Investigating the Stability of Individual Carboxylate Rich Alicyclic Molecules Under Simulated Environmental Irradiation and Microbial Incubation Conditions

Alexander J. Craig[1,2], Mahsa Norouzi[1], Paul Löffler[3], Foon Yin Lai[3], Rim Mtibaà[4], Eva Breyer[4,5], Federico Baltar[4,5], Lindon W. K. Moodie,[2] Jeffrey A. Hawkes[1]*

[1]Analytical Chemistry, Department of Chemistry BMC, Uppsala University, Uppsala 752 37, Sweden

[2]Drug Design and Discovery, Department of Medicinal Chemistry, Uppsala University, Uppsala 752 37, Sweden

[3]Swedish University of Agricultural Sciences, Department of Aquatic Sciences and Assessment, Box 7050, 750 07 Uppsala, Sweden

[4]Department of Functional and Evolutionary Ecology, University of Vienna, Vienna, Austria

[5]Shanghai Engineering Research Center of Hadal Science and Technology, College of Marine Sciences, Shanghai Ocean University, Shanghai, China

*Corresponding author: jeffrey.hawkes@kemi.uu.se

Keywords

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Synopsis

In this study, we test the stability of synthetic carboxylate-rich alicyclic molecule standards to both solar simulated irradiations and microbial incubations, alongside an additional set of biologically or chemically relevant molecules for comparison. We find that compounds that more resemble the features of 'old' dissolved organic matter are generally more stable in all of our tests.

Abstract

Understanding environmental dissolved organic matter (DOM) relies on the development of methods capable of navigating its inherent complexity. Although analytical techniques have continually advanced, leading to improved insight for both bulk and fractionated DOM, the fate of individual compound classes remains nearly impossible to track with current technology. Previously, we reported the synthesis of carboxylate rich alicyclic molecule (CRAM) compounds that shared more similar analytical features with DOM than previously available standards. Here, we adopt an alternative approach to the conventional use of DOM as a bulk material, by taking our synthesized CRAM compounds and subjecting them to simulated solar irradiation and microbial incubation experiments alongside an additional curated set of purchased molecules with chosen biological or chemical relevance. Irradiation experiments typically showed that compounds bearing only carboxylic acids and/or alcohols on a saturated carbon backbone were the most resistant to photochemical degradation, but also that some compounds with CRAM-like formulas and chemical functionality were notably more stable in the presence of DOM. Within microbial incubations, all of our synthesized CRAMs were entirely stable after 8-months in various aquatic settings. These sets of experiments provide support for the proposed stability of CRAM within the environment, as well as providing a platform from which a more diverse set of molecules can be used to assist in probing the stability of DOM.

1. Introduction.

Within all of Earth's bodies of water, dissolved organic matter (DOM) amounts to approximately 700 gigatons of carbon.[1] The most important role of DOM within the environment is as a vector for nutrient transport, specifically between microbes and the decomposition products of life. While the vast

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majority of DOM-turnover occurs through these labile DOM (LDOM) pool metabolites, any readily available materials are quickly harnessed and re-used by microbes. Conversely, approximately 95% of DOM at any one point in time is designated as recalcitrant (RDOM) and has a residence half-life in the ocean of between 4000 and 6000 years.[1] Both the sheer size and overwhelming chemical complexity of this low-reactivity pool of material has puzzled scientists for decades.[2]

The extremely long lifetime of RDOM is typically explained by three concepts.[2,3] Intrinsic chemical stability hypothesizes that the chemical structures that DOM contains are stable to degradation and sequestration.[4] The dilution hypothesis suggests that DOM is composed of an extremely diverse mixture of molecules present at tiny concentrations that are not viable for biological utilization, but would be if present at higher concentrations.[5] Finally, restricted or absent essential nutrients can limit organisms from metabolizing DOM.[6,7] Likely, it is all of these factors that contribute to the total stability of RDOM, which is chemically diverse. Previous attempts to understand the fluxes of RDOM have investigated the photochemical,[8–12] biological,[12–15] and sequestrative[16–19] processes that affect it. However, most of these types of studies have examined DOM as a bulk material, often using only chemical formula assignments to differentiate between chemical class and, by extension, molecular structure.[20–26] This simplification can be seen as necessary due to the limitations of analytical methods and instruments, but overlooks structural isomerism and its effects on stability, ultimately generalizing the findings of individual studies.

While the extensive use of mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR) in recent decades has improved the understanding of the chemical composition of DOM, these techniques have crucial limitations. Most importantly, both techniques provide an aggregate view of any individual DOM dataset, with outputs representing averages of its total composition. NMR data is frequently bucketed into a few broad and poorly defined regions, hindering nuanced structural insight.[27] Within MS analysis, regions in van Krevelen diagrams are frequently ascribed to classes such as sugars, proteins, or carboxylate rich alicyclic molecules (CRAM). However, one cannot definitively assign a specific molecular formula to a chemical class based only on MS data, despite this being common in the field. Critically, isomeric compounds can exhibit radically different reactivity, even if they belong to the same chemical class, and as a result, any generalization hinders accurate assessment of the properties of DOM. Compounding this, standards of common classes of RDOM molecules are mostly unavailable by synthesis or isolation. Without accurate structural description, the understanding of the chemical behavior of individual RDOM compounds is limited to theory alone, and pushes discussions on the fluxes and nature of DOM away from evidence and towards speculation.

