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Metal-Catalyzed Hydrolysis of RNA in Aqueous Environments

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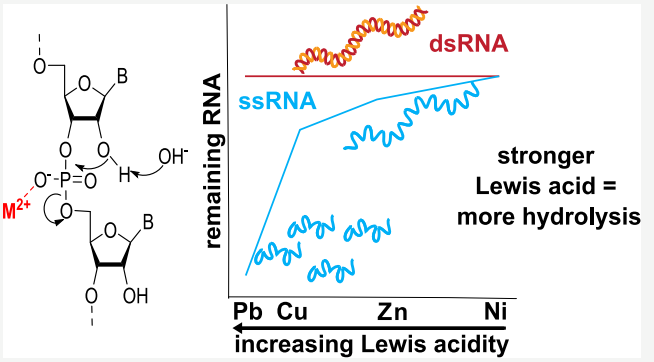
ABSTRACT: The stability of RNA in aqueous systems is critical for multiple environmental applications including evaluating the environmental fate of RNA interference pesticides and interpreting viral genetic marker abundance for wastewater-based epidemiology. In addition to biological processes, abiotic reactions may also contribute to RNA loss. In particular, some metals are known to dramatically accelerate rates of RNA hydrolysis under certain conditions (i.e., 37 °C or higher temperatures, 0.15−100 mM metal concentrations). In this study, we investigated the extent to which metals catalyze RNA hydrolysis under environmentally relevant conditions. At ambient temperature, neutral pH, and ∼10 μM metal concentrations, we determined that metals that are stronger Lewis acids (i.e., lead, copper) catalyzed single-stranded (ss)RNA, whereas metals that are weaker Lewis acids (i.e., zinc, nickel) did not. In contrast, double-stranded (ds)RNA resisted hydrolysis by all metals. While lead and copper catalyzed ssRNA hydrolysis at ambient temperature and neutral pH values, other factors such as lowering the solution pH and including inorganic and organic ligands reduced the rates of these reactions. Considering these factors along with sub-micromolar metal concentrations typical of environmental systems, we determined that both ssRNA and dsRNA are unlikely to undergo significant metal-catalyzed hydrolysis in most environmental aqueous systems.



KEYWORDS: *RNA interference (RNAi), wastewater-based epidemiology (WBE), single-stranded RNA, double-stranded RNA, abiotic hydrolysis, secondary structure, phosphodiester bond cleavage*

INTRODUCTION

Due to the proliferation of diverse technologies employing RNA in recent years, the ability to understand the behavior of RNA in the environment has become increasingly important. Novel biopesticides that use double-stranded (ds)RNA as the active agent have been developed to control agricultural pests, including insects[1−4] and viruses.[2,3] At the same time, both single-stranded (ss)RNA and dsRNA have been measured in wastewater for community tracking of viral outbreaks (e.g., COVID-19) by wastewater-based epidemiology (WBE).[5−10] In both cases, understanding the fate of RNA in environmental systems is necessary for the successful deployment of these technologies. In the first case, the safe use of dsRNA biopesticides requires an assessment of their ecological risks,[11] including evaluation of their fate in receiving environments such as agricultural soils[12,13] (including soil pore water) and surface waters.[12−14] In the second case, the persistence of RNA (particularly the significant fraction of free genomic RNA[9]) in wastewater determines the relationship between the loading of viral RNA to the sewage system and the ultimately measured abundance of RNA. Both the assessment of dsRNA biopesticide fate and the measurement of viral RNA for WBE benefit from an increasingly accurate understanding of RNA degradation rates and mechanisms in environmental systems.

