Molecular orientation at biological interfaces: Water and lipids studied through surface-specific vibrational spectroscopy
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4 Nuclear Quantum Effects at the Water-Air Interface

We start the experimental part of this thesis by looking at the fundamental physics of the bare air/water interface. Using combined theoretical and experimental approaches, we demonstrate that the bond orientation of water at the water-vapor interface depends markedly on the water isotope (H/D) composition. While the interfacial water structures of H$_2$O and D$_2$O are indistinguishable, the intramolecular symmetry breaking in HDO is directly reflected at the surface: the OD bonds preferably orient down towards the bulk water, whereas the OH bond tends to orient up into the vapor phase. Path integral molecular dynamics simulations show good agreement with surface-specific sum-frequency generation (SFG) spectroscopy results, revealing that the distinct interfacial bond orientations originate from nuclear quantum effects. The enhanced localization of the heavier D atom leads to stronger hydrogen bonds, giving rise to OD bonds pointing down into the bulk.

4.1 Introduction

Water in condensed phases is unique in its strong, anisotropic intermolecular interactions. These interactions occur through hydrogen bonds (HBs) between a H atom on one molecule and an O atom of another, and collectively lead to a structurally rich network. Nuclear quantum effects cause a non-negligible zero point energy (ZPE) for low mass nuclei, specifically for H atoms. The high density of H atoms in water makes nuclear quantum effects significant [71–75]. These effects show up when H atoms are replaced by heavier D atoms, giving rise to different macroscopic properties of H$_2$O and D$_2$O in a non-intuitive fashion: D$_2$O melts at a temperature 3.8 K higher than H$_2$O; the temperature of the maximum density increased by 7.2 K in D$_2$O compared with H$_2$O; protein stability is increased in D$_2$O [74]; D$_2$O ice is expanded relative to H$_2$O [75]. Isotopic substitution of water has provided an indispensable tool for studying nuclear quantum effects on water properties, structure and dynamics.

While significant understanding has been reached regarding the different HB interactions due to the isotope effects in bulk water [71–76], these effects have not been fully examined at aqueous interfaces. Aqueous interfaces require the interruption of the HB network, exhibiting specific anisotropic properties. At the water-vapor interface, this results in some interfacial water molecules having
free’ non-H-bonded OH groups sticking into the vapor phase. The water-vapor interface is of intrinsic interest owing to its ubiquity in nature, and also serves as a paradigmatic model system for extended hydrophobic surfaces. In addition to these fundamental interests, nuclear quantum effects at aqueous interfaces are relevant for interface-specific vibrational spectroscopy studies such as sum-frequency generation (SFG), where isotopic dilution has been routinely used to avoid intra- and intermolecular couplings of interfacial water [23, 24, 77–80]. Knowledge of the isotope effect is essential to verify the implicit assumption that interfacial water structures for the mixture of H$_2$O, D$_2$O, and HDO are identical.

In this chapter, we explore the nuclear quantum effects on the water structure near the vapor interface. We perform path integral molecular dynamics (PIMD) simulations to include the nuclear quantum effects explicitly [81]. Our simulation shows that the broken symmetry of the HB interactions in HDO gives rise to distinct OH and OD bond orientations at the vapor interface. We confirm distinct bond orientations by measuring SFG spectra experimentally and comparing them with the PIMD simulations.

### 4.2 Theory

PIMD simulations were performed with 80 water molecules by using the CP2K package program. To simplify the discussion, we used pure H$_2$O, D$_2$O, and HDO instead of their mixture. The simulation cell was set to 13.2 for the $x$ and $y$ axes and 70 for the $z$ axis, where the $xy$ plane was parallel to the interface and the $z$ axis was parallel to the surface normal. Periodic boundary conditions were used. First, we ran classical MD simulations to sample 30 configurations for the HDO-vapor interface at 285, 300, and 315 K and 15 configurations for the H$_2$O-vapor and D$_2$O-vapor interfaces at 300 K. Then, the PIMD simulations based on generalized Langevin equations (GLE) [82] were performed at each temperature, where the independent Gaussian number $N_s+1=5$, the minimum frequency of thermostating $\omega_{\text{min}}=1$ cm$^{-1}$, and the maximum frequency $\omega_{\text{max}}=10000$ cm$^{-1}$. The number of beads for the PIMD simulation, $P$, was set to 24. $P = 24$ is sufficient for reproducing the quantum distribution function and energy reasonably [76]. The time step was set to 0.1fs. 100ps PIMD runs were conducted to equilibrate the systems. Sequentially, we ran over 200ps PIMD simulations to sample water configurations, which were used for calculating the bond orientations, the HB number, and the SFG spectra.