Recently, our group disclosed the synthesis of simple CRAM analogues.[28] As a compound class, CRAM is hypothesized as one of the largest pools of material within RDOM, and consists of molecules predominantly built from fused alicyclic rings and furnished with several carboxylic acid functionalities.[29] In our initial work, we showed that the chemical features of eight novel synthetic analogues more accurately aligned with the postulated features of the theorized environmental CRAM compound class than previously used compounds, such that they could be used in future experiments to study the recalcitrant properties of CRAM. Here, we test the stability of our first CRAM standards alongside an additional curated set of molecules with chosen biological or chemical relevance. The compounds were subjected to irradiation experiments using a solar simulator, and incubations with lake and coastal water microbial communities, as well as isolated pelagic fungal strains. The behavior of the synthesized molecules alongside other pure standards in these settings provides previously inaccessible information about the stability of individual compounds with CRAM appropriate functionality and molecular formulas.

2. Methods and Materials. Expanded information is available for all methods and materials sections in the Supporting Information, including information about quality control.

2.1. Materials. In addition to our 8 synthetic CRAM diastereomeric mixtures, which were combined to comprise compounds **1-4** purities; **1a** - 92%, **1b** – 99%, **2a** – 90%, **2b** – 98%, **3a** – 99+%, **3b** – 95%, **4a** –

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94%, **4b** – 97%),[28] where compounds denoted **a** have *syn* stereochemistry at the 1,2-diacid, and compounds denoted **b** have *anti* stereochemistry. Nine commercially-available compounds were selected to represent general classes of biological molecules; cholic acid (**5**, 97% purity, terpenoid),[28] oleanolic acid (**6**, >98% purity, terpenoid), Leu-Gly-Gly (**7**) (≥98%, peptide), syringic acid (**8**, ≥95%, phenol/tannin monomer), fraxin (**9**, ≥95%, coumarin-glycoside conjugate), glycyrrhizic acid (**10**, ≥95%, terpenoid-glycoside conjugate), raffinose (**11**, ≥98%, oligosaccharide), and guanosine monophosphate (**12,** ≥95%, nucleotide). An additional compound, 2-(4-(2,2-dicarboxy-ethyl)-2,5-dimethoxy-benzyl)-malonic acid (**13**, purity not listed), was selected as an aromatic CRAM-like equivalent. The CRAM-like compounds **1**-**4** were designed based on putative structures described by Hertkorn and colleagues,[29] as described previously.[28] Cholic acid (**5**), oleanolic acid (**6**) and glycyrrhizic acid (**10**) were selected as commercially available polycyclic terpenoid molecules of biological origin, which have backbone similarities to proposed CRAM scaffolds, but different functionality, including a glycoside linkage to a disaccharide moiety in the case of **10**. A peptide (**7**), sugar (**11**) and nucleotide (**12**) were included as expected labile biological metabolites, and natural products syringic acid (**8**) and fraxin (**9**) were included as aromatic plant metabolites. The aromatic carboxylate rich molecule (**13**) was included due to its similarity to proposed CRAM structures and also after its continued use as a DOM-like standard in the DOM HRMS literature.[30–34]

Four sample matrices were used in the experiments, Milli-Q water (MQ), artificial seawater (ASW),[35] surface lake water (LW) taken in late summer from Långsjö, near Bjorklinge, Sweden: 60°02'31.97" N, 17°33'36.40" E, and coastal seawater (CSW), taken in late summer from the jetty at Tjärnö Marine Laboratory, Sweden, see supporting information for more details. LW and CSW were filtered (0.7 µm, GF/F filter), to remove microbes.

Långsjö is regularly sampled as part of a long-term monitoring program in Sweden,[36] and water parameters have been very stable over several years prior to our sampling. Water quality parameters were measured 9 days before our sampling date, and included TOC 6.3 mg/L, total nitrogen 18 µg/L and total phosphorus 16.4 µg/L. At Tjärnö, seawater is also measured nearby at Kosterfjörden, and in the same month as sampling, TOC was measured at 2 mg/L, nitrate+nitrite and phosphate were measured at 2.66 µM and 0.08 µM (at surface, respectively).[37]

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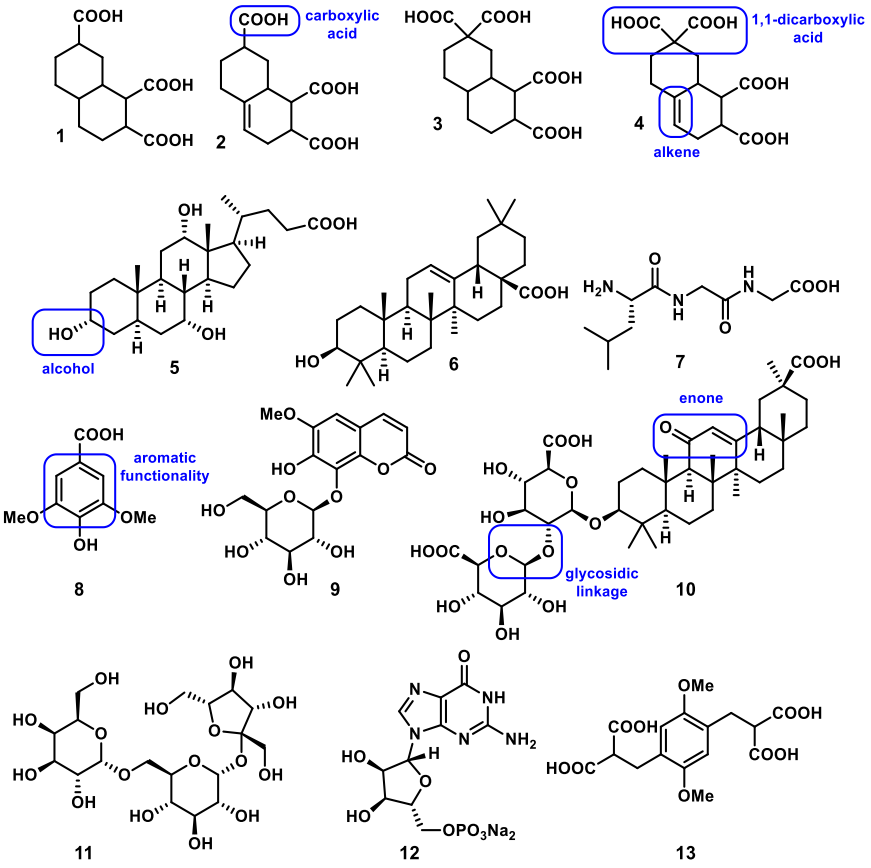

