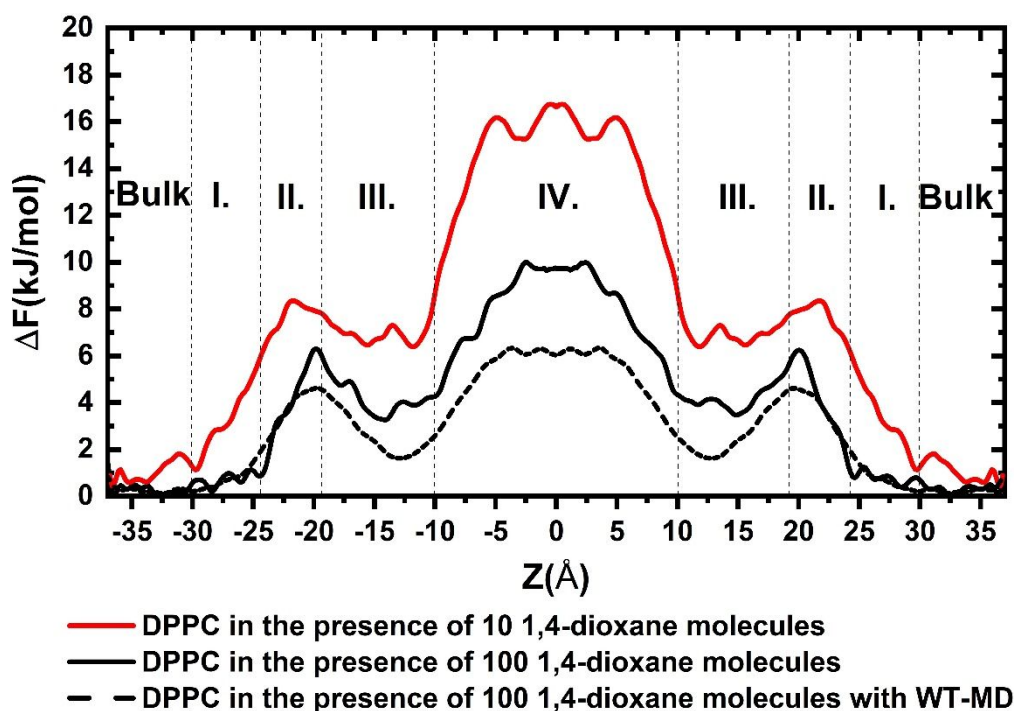
As **Figure 11** shows, orientational maps of 1,4-dioxane molecules have very similar features for DPPC and IPPC membranes in each membrane/water region. As expected, dioxane molecules have random orientations in the bulk phase. In Region I and II, as it was also perceived from the orientational function profiles (see the middle panel of **Figure 8**), the O-O vectors of the pollutant molecules are parallel with the bilayer surface, but have no preference according to  $\delta$ . Similar is observed in Region III, but after the spin of the dioxane around its axis  $y$ .

In the central region (Region IV), the preferred orientation of dioxane molecules is within  $0^\circ \leq \delta \leq 30^\circ$  and  $0 \leq \cos(\alpha) \leq -1.0$ , which means that the O-O vector is enclosing  $90^\circ$ - $45^\circ$  with the bilayer normal, while its normal vector is pointing towards the bilayer inner surface, which means a close to

planar position of the pollutant molecule with the bilayer surface.

### Free energy profile of the dioxane penetration through the membrane

Since DPPC and IPPC composed biomembrane models are changed similarly in the presence of 1,4-dioxane, metadynamics simulation of the dioxane penetration was carried out only in the case of DPPC composed membranes. The obtained free energy profiles along axis Z are shown in **Figure 12**.