While RNA dissipation rates have been measured in several environmental systems, the determination of specific processes that dominate the loss of measurable RNA remains inconclusive. In surface water, dsRNA was no longer measurable after 4−7 days at room temperature.[15,16] The dissipation rate of RNA was unaffected by autoclave sterilization of surface water, indicating that RNA loss may occur in the absence of active microbial communities.[16] Similarly, dsRNA in solutions collected from soil slurries degraded within 24 h.[17] Although pretreatment of soil with X-ray radiolysis, which was confirmed to reduce the abundance of viable microorganisms, prevented the formation of products associated with microbial utilization, dsRNA still degraded within 24 h.[17] Finally, viral ssRNA from severe acute respiratory syndrome coronavirus 2 (SARS-COV-2) dissipated with a half-life of ∼4 days in both native and sterilized wastewater at 25 °C.[18] Overall, these studies suggest that RNA loss occurs in diverse aqueous systems independently of the

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presence of active microorganisms. One explanation is that sterilization may incompletely deactivate RNA-degrading nucleases in environmental samples.[17] Alternatively, abiotic pathways may contribute to RNA degradation in sterilized samples.

Abiotic mechanisms (e.g., chemically catalyzed hydrolysis) have been found to cause significant and rapid RNA degradation in certain contexts. For example, acids and bases catalyze rapid hydrolysis of phosphodiester bonds in acidic (pH < 2) and alkaline (pH > 8) solutions at 90 °C.[19] Additionally, ammonia catalyzes viral ssRNA degradation in simulated wastewater matrices across a pH range of 7.5−9.5 at temperatures ranging from 4 to 35 °C.[20] Beyond these established mechanisms, metal-catalyzed hydrolysis has the potential to contribute to RNA degradation in the environment. Zinc and lead ions have been reported to increase the hydrolysis rates of phosphodiester bonds by 2 or 3 orders of magnitude, respectively, relative to a metal-free control.[21] The ability of metal ions and complexes to catalyze phosphodiester bond hydrolysis is proposed to result from the electrophilic activation of the phosphorus center by the metal, facilitating a subsequent nucleophilic attack by the deprotonated 2′-hydroxyl group as the first step in phosphodiester bond cleavage.[22]

While at least one recent study has raised the potential for metal ions to catalyze RNA in environmental aqueous systems,[20] the true relevance of this mechanism to environmental RNA degradation is unclear due to the major differences between conditions used in prior studies and those relevant to environmental systems. For example, while metal-catalyzed hydrolysis has been typically evaluated with metals (e.g., nickel, zinc, lead, magnesium, copper, lanthanides)[21−29] in concentrations ranging from 0.15 to 100 mM, metals including copper, nickel, and zinc typically occur at much lower concentrations in the environment (i.e., up to 0.06−0.2 μM in river water,[30] 0.1−1 μM in municipal wastewater[31]). Lead concentrations are even lower by a factor of ∼3−200.[30,31] Further, while previous studies have evaluated metal-catalyzed RNA hydrolysis at temperatures of 37 °C or higher,[21−29] surface water and wastewater exhibit a range of 0−33[32] and 7−34 °C,[33] respectively. Additionally, although ligands have also been shown to influence metal-catalyzed hydrolysis by both slowing (e.g., polyaza-macrocycles,[24] acetylacetone[29]) and accelerating (e.g., imidazole[34]) the reaction rate, these ligands are not representative of ligands found in environmental aqueous systems (e.g., chloride, sulfate, organic matter).[35] Finally, previous studies typically evaluated the metal-catalyzed cleavage of phosphodiester bonds in shorter RNA molecules, such as diribonucleotides,[21,22,25,28] cyclic monophosphates,[22,29] and oligonucleotides[23,27] rather than long RNA molecules. In particular, the duplex structure of dsRNA as well as some regions in ssRNA may resist hydrolysis:[36−39] some reports indicate that metal-catalyzed cleavage of duplex RNA occurs exclusively at bulged sites formed due to base pair mismatches.[36,38]

In this study, we evaluated the potential for metals to significantly contribute to RNA hydrolysis at environmentally relevant conditions. To characterize the mechanism of metal-catalyzed hydrolysis, we first measured the kinetics and extents of RNA loss and product formation at ambient temperatures. We also applied these measurements to test our hypotheses that the extent of RNA hydrolysis would be controlled by the Lewis acidity of metal ions[40] and that dsRNA would hydrolyze to a lesser extent than ssRNA. Next, we further investigated the potential relevance of this degradation mechanism to environmental systems by determining the extent of RNA hydrolysis at varying pH conditions, ligand types, and metal concentrations. By integrating our results with known environmental metal concentrations and RNA dissipation rates, we evaluate the potential importance of metal-catalyzed hydrolysis for the environmental fate of dissolved RNA molecules, including released dsRNA biopesticides and viral RNA.