Figure 1: *Compounds used in this study; CRAM-like diastereomeric mixtures **1**-**4** and commercially available compounds **5**-**13**. Functionality discussed later in paper is highlighted in blue.*

2.2. Preparation of Compound Mixture and Control Samples. For the 427-hour solar incubation, microbial community, and isolated fungal experiments, a mixed stock solution was prepared from **1**-**13** in dimethyl sulfoxide (at 0.1 mg/ml each) initially into 50:50 methanol:water at 5 ppm concentration each, before being diluted into MQ, ASW, LW, or CSW to a concentration of 10 ppb (**5**-**13**) or 20 ppb (**1**-**4**), due to the combination if 10 ppb of each isomer (i.e. 1a and 2a). For the 93-hour solar incubations, a mixed stock solution of **1**-**13** in MQ was diluted into MQ to a concentration of 10 ppb (**5**-**13**) or 20 ppb (**1**-**4**), avoiding organic solvents.

2.3. Irradiation Experiments. Irradiation experiments were conducted using a Suntest XXL+FD (Atlas, Linsengericht-Altenhaßlau, Germany) at 25 °C with an irradiation intensity of 65 W m^{-2}, adjusted over 300-400 nm. The chamber has three xenon lamps whose spectral distribution aligns well with the international standard CIE 85 (Figure SI1). For the first, longer experiments, samples were continuously irradiated for 427 hours, compared with 93 hours in the second, shorter experiment. The cumulative irradiant exposure was 10000 kJ m^{-2}, and 2180 kJ m^{-2}, respectively, corresponding to approximately 132 days and 29 days at sea level, respectively. More information about the irradiation experiments can be found in the Supporting Information.

2.4. Biological Community Incubation. Samples were prepared using LW and 2% unfiltered lake water inoculum (experiments LW22 and LW259), or CSW and 2% unfiltered coastal sea water inoculum (experiment CSW251), before being placed at 20 °C in a dark, temperature-controlled room for 22 days (LW22), 259 days (LW259), and 251 days (CSW251).

2.5. Marine Fungi Incubation. The marine pelagic fungal cultures used were isolated from open ocean waters and included *Rhodotorula sphaerocarpa, unknown fungal strain ECO1-30, Cladosporium sp, and*

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Sakaguchia dacryoidea.[38] Fungal liquid cultures were performed using ASW inoculated with 1% fresh fungal suspension over 102 days at 20 °C in the dark.

2.6. Sample Extraction and Analysis. Detailed procedures for extraction, LCMS analysis, and data analysis for all experiments are provided in the Supporting Information.

2.7 Experimental Considerations. Of the 13 compounds subjected to testing, it was found that **6**, **11**, and **12** were not observed in any control or post-experiment samples (Figure SI2). For **11** and **12**, this is likely due to their hydrophilicity preventing their retention during solid-phase extraction (SPE) extraction (see Figure SI2). Conversely, **6** likely was too insoluble in water to be present in sufficient amounts for analytical detection.[39] Additionally, **8** was not observed in either control or post-experiment samples for either the 250-day LW incubations or the 100-day fungal incubations, likely due to poor ionization efficiency (see supporting information). The analytical experimental values shown in Figures 2-4 have only moderate precision and accuracy due to various factors, including 1) biological variability and general bottle effects, 2) the summing of several isomers for a single reported value, 3) lack of accurate internal standards for quantification, 4) the experiments were performed on a mixture 5) the low concentrations used. For these reasons, we focus our discussion on whether individual compounds were largely unaffected, were partially degraded, or were completely removed.

3. Results and discussion

3.1 Irradiation Experiments. In the first experiment, compounds **1**-**13** were irradiated for 427 hours under simulated solar conditions. Initial stock solutions were prepared using small amounts of DMSO and methanol to attempt to solubilize hydrophobic compounds before their dilution to 10 ppb concentrations in water.

To interpret the results of the stability experiments, we have prepared bar graphs that show % compound remaining relative to the control samples (Figure 2-3 for irradiation experiment, Figure 4 for biological incubation). In these figures, the data shown was first normalized to an internal standard and then the volume of sample extracted, before averaging of intensities between three replicates and then comparing the mean value to the control mean (at time zero) as a percentage. The error bar in such a calculation is determined as the standard error of the difference of means (see supporting information), which is scaled to the same % scale for the graph. The error bars therefore include biological, sample preparation and analytical errors for both the experiment and the control samples, and are larger than the standard error of either the experiment or control means, accounting for the uncertainty of comparing the two.