**Figure 12** Free energy profile of 1,4-dioxane penetration into a DPPC bilayer. The black curve represents the results of the metadynamics simulations for the system having 100 1,4-dioxane molecules in the simulation (the average dioxane concentration is 75.102 g/dm<sup>3</sup>), while red is for 7.510 g/dm<sup>3</sup> dioxane concentration). The dashed black curve indicates the results of the WT-MD simulation carried out for the system having 100 1,4-dioxane molecules. The estimated error of  $\Delta F$  is 4 kJ/mol.

The lowest free energy corresponds to the bulk water region ( $|Z| > 30 \text{ \AA}$ ) which is therefore the preferred state for 1,4-dioxane molecules and it has been chosen as the reference line. As 1,4-dioxane approaches the bilayer surface  $\Delta F$  starts to grow sharply until it reaches the head groups in Region II where the free energy profile reaches a local maximum. The starting point of the growth of free energy appears to be closer to the bilayer center for the more concentrated system by 5  $\text{\AA}$  while the relative free energy values appear to be somewhat higher for the diluted system ( $\Delta F_{diluted}$ ) than that of the concentrated one ( $\Delta F_{conc}$ ). This means that the penetrating dioxane molecules make the membrane more accessible for additional pollutant molecules. The height and the position of the local free energy barrier at the sight of the head groups shows only a weak concentration dependence:  $\Delta F_{diluted}=8.3 \text{ kJ/mol}$  at  $|Z| = 21.8 \text{ \AA}$  and  $\Delta F_{conc}=6.5 \text{ kJ/mol}$  at  $|Z| = 19.7 \text{ \AA}$  for the concentrated system. As dioxane penetrates inside the membrane (Region III) the free energy profile reaches a metastable

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2 state where  $\Delta F_{conc}=6.4$  kJ/mol and  $\Delta F_{diluted}=3.3$  kJ/mol, which corresponds to the site of the ester  
3 groups ( $|Z| = 16-11$  Å). The local maximum in dioxane density - which was experienced previously  
4 in the case of mass density profiles (bottom of **Figure 3**) - is consistent with this free energy basin.  
5 As 1,4-dioxane approaches the bilayer center (Region IV) free energy starts to grow steeply again  
6 until it reaches the global free energy maximum in the bilayer center where  $\Delta F_{conc}=16.7$  kJ/mol and  
7  $\Delta F_{diluted}=10.4$  kJ/mol.  
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10 To test the results of the metadynamics simulations an additional WT-MD simulation have  
11 been carried out for the higher dioxane concentration as seen in **Figure 12** (shown as dashed line).  
12 Since WT-MD profile agrees with that of the previous metadynamics simulation (shown as solid  
13 black line in **Figure 12**) within the chemical accuracy, the barrier height of the penetration at the  
14 bilayer center appears at 6 kJ/mol.  
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17 Molecules with both hydrophilic and hydrophobic components demonstrate a sinusoidal  
18 pattern where the free energy decreases near the interface (e.g. Regions II and III) and increases at  
19 the center of the membrane bilayer. This pattern is commonly witnessed among biomolecules such  
20 as methanol<sup>117,118</sup> or acetone<sup>119</sup>, which is similar to the obtained results for 1,4-dioxane.  
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23 Due to the relatively small height of the free energy barrier, DPPC membranes can be  
24 permeable for 1,4-dioxane which effect can be further enhanced by increasing dioxane concentration.  
25 This allows us to speculate that the presence of dioxane might also make the membrane more  
26 permeable for additional molecules, similar to the effects of alcohol<sup>120</sup>.  
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## 38 **Conclusion**