MATERIALS AND METHODS

Material Sources and Preparation. Materials and supplies are detailed in Section S1. We prepared 1006 nucleotides (nt) ssRNA and 1000 base pairs (bp) dsRNA (sequences available in Section S2) according to a previously described protocol[41,42] (details in Section S3.1). To conduct experiments isolating RNA degradation by abiotic processes, we used previously established protocols to avoid the presence of ribonucleases and microbes[41] (details in Section S3.2).

Incubation of RNA with Metals. Incubation experiments were performed in 1.5 mL Protein LoBind tubes because dsRNA adsorbs negligibly to the tube walls.[17] The incubation mixtures were prepared by either combining 3-(*N*-morpholino)-propanesulfonic acid (MOPS) or 2-(*N*-morpholino)-ethanesulfonic acid (MES) buffer and sodium chloride (NaCl) with RNA with final concentrations of buffer at 2.34 mM. We controlled ionic strength using 10 mM NaCl and initial RNA concentrations of 25 ng/μL across all experiments unless indicated otherwise.

The metal stock solutions were filtered (to ensure removal of any precipitates) using sterile syringes and 0.22 μM polyvinylidene difluoride (PVDF) filters into 15 mL centrifuge tubes prior to experiments. If a diluted stock was necessary for experiments with lower metal concentrations, the filtered metal stock was diluted with autoclaved ultrapure water. The metal stock was then added to the buffer−RNA mixture to a total volume of 40 μL. Solutions were vortexed and then centrifuged briefly to spin down the liquid and incubated without shaking at 22−23 °C for 24 h, unless indicated otherwise.

Analysis of RNA Concentrations. We used a spectrophotometer (NanoDrop 2000c, Thermo Fisher Scientific) to measure RNA concentrations via ultraviolet (UV) light absorbance at 260 nm (A_{260nm}). We employed extinction coefficients of 0.0266 and 0.0214 $(ng/\mu L)^{-1}$ cm^{-1} for ssRNA and dsRNA, respectively.[43]

We used gel electrophoresis to measure RNA degradation by hydrolysis (protocol described in Section S3.3). In addition to gel electrophoresis, loss of detectable ssRNA was analyzed using the quantitative reverse transcription-polymerase chain reaction (RT-qPCR, protocol[42] described in Section S3.4).

The end products of ssRNA hydrolysis mediated by lead and copper were quantified using liquid chromatography-mass spectrometry (LC-MS; protocol described in Section S3.5).

Quantification of Metal Concentrations. Metal stock solutions and final samples were first diluted with ultrapure water (>18.2 MΩ cm) to have metal concentrations within the inductively coupled plasma mass spectrometry (ICP-MS) measurement range (0−200 ppb) and solution volumes higher than the intake volume (>1 mL). The diluted stocks and samples were subsequently filtered with 0.22 μm PVDF syringe filters and then acidified with nitric acid (1% v/v) for analysis of total dissolved metals by ICP-MS (PerkinElmer NexION 2000).