For the 427-hour experiment in MQ (Figure 2), all compounds except for CRAMs **1** and **2**, cholic acid (**5**), and Leu-Gly-Gly (**7**) were completely degraded. For **5**, its reduced carbon backbone and seemingly unreactive carboxylic acid and alcohol functionalities appear to be stable to degradation. CRAM **1**, as the next most stable compound, contains only carboxylic acid functionalities on a fully reduced carbon backbone. As such, the greater degradation of **1** compared to **5** could be attributed to either its increased number of carboxylic acid functionalities, or its lack of hydroxyl groups. For CRAMs **2**, **3**, and **4**, the presence of an alkene, a 1,1-dicarboxylic acid functionality, or both of these features likely led to their relative instability within this context. Similarly, **10** remained only in trace quantities, with both its enone functionality and glycosidic linkages likely diminishing its stability. Analogous results are observed in the ASW samples, with the only key differences being the retention of small amounts of CRAM **3** and **4**, and the loss of peptide **7**. The loss of **7** is unusual, and while its loss was consistent across all three replicates for ASW (and LW, vide infra), we have no chemical reasoning for this loss and consider it likely to be an artefact.

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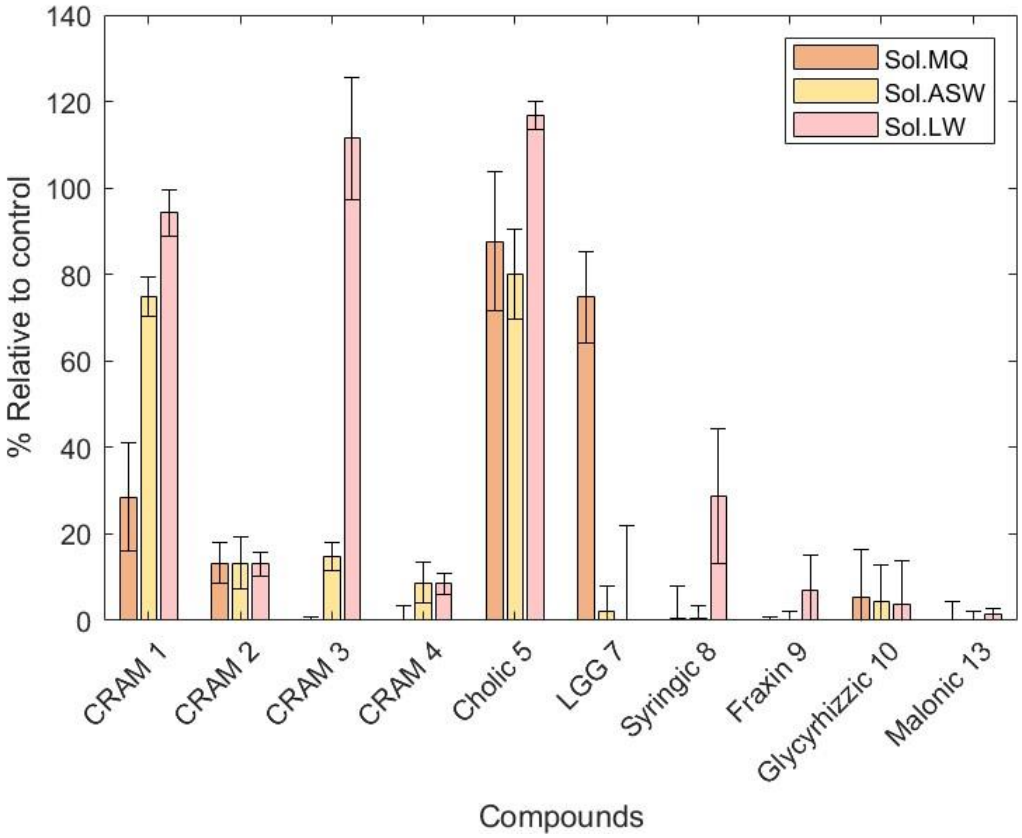

Figure 2: *Percent remaining values for the irradiation treatments relative to time zero for 10 compound groups detected by ultra-performance liquid chromatography electrospray ionization high resolution mass spectrometry (UPLC-ESI-HRMS) after solid phase extraction. Sol.MQ = Solar Milli-Q water, Sol.ASW = Solar artificial sea water, Sol.LW = Solar lake water. Compound names and numbers refer to those in Figure 1.*

For the irradiation experiment in LW, CRAMs **1** and **3**, and steroid **5** appeared entirely stable, while syringic acid (**8**) underwent significantly less degradation than it did in the MQ and ASW experiments. CRAM **2** and **4** were observed in low quantities, while all other compounds were completely removed. The remaining levels of **1**, **3**, **5** and **8** in LW is considerably higher in comparison to both MQ and ASW, and suggests that the DOM from the sampled LW is protecting these compounds from degradation. Possibilities for this include the potential for light-screening by DOM (molecules within DOM acting as quenchers for the tested molecules that could be photochemically excited),[40,41] or more reactive compounds within the sampled DOM preferentially reacting with any generated reactive oxygen species (ROS) and undergoing modification or mineralization.[21,42] While this pool of more reactive material from DOM that could quench ROS would eventually be exhausted here, in nature it would be continually replaced by newly leached DOM from catchment soils, depending on the balance of water residence time of the lake and the amount of sunlight exposure. Assessment of DOM changes from this sample under the same conditions can be found later in section 3.3.