The possible anisotropy of water at interfaces can be characterized by the OH (OD) bond orientation [83], which is readily qualified by the axial profile of the angle $\theta$ between the OH (OD) bond and the surface normal. Note that two angles $\theta$ for each H$_2$O (D$_2$O) molecule were calculated. The origin point of the $z$-coordinate was set to the center of mass for the system. The Gibbs dividing surface is located at $|z|=6.8$ (see supporting information). Figure 4.2 depicts the average angle $\langle \cos \theta \rangle$ for H$_2$O, D$_2$O, and HDO. The H$_2$O and D$_2$O...
4.2 Nuclear Quantum Effects at the Water-Air Interface

Figure 4.1. (Left) Pictorial representation of the angle $\theta$ between the OH (OD) bond and the surface normal. (Right) Axial distribution of average angles $\langle \cos \theta \rangle$. Lines are guides for the eye. The water molecules illustrate the average orientations of HDO (D dark grey, H light grey) in the different regions. The positive and negative $z$-axis points up to the vapor region and to the bulk water region, respectively.

Curves overlap, indicating that the OH bond orientation of H$_2$O is similar to the OD orientation of D$_2$O. On average both the OH and OD bonds orient to the vapor phase for $|z|>6.7$ and orient to the bulk for $3.0 < |z| < 6.7$, while specific orientation vanishes for $|z|<3.0$. While H$_2$O and D$_2$O give indiscernible results, the OH bond orientation of HDO is markedly different from the OD bond. In the bulk region ($|z|<2.0$), the OH and OD bonds show no specific orientations. Approaching the interface, the OH bond shows slightly stronger orientation toward the bulk than the OD bond for $2.0 < |z| < 4.2$, while the opposite tendency can be found for $4.2 < |z| < 6.1$. In $6.1 < |z| < 7.4$, the OH and OD bonds orient to the vapor and the bulk, respectively. Both the OH and OD bonds orient to the vapor region in $|z|>7.4$, but $\cos \theta$ is larger for the OH bond than the OD bond. This distinct orientation arises from nuclear quantum effects, as evidenced by its disappearance as reducing the number of beads for the PIMD simulation, $P$, and by its temperature dependence. At 285 K the distinct OH and OD bond orientations were enhanced while they decreased at 315K (see supporting information), which is consistent with the enhanced nuclear quantum effect at lower temperature. The symmetry is broken due to the nuclear quantum effects, so that the OH and OD bonds tend to orient toward the vapor and the bulk regions, respectively.

To elucidate the intermolecular interactions underlying the asymmetric bond orientation of HDO, we calculated the numbers of OH...O and OD...O HBs. The presence of the HB was defined as $1.59 < r_{H...O} < 2.27$ and $\alpha<40^\circ$, where $r_{H...O}$ denotes the intermolecular OH (OD) distance and $\alpha$ the angle between intra- and intermolecular OH (OD) bonds (see the sketch in the left side of
4.2

Figure 4.2. (Left) Pictorial representation of the distance $r_{H:::O}$ and the angle $\alpha$ for the HB definition. (Right) Depth profiles of the HB numbers for the OH O $\langle n_H \rangle$ and OD...O $\langle n_D \rangle$ at the HDO/vapor interface. The free energy loss $-T\Delta S/k_b$ originated from the entropy decrease due to the distinct OH and OD bond orientations are plotted in the inset. The lines are guide for the eyes.