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40 The effect of 1,4-dioxane on biomembrane models composed of DPPC - and its branched  
41 isomer - IPPC was thoroughly analyzed by the means of MD simulations. We have been focusing on  
42 structural changes induced by the pollutant – inside the membrane and also in its vicinity by creating  
43 orientational maps of water and 1,4-dioxane molecules - as well as energetics of its penetration.  
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46 We have thoroughly analyzed the effects of 1,4-dioxane on the membrane structural  
47 parameters, such as mass density distribution, APL, VPL,  $S_{CD}$ ,  $D_{HH}$ ,  $K_A$ . In pollutant free environment,  
48 as an effect of the branching found in one of the IPPC acyl chains, IPPC composed membranes have  
49 significantly higher APL, VPL and  $K_A$  values compared to those of DPPC, while its  $D_{HH}$  and  $S_{CD}$   
50 values are significantly lower. To the presence of 1,4-dioxane both membranes respond the same  
51 way. As observable from the mass density profiles, 1,4-dioxane can penetrate inside the membranes,  
52 where it appears at more than half of its bulk phase density at the inner site of the ester groups of the  
53 fatty acid chains. As an effect the pollutant pushes away the phospholipids from each other, leading  
54 to the swelling of the membrane. This phenomena can also be observed from the growth of local APL  
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2 in the vicinity of 1,4-dioxane. This created disturbance in the distribution of phospholipids leads to  
3 an overall lateral growth of the biomembranes, a more disordered state of the lipid tails and thinning  
4 of the bilayer. This overall change reduces the stress resistance of the biomembrane ( $K_A$ ), which may  
5 initiate a serious cell-damaging process induced by 1,4-dioxane pollution by increasing its  
6 permeability.  
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10 Four regions of the membrane/water interface were distinguished by the orientational  
11 functions based on the orientational preference of the local vectors (dipole, H-H and normal vector)  
12 bound to the water molecules, which are equivalent to the membrane regions proposed by Marrink  
13 and Berendsen. In the interfacial regions (Region I, II, III and IV) starting from about 30 Å from the  
14 center of the bilayer, not only water but also dioxane molecules can be found in highly ordered states  
15 which have been analyzed *via* orientational maps. From these maps it can also be understood clearly  
16 that dioxane is hydrated throughout its dissolution, as water density also increased in the membrane  
17 interior. The free energy of 1,4-dioxane penetration into DPPC membranes at two different  
18 concentrations (75.102 g/dm<sup>3</sup> and 7.5102 g/dm<sup>3</sup>) was obtained using metadynamics parallel with  
19 well-tempered metadynamics to understand the method dependence of the profile. The results of the  
20 two methods are found to be consistent with each other.  
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30 By comparing the results of the orientational maps and the free energy calculations the  
31 penetration mechanism of 1,4-dioxane into DPPC composed biomembranes can be described in detail.  
32 The pollutant molecule can be found with the lowest free energy in the bulk phase which is parallel  
33 with the mass density profiles obtained from classical molecular dynamics simulations, where the  
34 highest concentration of dioxane was found to be at this region. In Region I the plane of dioxane is  
35 preferred to be parallel to the membrane surface, and at the same time a growth in free energy is  
36 observed. In Region II the pollutant molecule twists around its normal vector, the free energy of the  
37 mechanism reaches a peak, and dioxane density drops down. In Region III 1,4-dioxane is found to be  
38 parallel with the bilayer surface. Meanwhile, its density increases, and the free energy gets lower,  
39 indicating, that this is a metastate for the penetration mechanism. Here, dioxane can establish weak  
40 induced dipole-dipole interactions with the ester groups, pushing away the phospholipid molecules  
41 from each other. As the pollutant reaches Region IV, it turns around its normal vector, so that the  
42 O-O vector is pointing towards the bilayer surface. In the center free energy reaches a global  
43 maximum, while density of 1,4-dioxane reaches a global minimum. The obtained free energy profiles  
44 of the penetration shows concentration dependence. The relative free energy value is found to be  
45 distinctly higher for the diluted system than that of the concentrated one which means that 1,4-dioxane  
46 makes the membrane more accessible for additional pollutant molecules.  
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### 19 **Supporting Information Available**

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21 Included is a diagram of the comparison of calculate and experimental electron density profile of  
22 DPPC membranes, the radial distribution function of the penetrated 1,4-dioxane molecules and the  
23 comparison of the calculated and experimental  $S_{CD}$  profiles of DPPC membranes.  
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