Examination of the tested compounds by UV-vis spectrophotometry showed that compounds **1-5**, **7**, and **11** displayed little or no absorbance that overlapped with the wavelengths of the solar light filter (see Supporting Information). Conversely, compounds **8**-**10** and **12**-**13** absorbed light at wavelengths between 270 nm and 430 nm. As a potential explanation for the degradation of non-absorbing molecules, it was considered that **8-10** and **12**-**13** could become excited and form reactive intermediates that could immediately react with compounds **1**-**7** and **11**, or go on to form other

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reactive intermediates. Similarly, after initial experimentation, we found past experiments we attempted to dissolve all compounds solely in water, regardless of their solubility.

To investigate various points of contention from the 427-hour experiments, a series of shorter tests over 93 hours in MQ alone were designed (Figure 3 and SI3). CRAMs **1**, **2**, and **3** were irradiated individually, as was a mixture of CRAMs **1**, **2**, **3** and **4**, without additional compounds **5**-**13**, to test whether the addition of compounds **5**-**13** was leading to degradation in MQ. Additionally, two separate experiments were performed on the full mixture of compounds **1**-**13**, one in which no DMSO or methanol was added, and one where much higher concentrations of both DMSO and methanol was added compared to the initial experiments (1 molar vs 2.5 mM methanol, 0.14 mM DMSO from the 427-hour experiment). The very high concentration was chosen to provide conditions in which DMSO and methanol could not be consumed by any produced ROS, and check whether their presence was hindering or accelerating degradation. Finally, for all six of these experiments (three single compounds, one CRAM mixture, two compound **1**-**13** mixtures), additional dark control experiments were performed to check for sorption onto glass, or potential hydrolysis in water.

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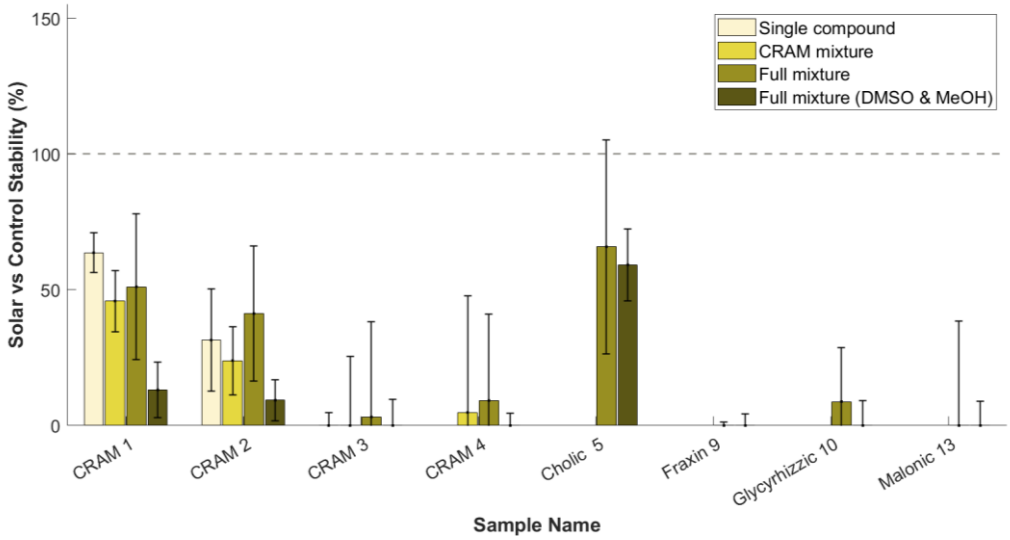

Figure 3: *Solar vs dark control for 93-hour experiments in Milli-Q water. These experiments were to check the CRAM compound behavior in isolation from the other more reactive compounds in the full mixture. CRAMs were also irradiated individually, with consistent results. Having 1M DMSO and methanol in the solvent increased the degradation of CRAMs.*

For individual irradiation experiments, triacid CRAMs **1** and **2** were only partially degraded, while tetraacid alkane **3** underwent complete degradation. This aligns with CRAMs **1** and **2** being the only two CRAM compounds (alongside cholic acid (**5**)) remaining in the 427-hour irradiation experiment. When all four CRAM compounds were mixed in the absence of compounds **5**-**13**, the results were nearly identical, with triacids **1** and **2** remaining after 93 hours of irradiation, while tetraacids **3** and **4** fully decomposed. Peaks corresponding to decarboxylated triacid products of compound **3** (i.e. diastereomers of compound **1**) were observed, suggesting degradation proceeds via decarboxylation of the more labile 1,1-diacid functionality.

For all dark control experiments, compounds **1**-**5**, **7**-**10**, and **13** were returned at 100% intensity after the 93-hour incubation (see Supporting Information), indicating hydrolysis and sorption effects were minimal. Notably, the absence of UV-visible absorbance overlaps of **3** or **4** with the experimental irradiation wavelengths (See Supporting Information) suggests that degradation proceeds through some additional excited species. Although trace impurities could be responsible, LC analysis with charged aerosol detection showed all tetraacid compounds were at least 94% pure, [28] making extensive degradation unlikely, assuming reaction occurred on a stoichiometric basis. This would require trace impurities at sub-part-per-billion levels to react sequentially and degrade the compounds fully. While the specific mechanism of tetraacid degradation remains unclear, the identification of decarboxylated products and the lack of degradation in dark controls confirm their instability under the simulated solar irradiation conditions.