Figure 4.2) [84]. Figure 4.2 displays the axial profiles of the HB numbers for OH...O $\langle n_H \rangle$ and for OD...O $\langle n_D \rangle$. In the bulk region $(|z|<2.0)$, $\langle n_D \rangle$ is 2% larger than $\langle n_H \rangle$, which is consistent with a more diffusive OH...O angle compared to the OD...O angle in bulk water [73]. Moving from the bulk to the vapor region, the difference between $\langle n_D \rangle$ and $\langle n_H \rangle$ increases, indicating that increased OD...O HBs stabilizes the interfacial water structure at the cost of the concomitant entropy decrease. More OD...O HBs than OH...O HBs make the enrichment of the free OH bonds pointing toward the vapor phase. Our finding can be connected with the previous study of isotopic fractionation of H$_2$O-HDO water [85]. By decomposing the kinetic energy into the components arising from the OH bond vector and two vectors orthogonal to the OH bond, Markland and Berne have showed that the components arising from the vectors orthogonal to the OH bond stabilizes the D excess in liquid compared to gas. This indicates that the free OH bond and hydrogen-bonded OD bond is favored compared with the free OD bond and hydrogen-bonded OH bond, which is consistent with our observation. This stronger ‘H-bond’ of a D atom arises from the increased moment of inertia of D$_2$O molecule: the librational mode does not respond as fast to changes in the environment as that of H$_2$O. This entails that it is more stable and has less rotational uncertainty, making it less prone to quantum tunneling effects that quickly make and break bonds [86,87].

The entropic cost of creating this asymmetry should be balanced by the OH...O and OD...O HB energy difference. This HB energy difference results in a melting point difference of 3.8 K between bulk H$_2$O and D$_2$O. In the following we will demonstrate that an energy difference corresponding to a few K originating from the nuclear quantum effects is enough to generate the distinct OH and OD bond orientations at the HDO-vapor interface. Our calculation
methodology is as follows: The entropy would be maximal when the OH and OD bond orientations are the same. The entropy for this hypothetical case is denoted by $S_1$. On the other hand, our PIMD simulations show different preferred orientations of the OH and OD bonds. The entropy of this case is called $S_2$. The entropy decrease due to the distinct bond orientations can then be calculated as $\Delta S = S_2 - S_1$. $S_1$ and $S_2$ were calculated from the histograms of the OH and OD bond orientations (see supporting information). The axial profile of $-T\Delta S/k_b$ for one HDO molecule is plotted in the inset of figure 4.2, indicating that $-T\Delta S/k_b$ increases with approaching the vapor region and reaches a maximum value of a few K, i.e. the same magnitude as the melting point difference between H$_2$O and D$_2$O. This shows that energetic gain and entropic costs for the preferred bond orientations are balanced.

### 4.3 Experiment

![Figure 4.3](image)

Figure 4.3. (a, b) Experimentally measured SFG spectra $\left|\chi_{ssp}^{(2)}(\omega)\right|^2$ (red and green) and their fitted curves (blue), (c, d) $\text{Im}\left(\chi_{ssp}^{(2)}(\omega)\right)$ constituted by the fitted parameters, and (e, f) simulated $\text{Im}\left(\chi_{ssp}^{(2)}(\omega)\right)$. Top panels (a), (c), (e) and down panels (b), (d), (f) display the spectra in the OD and OH stretching regions, respectively. Filled areas in (c), (d), (e), and (f) represent the free OH or OD bond peak areas (see main text).

Our PIMD simulations predict that more free OH bonds than free OD bonds are present at the isotopically diluted water-vapor interface due to nuclear quantum effects. We used vibrational SFG spectroscopy to establish this enrichment of free OH bonds experimentally. As SFG is a second-order process, it is forbidden in bulk media and therefore provides an excellent tool to probe the surface
composition \cite{25}. In our study, SFG spectra at the water-vapor interface were recorded for a range of different isotopic dilutions, two of which are plotted in figure 4.3 (a) and (b), under ssp polarization (SFG and visible s polarized, infrared p polarized). The free OH (OD) stretch vibration shows up in the SFG spectra of the water-vapor interface as a narrow resonant peak at $\sim 3700 \text{ cm}^{-1}$ \cite{23, 25, 77–80}. To probe the OH bond enrichment at the vapor interface, the SFG spectra for the OH and OD stretch regions were measured as a function of isotopic dilution. To decompose the SFG intensity to the free OH (OD), hydrogen bonded OH (OD), and non-resonant contributions, we fit the SFG intensities by using

$$I_{\text{ssp}}(\omega) \propto \left| \chi^{(2)}_{\text{ssp}}(\omega) \right|^2 = A_{NR} e^{i\varphi_{NR}} + \sum_{i} \frac{A_i}{\omega - \omega_i + i\Gamma_i}^2,$$  \hspace{1cm} (4.1)

where $A_{NR}$ is the non-resonant amplitude, $\varphi_{NR}$ the non-resonant phase, and $A_i$, $\omega_i$, and $\Gamma_i$ the peak intensity, center frequency, and width of the $i$th Lorentzian, respectively. Details of the fitting procedure can be found in the supporting information. The fitted curves in figure 4.3 (a) and (b) accurately reproduce the SFG spectral shapes. $\text{Im}(\chi^{(2)}_{\text{ssp}}(\omega))$ constructed from these fitting parameters are shown in figure 4.3 (c) and (d). Since $\text{Im}(\chi^{(2)}_{\text{ssp}}(\omega))$ is directly related to the spectral shape of the resonances, we used it to calculate the free OH (OD) peak area $A_{OH}(x)$ ($A_{OD}(x)$) for the concentration $x$ of H$_2$O (D$_2$O), which was defined as the peak area of the fitted $\text{Im}(\chi^{(2)}_{\text{ssp}}(\omega))$ above a baseline defined for the OH (OD) stretch region at 3770 (2800) cm$^{-1}$. Subsequently, in order to enable direct comparison between the SFG experiments and the PIMD simulations, we simulated $\text{Im}(\chi^{(2)}_{\text{ssp}}(\omega))$ by using the PIMD snapshots and calculated the normalized peak areas. The simulation details are written in the supporting information. The simulated SFG spectra of H$_2$O, D$_2$O, and HDO are shown in figure 4.3 (e) and (f). Since diluted water consists of a mixture of H$_2$O, D$_2$O, and HDO, $\text{Im}(\chi^{(2)}_{\text{ssp}}(\omega))$ for the mixtures can be calculated by

$$\chi^{(2)}(\omega) = [\text{H}_2\text{O}]\chi^{(2)}_{\text{H}_2\text{O}}(\omega) + [\text{HDO}]\chi^{(2)}_{\text{HDO}}(\omega) + [\text{D}_2\text{O}]\chi^{(2)}_{\text{D}_2\text{O}}(\omega), \hspace{1cm} (4.2)$$

where $[\text{H}_2\text{O}]$, $[\text{D}_2\text{O}]$, and $[\text{HDO}]$ are the concentrations of H$_2$O, D$_2$O, and HDO, respectively. By utilizing the equilibrium constant $K = [\text{HDO}]^2/[\text{H}_2\text{O}]\text{D}_2\text{O}$ of 3.86 \cite{88}, we calculated $\text{Im}(\chi^{(2)}_{\text{ssp}}(\omega))$ for different H$_2$O/D$_2$O concentrations. As with the experimental data, the normalized free OH (OD) bond peak areas, $A_{OH}(x)$ ($A_{OD}(x)$), were calculated by integrating the peak areas above the baseline $\text{Im}(\chi^{(2)}_{\text{ssp}}(\omega)) = 0$.

In figure 4.3, the experimentally obtained normalized bond areas for the different isotopic dilutions, $A_{OH}(x)/A_{OH}(x=1.0)$ and $A_{OD}(x)/A_{OD}(x=1.0)$, are plotted as squares while the simulated peak areas are represented by solid lines. Good agreement can be seen between the simulated normalized peak areas and the experimentally measured data with exceptions at the high concentration of
the OD stretch mode. For high D$_2$O concentration, the free D$_2$O shoulder peak at $\sim$2665 cm$^{-1}$ contributes to the signal, complicating the estimation of the free OD bond peak areas. Nevertheless, both the SFG spectra and the PIMD simulations indicate that the normalized free OH peak areas consistently lie above the normalized free OD peak areas. Since without distinct bond orientations SFG signals for both the free OH and OD stretches would be reduced with the concentration in the same way [23, 78], this result confirms that more free OH bonds than free OD bonds are present at the vapor interface in isotopic mixtures. Note that we assumed that the concentration of the H$_2$O, D$_2$O, and HDO is in the same manner with the bulk water in this study. We will address the isotope effect of the distribution of water species in the mixture of H$_2$O, D$_2$O, and HDO.