For the 93-hour incubations comprising all compounds **1**-**13**, the exclusion of methanol and DMSO led to similar results to the 427-hour MQ experiments. CRAM **1** and cholic acid (**5**) were the least degraded, CRAM **2** was slightly more degraded than CRAM **1**, and all other compounds detectable in the corresponding control samples were entirely degraded. In comparison, the inclusion of high concentrations of methanol and DMSO led to far more extensive degradation of all compounds, including **5**, which was completely unaffected in all 427-hour experiments. This, likely, is due to the generation of other reactive species in the irradiation experiments from these co-solvents. While concentrations in the 427-hour experiments were low (methanol 2.5 mM, DMSO 0.14 mM), they could still act as additional reactive species until their consumption if ROS were being produced by other photo-excitable molecules. As such, these shorter control experiments suggest the presence of these

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co-solvents in the 427-hour experiment might result in slightly overestimated degradation relative to comparable environmental conditions. Nevertheless, the trends observed in the 427-hour MQ experiments are reinforced by the 93-hour experiments in which both methanol and DMSO were excluded.

Further experimentation is ultimately required to determine the mechanisms of degradation for CRAM compounds **2**-**4** under these conditions. This will involve testing the breakdown pathways of these molecules individually. At the environmentally-analogous concentrations (20 ppb combined between several stereoisomers) used in these experiments, detection of degradation products by LCMS is difficult. As such, experiments exploring the mechanistic pathways that lead to their breakdown in this setting will require testing at higher concentrations, extraction volumes, or more sensitive equipment, but are an important direction for future exploration. Furthermore, expanding the set of available CRAM-like molecules to include compounds with broader functional group and carbon-backbone diversity will help to examine whether all reduced CRAM-like scaffolds are stable under these irradiation conditions, or whether the decalin (two fused 6-membered rings) structure employed here is part of a sub-group of stable scaffolds.

3.2 Biological Incubations. For the biological incubations in both LW and CSW, CRAMs **1**-**4** and tetra-carboxylic acid **13** were unaffected by microbial communities. Due to challenges in accurate quantification (see Section 2.7 and Supporting Information), we treat any result over 100% as indicating that a compound remains, rather than that compounds of identical mass and retention time have been produced during the experiment. However, establishing this definitively would require further experimentation. In contrast, all other detected compounds were completely removed in community incubation experiments, with the exception of **9**, which was observed only in trace quantities in the LW community after 22 days. It is noteworthy that nutrient conditions of the selected waters were on the oligotrophic side (particularly for phosphate), see section 2.1, but these lower nutrient conditions did not limit the usage of compounds **5** and **7-10**, indicating an active microbial community was able to consume labile compounds in these experiments. It is perhaps unsurprising that compounds **5** and **7-10** are degraded in biological incubations; microbial life has evolved a range of mechanisms that utilize natural products as substrates for enzymatic reactions, leading to their degradation or modification.[46–49] Tripeptides such as **7** are derived from universal amino acid monomers, **9** and **10** contain energy rich sugar functionalities, **8** is a common plant metabolite, and bile acids such as **5** are readily degraded by soil and water bacteria.[47]

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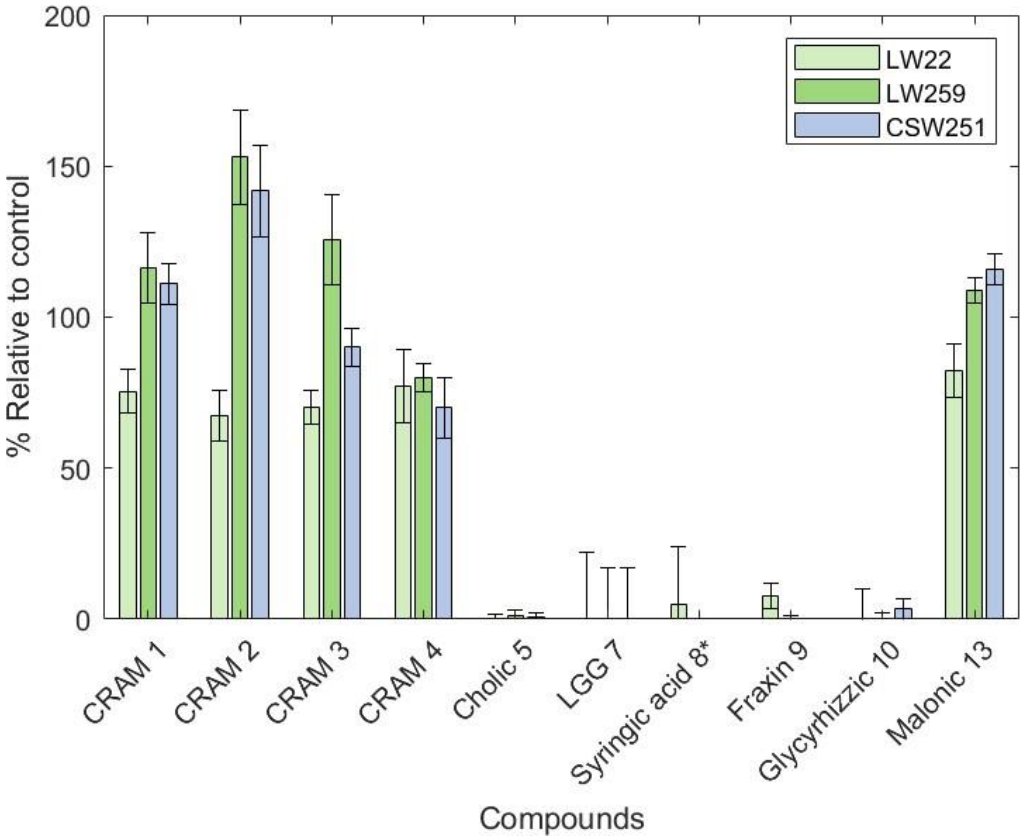

Figure 4: *Percent remaining values compared to time zero control for 10 compound types detected by UPLC-ESI-HRMS after solid phase extraction for the biological incubations. *Syringic acid **8** only detected in the LW22 test and its control, and not in the LW259, CSW251 or their control samples. LW22 = lake water 22 days, LW259 = lake water 259 days, CSW251 = coastal sea water 259.*

In comparison to the microbial community experiments, few isolated fungal species appeared capable of degrading any of the tested compounds (Figure SI4). Ultimately, **7** and **9** were reliably degraded by the fungal strains *Rhodotorula sphaerocarpa* and unknown fungal strain ECO1-30, and **10** was partially degraded by *Cladosporium sp.* Syringic acid (**8**) was not detected in the controls or tests in this dataset. While the fungal degradation of bile acids is generally known,[50,51] and fungi possess cytochrome P450 (CYP) enzymes capable of the oxidation of a wide-number of substrates,[52,53] the strains used here were unable to degrade cholic acid (**5**) in this context, a clear difference from the community experiments. It should be noted that natural microbial communities often collaborate by co-degrading organic matter and utilizing each other's breakdown by-products.[54] As the fungal species in this study were incubated individually, this community effect was absent, limiting the diversity of enzymes required for the degradation of complex material. However, the inability for both environmental microbial communities and isolated fungal strains to degrade CRAM-like analogues **1**-**4** or tetra-carboxylic acid **13** provides valuable evidence for their biological recalcitrance. Ultimately, CRAMs **1**-**4** represent only a small portion of the scaffolds and functional group compositions that make up natural CRAM, and as such, a broader range of compounds, as well as additional microbes, must be tested to determine the extent of environmental CRAMs biological recalcitrance.

3.3 Experimental effects on Lake DOM

In the lake water experiments (i.e. Sol.LW, LW22 and LW259), the lake water used as a matrix for study of compound stability contained thousands of molecular formulas from DOM in the sample. The identity of the compounds making up these molecular formulas is unknown, as explained in the

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introduction, and each molecular formula is constituted by an unknown number of structural isomers. Despite the lack of knowledge of the chemical structures making up DOM, it is possible to measure changes to the mixture, at least to the portion that is ionizable to deprotonated ions by electrospray, using direct infusion HRMS or LC-HRMS. Using the data already in hand from LC-HRMS analysis of compounds **1**-**13**, we assessed overall 'molecular formula level' changes to the DOM mixture in the triplicate samples at experimental conditions LW-control (time zero), Sol.LW and LW22, as these were measured in the same analytical run.

Assessment of the lake water DOM changes after experiments showed minor but statistically significant changes in the irradiation experiment (Sol.LW), and essentially no detectable changes in the biological degradation experiment (LW22), in line with recent findings regarding biodegradability of SPE-DOM by ESI-MS from the same lake (Table 1).[55] This lack of apparent change is partly due to the limitations of SPE and ESI in measuring the hydrophilic, labile species, rather than due to lack of degradation of total DOC. The changes found to the more hydrophobic part of the DOM mixture in the irradiation experiment were in line with expectations, with higher molecular weight, unsaturated compounds being the most sensitive to loss (Figure 5).

Table 1: *Weighted average metrics from the first lake water experiments. Shown are average oxygen:carbon ratio (O/C), hydrogen to carbon ratio (H/C), mass to charge ratio (m/z) and number of peaks detected, as intensity-weighted averages, with standard deviations shown (n=3). Results that are statistically significantly different from the control (Student's t-test) are shown in bold.*

Sample	O/C_{wa}	H/C_{wa}	m/z_{wa}	Number of Formulas
Control LW	0.488 ± 0.005	1.289 ± 0.006	359 ± 3	4041 ± 130
LW22	0.482 ± 0.003	1.292 ± 0.007	354 ± 3	4252 ± 143
Sol.LW	0.494 ± 0.004	**1.318 ± 0.006**	**344 ± 7**	4224 ± 144