In summary, we have simulated the H$_2$O, D$_2$O, and HDO-vapor interfaces by using the PIMD simulations to incorporate the nuclear quantum effects explicitly. The OH bond orientation at the H$_2$O-vapor interface was similar to the OD bond orientation at the D$_2$O-vapor interfaces. In contrast, our PIMD simulations indicate that the OH and OD bonds have distinct orientation at the HDO-vapor interface; the OD bond tends to orient toward the bulk region, while the OH bond tends to orient toward the vapor region. This distinct OH and OD bond orientations have been identified by experimentally observed SFG spectra and the simulated SFG spectra from the PIMD simulations. The current study confirms that nuclear quantum effects generate distinct OH and OD bond orientations at the water-vapor interface and indicates that nuclear quantum effects are non-negligible for quantitative analyses in interface-specific studies, e.g. SFG at isotopic diluted samples.

Figure 4.4. Normalized free OH and OD bond peak areas $A(x)/A(x=1.0)$ as a function of the concentration $x$ from the SFG experiments (squares) and the PIMD simulations (lines).
4.4 Supporting Information

Simulated Density Profile

The density profiles of H$_2$O, D$_2$O, and HDO are shown in figure 4.4. The Gibbs dividing surface is located at $|z| = 6.8$. All the density profiles are identical after rescaling molecular masses.

![Density profile graph](image)

**Figure 4.5.** Axial distribution of the density at HDO, H$_2$O, and D$_2$O-vapor interfaces. Lines are guides for the eye.

Temperature Dependence for the Orientation Distribution

The temperature dependence of the OH and OD orientations were surveyed, by performing PIMD simulation for the HDO-vapor interface at 285, 300, and 315K. The results are summarized in figure 4.4. This figure shows that the distinction of the OH and OD bond orientations are enhanced at lower temperatures at the vapor interface. The nuclear quantum effect is enhanced at the lower temperature [89], and one can expect that the enhanced difference of the OH and OD bond orientations due to the nuclear quantum effect is also enhanced at lower temperature. This figure is consistent with the nature of the nuclear quantum effect.

Entropy Calculation from the Distribution of $\cos \theta$

The normalized distribution of $\cos \theta$ for each layer is displayed in figure 4.4. The entropy can be calculated

$$S = -k_B \sum_i p_i \ln p_i$$  \hspace{1cm} (4.3)
where \( p_i \) is the probability for \( \cos \theta \). \( S_2 \) is the entropy for the OH and OD bond orientations of HDO obtained from our PIMD simulation. \( S_2 \) for one HDO molecule can be calculated

\[
S_2 = -k_B \left( \sum_i p_i^{OH} \ln p_i^{OH} + \sum_i p_i^{OD} \ln p_i^{OD} \right)
\]  

(4.4)

\( S_1 \) is the entropy by assuming that the OH and OD bond orientations are the same. \( S_1 \) is, thus, given by

\[
S_1 = -2k_B \sum_i \frac{p_i^{OH} + p_i^{OD}}{2} \ln \frac{p_i^{OH} + p_i^{OD}}{2}
\]  

(4.5)

The entropy decreases due to the distinct bond orientations, \( \Delta S = S_2 - S_1 \), was calculated from \( p_i^{OH} \) and \( p_i^{OD} \) given in figure 4.4. The depth profile of \( T\Delta S/k_b \) is shown in the inset of figure 4.4.

**SFG Spectral Calculation from PIMD**

We simulated the free OH and OD stretch regions of the SFG spectra at the \( \text{H}_2\text{O}, \text{D}_2\text{O}, \) and HDO-vapor interfaces by using the snapshots obtained from our PIMD simulation. In the homogeneous limit, the resonant part of the \( x\)-directed SFG response function generated by the \( z\)-directed infrared (IR) and \( x\)-directed visible pulses can be written

\[
\text{SFG spectral calculation from PIMD}
\]
Figure 4.7. Normalized distribution of $\cos \theta$ for the OH bond (dark grey) and OD bond (light grey).

$$\chi^{(2)}_{xxz}(\omega) = \sum_n \left( \frac{a_{n,xx}(0)m_{n,z}(0)}{\omega_n(0) - \omega - i/2T_1} \right),$$