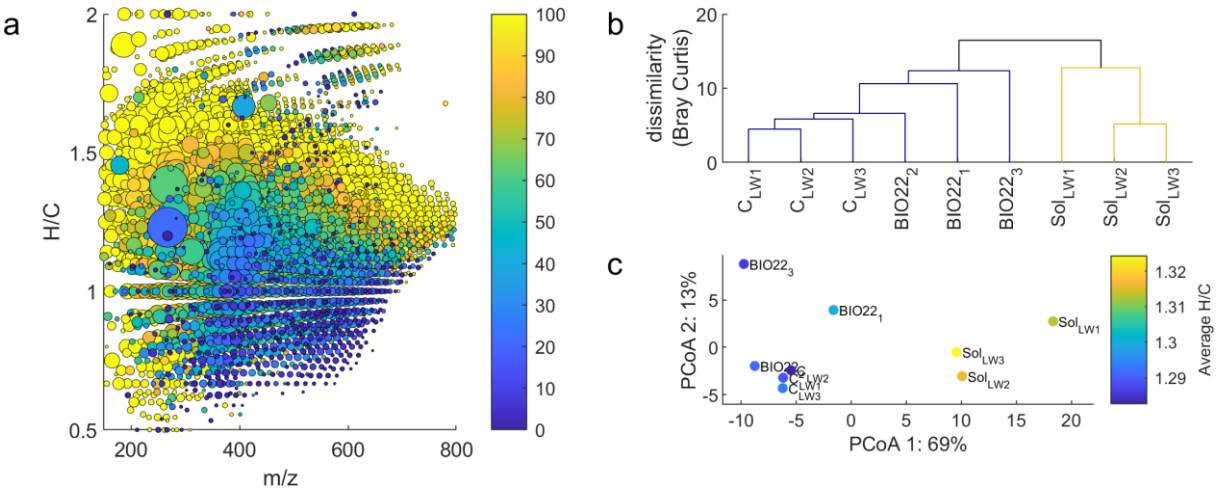

Figure 5*: a) Compounds detected in lake water DOM, colored according to relative loss in the longer irradiation experiment (Sol.LW). Each compound is plotted at the determined H/C ratio vs. m/z, and all compounds detected in the lake water control sample are shown. Point size is shown according to square root of intensity (mean, n=3), for scaling purposes (i.e. in order to see more peaks). Color is shown according to the difference in relative intensity between the irradiated and control sample (irradiated/control x 100), where initial values were normalized per sample to sum to 100. This means that the difference is not quantitative (i.e. showing % loss), but rather qualitative, showing extent of change relative to other peaks in the sample. The color scale is limited to 100 to focus on the peaks that had a relative loss of intensity, and the points were plotted in decreasing color order, to highlight the peaks that were lost. b) Bray Curtis Dissimilarity based cluster dendrogram with cutoff set to 15%*

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dissimilarity, c) Principal coordinate analysis (PCoA) based on a Bray Curtis dissimilarity matrix, showing scores in the first two dimensions (totaling 82% of data variability), with H/C_{wa} indicated by colour.

3.4 Research Outcomes and Future Directions. In the irradiation experiments, tests probing the physical and chemical stability of our initial CRAM-analogues **1**-**4** within a small curated chemical library highlighted that specific chemical functionalities were the strongest indicator of stability. CRAM **1** and cholic acid (**5**) were the only compounds that remained in significant quantities in after irradiation. The majority of compounds that contained 1,1-diacid, enone, and aromatic functionalities were either completely degraded or remained only in trace quantities in all experiments, and the presence of DOM only partially protected some of those same compounds. As an exception, CRAM-like compound **3**, which contained a 1,1-diacid, and had a fully reduced backbone remained unaffected after irradiation in the presence of DOM, as did triacid CRAM **1** with its fully reduced carbon backbone. Notably, CRAM alkenes **2** and **4** were not protected to the same extent by lake water DOM. Future experiments will probe the mechanisms through which DOM protects alkane CRAM-like compounds by assessing the extent to which it screens light, preferentially reacts with ROS, and whether it can directly quench photo-excited compounds with molecular compositions relevant to DOM. Furthermore, select compounds will have their breakdown products tracked, to examine potential degradation pathways for compounds with CRAM-like chemical formulas in the environment.

Within the set of biological experiments, a compound's existence as a direct biological metabolite was the best indicator of its stability to challenges by either microbial communities or isolated fungal strains. It is notable that no member of either microbial community was able to harness any of the CRAM analogues **1-4** or tetra-carboxylic acid **13** over approximately eight months under warm conditions (20 °C). This, of course, could be simply due to the fact that these compounds are not known biological substrates. Similarly, it is likely that a significant portion of recalcitrant DOM is modified by abiotic processes,[3] being derived from the geochemical degradation of biological compounds through reaction with light and ROS.[56] Thus, the stability of these synthesized compounds in this context is in part indicative of the broader recalcitrance to biological challenges of geochemically-processed compounds with CRAM-like functionalities and molecular formulas. Further investigation of biological recalcitrance will focus predominantly on the diversification of CRAM-analogue scaffolds, to test whether specific carbon backbones may render these compounds biologically available. Additionally, tests tailored towards the aggregation and complexation of these compounds are envisioned to expand this initial set of recalcitrance experiments upon isolated CRAM-like compounds.

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Supporting Information: Experimental file includes additional experimental details, including materials, sample preparation, experimental set-up, data analysis, quality control, and experimental considerations. Also available is a feature data file, which is an excel sheet with processing steps to convert feature data from mzMine to the graphs found in the paper according to equations 1-3 from the supporting information.

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Credit system for author contributions:

Term	Definition	Initials
Conceptualization	Ideas; formulation or evolution of overarching research goals and aims	JH AC
Methodology	Development or design of methodology; creation of models	JH AC EB PL
Software	Programming, software development; designing computer programs; implementation of the computer code and supporting algorithms; testing of existing code components	JH
Validation	Verification, whether as a part of the activity or separate, of the overall replication/ reproducibility of results/experiments and other research outputs	JH MN
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Investigation	Conducting a research and investigation process, specifically performing the experiments, or data/evidence collection	JH AC MN
Resources	Provision of study materials, reagents, materials, patients, laboratory samples, animals, instrumentation, computing resources, or other analysis tools	JH EB FB
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Term	Definition	Initials
Project administration	Management and coordination responsibility for the research activity planning and execution	JH
Funding acquisition	Acquisition of the financial support for the project leading to this publication	JH FB LM FYL

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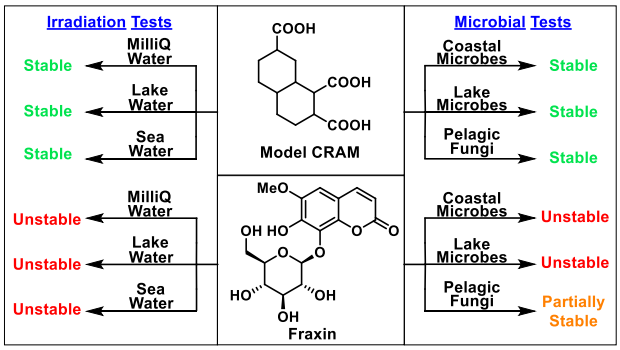

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