(4.6)

where $a_{n,xx}(0)$, $m_{n,z}(0)$, and $\omega_n(0)$ is the $xx$-tensor element of the transition polarizability, the $z$-component of the transition dipole moment, and the transition frequency of chromophore $n$, respectively [90–92]. Note that $\chi^{(2)}_{ssp}(\omega) \propto \chi^{(2)}_{xxz}(\omega)$ [66]. The excited vibrational lifetime $T_1$ was set to 1.3 ps [90, 93]. The $z$-component of the transition dipole moment can be written as

$$m_z = \mu'x_{10}\vec{u}_{bond}\cdot\vec{u}_z,$$

(4.7)

where $\mu'$ is the magnitude of the dipole derivative, $x_{10}$ is the 1-0 matrix element of the OH stretch coordinate, $\vec{u}_{bond}$ is the OH or OD bond unit vector, and $\vec{u}_z$ is the surface normal unit vector [90]. Similarly, the $xx$-tensor of the transition polarizability can be given by

$$a_{xx} = x_{10} \left( (\alpha'_{||} - \alpha'_{\perp})(\vec{u}_{bond}\cdot\vec{u}_x)^2 + \alpha'_{\perp} \right),$$

(4.8)

where $\alpha'_{||}$ and $\alpha'_{\perp}$ are the magnitudes of the polarizability derivatives parallel and perpendicular to the OH bond, respectively [90]. $\mu'$, $\alpha'_{||}$, $\alpha'_{\perp}$, $x_{10}$, and $\omega$ have been parameterized as a function of the electric field, $E$, along the OH (OD) bond at H (D) atom [90,93]. We set $\alpha'_{||}/\alpha'_{\perp} = 5.6$ [90]. $E$ was calculated from all the water molecules within 6.6 by setting the SPC/E point charges to
the geometries obtained from our PIMD simulation. Note that we assume that the difference of the equilibrated structures between the SPC/E and q-SPC/Fw models is tiny and the electrostatic map for the SPC/E model can be applied for the geometry obtained from our PIMD simulations with the q-SPC/Fw water model. The intramolecular coupling shifts the frequencies of the OH (OD) stretching modes in H$_2$O-vapor and D$_2$O-vapor interfaces. The intramolecular coupling $\omega_{12}$ can be calculated from ref. [79]. The frequency shift due to the intramolecular coupling is given by [80]

$$\Delta = \sqrt{\frac{(\omega_1 - \omega_2)^2}{4} - \omega_{12}^2 - \frac{|\omega_1 - \omega_2|}{2}} \quad (4.9)$$

Then, the stretching frequencies are given by $\omega_1 + \Delta$ and $\omega_2 - \Delta$ for $\omega_1 > \omega_2$, and $\omega_1 - \Delta$ and $\omega_2 + \Delta$ for $\omega_1 < \omega_2$ for each water molecule.

**EXPERIMENTAL SETUP**

In the SFG experiments, 1 mJ of the output of a regeneratively amplified Ti:sapphire system (Spitfire Ace, Spectra Physics, Inc.) producing $\sim 35$ fs pulses centered at 800 nm was used to generate tunable mid-IR pulses using a home-built optical parametric amplifier and difference frequency generation unit [68]. 0.5 mJ of the amplifier output was spectrally narrowed to $\sim 15$ cm$^{-1}$ using a Fabry-Perot etalon. The IR beam passes through a half wave plate and polarizer before being focussed onto the sample together with this spectrally narrowed visible beam. A focal length of 50 mm and 200 mm were used in this reflection geometry, with angles of incidence of 45$^\circ$ and 40$^\circ$ with respect to the surface normal and a remaining power of 5 mW and 25 mW (IR and visible, respectively). The SFG signal was focused into a spectrograph (Acton, Princeton Instruments) in which it was dispersed, via a grating, and focused onto an electron multiplied Charge Coupled Device (emCCD) camera (Newton, Andor). All spectra reported in this study were collected under the ssp-polarization condition (SFG and visible $s$, IR $p$ polarizations). The measured SFG spectra are shown in figure 4.4.

**FITTING PROCEDURE OF THE SFG SIGNAL**

As described in the main text, the SFG spectra were fitted with a Lorentzian lineshape model. We fitted spectra from 2490 to 2840 cm$^{-1}$ for the OD stretch region and from 3420 to 3820 cm$^{-1}$ for the OH stretch region. $A_{NR}$ and $\varphi_{NR}$ were kept constant throughout the fits of all spectra, both in the OH and OD region. To fit the OH stretch region three Lorentzians were used. Two positive peaks corresponding to the free OH bonds of HDO and H$_2$O molecules make up the free OH itself, largely overlapping with central frequencies of 3682 cm$^{-1}$ and 3700 cm$^{-1}$, respectively. The third negative peak corresponds to the H-bonded OH stretch frequency around 3450 cm$^{-1}$. With these three resonances, an accurate fit of the experimental data could be achieved for all isotopic dilutions (see figure 4.4). The amplitudes of the two free OH (OD) stretch peaks overlap,
making them to a large extent exchangeable in the fittings. However, since we are interested in the peak areas of the free OH (OD) stretch peak, only the sum of these contributions to the SFG spectra are necessary to identify the distinct bond orientations. The peak area was insensitive to these exchanges.

In the OD stretch region, three analogous resonances were fitted to the data: the negative hydrogen bonded OD around 2550 cm$^{-1}$, the positive free HDO at 2722 cm$^{-1}$ and the positive free D$_2$O at 2737 cm$^{-1}$. However, two additional peaks were needed to fit the data with equal accuracy. Firstly, the negative H-bonded OH peak, although lying outside the scope of our experimental data, was added to the fitting expression because the red tail of the resonance was found to influence the free OD stretch region, especially for low OD:OH dilutions. Secondly, a broad feature at 2665 cm$^{-1}$ (free D$_2$O shoulder peak) was evident in the OD data. This peak has been described previously [80].

In fitting the data, all center frequencies and widths were fixed, except for the H-bonded OH and OD frequencies. These were slightly shifted to lower frequency with increasing HDO, which has been reported in previous studies [24,94]. It should be noted that the exact fitting results and the obtained areas of the free OH (OD) peak plotted in figure 4.3 of the main text depend subtly on the starting choice of fitting parameters. However, the OD data points lie below the OH points for all different fitting attempts. Fitting parameters are summarized in Table S1 and S2. All the fitted curves are shown in figure 4.4 along with the measured SFG spectra. The signs of the different peaks have been obtained from ref. [23,94].

We would like to note that fitting the data with a series of Lorentzians helps
in identifying the spectral overlaps, but the need for a non-resonant background
and phase term introduces uncertainty to the fitting process. To bypass the un-
certainty of the fitting procedure, the results were reproduced by means of an
alternative experimental method. By increasing the delay between the infrared
and visible pulse, the short-lived non-resonant background and broad spectral
features originating from the hydrogen bonded OH (OD) stretching mode are
suppressed, leaving only the narrow, longer-lived free mode remaining. This
method has been previously applied by Stiopkin et al. [80]. The area of the
remaining free OH (OD) peak is proportional to the square of the free OH
(OD) bond peak areas. Using this method, the nuclear quantum effects were
qualitatively reproduced. The downside of this method is a decrease of the
signal-to-noise ratio, increasing the uncertainty of extracted resonance intensi-
ties. Moreover, as the lifetime of the OH and OD are different [95], there is an
intrinsic error in this procedure.

<table>
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<th>H75</th>
<th>H65</th>
<th>H50</th>
<th>H35</th>
<th>H25</th>
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<td>110</td>
<td>110</td>
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<td>110</td>
</tr>
</tbody>
</table>
| Free HDO ampli-
| tude           | 0     | 0.200 | 0.278 | 0.350 | 0.341 | 0.250 | 0.150 |
| Free HDO frequency | 3682  | 3682  | 3682  | 3682  | 3682  | 3682  | 3682  |
| Free HDO width  | 18    | 18    | 18    | 18    | 18    | 18    | 18    |
| Free H$_2$O ampli-
| tude           | 1.684 | 1.301 | 1.013 | 0.719 | 0.519 | 0.310 | 0.228 |
| Free H$_2$O frequency | 3700  | 3700  | 3700  | 3700  | 3700  | 3700  | 3700  |
| Free H$_2$O width | 23    | 23    | 23    | 23    | 23    | 23    | 23    |

**Table I.** Fitting parameters for the OH stretch region. Phases are in radians, while frequencies and widths are expressed in cm$^{-1}$. 
<table>
<thead>
<tr>
<th>Parameter</th>
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<th>D85</th>
<th>D75</th>
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Table II. Fitting parameters for the OD stretch region. Phases are in radians, while frequencies and widths are expressed in cm$^{-1}$. 