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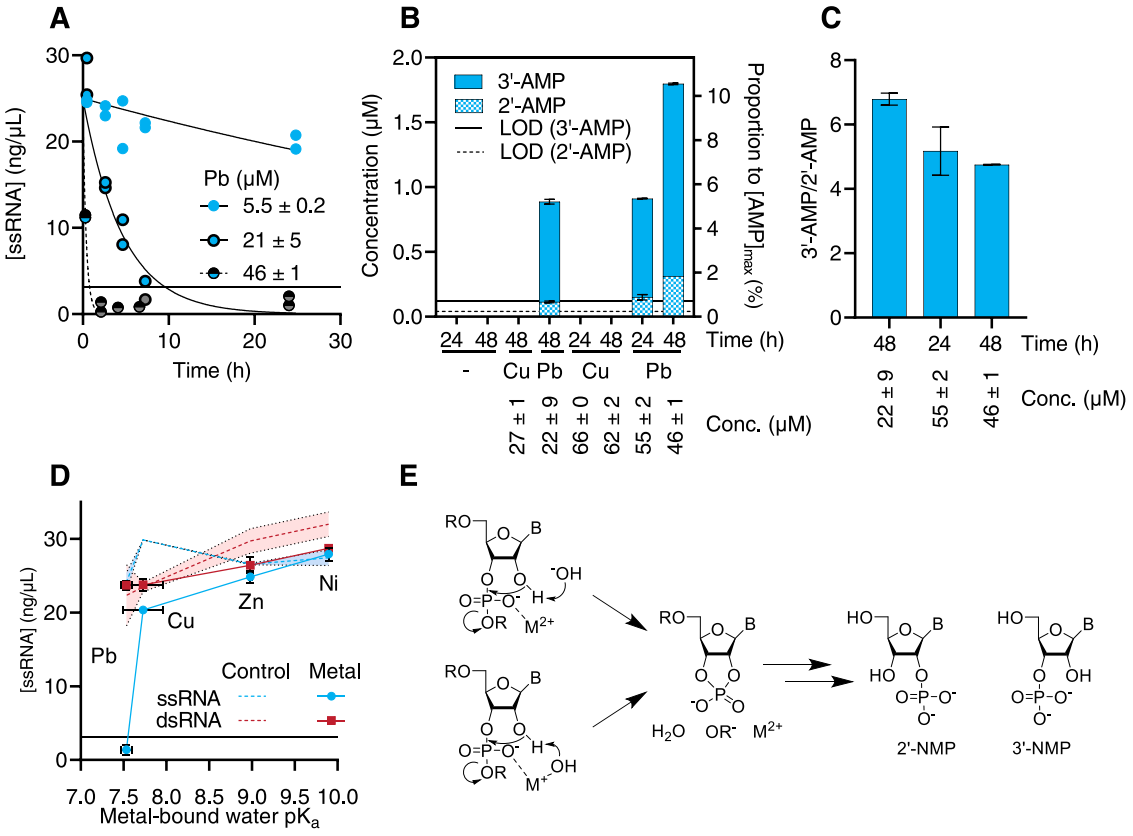

Figure 1. Metal-catalyzed RNA hydrolysis. Experiments were conducted with pH controlled at 7.0 by 2.34 mM MOPS buffer and ionic strength with 10 mM NaCl (A) ssRNA degradation catalyzed by lead (Pb) at 22−23 °C over a period of 24 h at specified measured concentrations of Pb (expected concentrations in Table S3) and ssRNA at an expected initial concentration of 25 ng/μL. Individual replicates are fitted with exponential decay curves. The individual replicates below the lowest quantifiable standard (black solid line, 3.1 ng/μL) are marked in gray color and were excluded from rate constant determination (Figure S6). (B) Hydrolysis end products: 2′-adenosine monophosphate (2′-AMP) and 3′-adenosine monophosphate (3′-AMP) generated in ssRNA samples incubated at an expected initial concentration of 25 ng/μL. Experiments were conducted at 25−26 °C over a period of 48 h with lead (Pb) and copper (Cu) at different measured concentrations (conc., expected concentrations in Table S8). Error bars represent the range of two replicate experiments. (C) Hydrolysis end product molar ratios in ssRNA incubated with lead. Experiments were conducted at 25−26 °C over a period of 48 h with lead (Pb) at different measured concentrations (conc., expected concentrations in Table S8). Error bars represent the range of two replicate experiments. (D) RNA concentrations after incubation with metals as a function of metal-bound water pK_a. The plotted pK_a values represent the midpoint of the ranges (represented as horizontal error bars) available in literature (Table S6). Experiments were conducted at 22−26 °C over a period of 24 h at measured metal concentrations close to 10 μM (expected concentrations in Table S9). Vertical error bars represent the range of two replicate experiments, and the black solid line indicates the lowest quantifiable standard (3.1 ng/μL). (E) Metal-catalyzed hydrolysis of phosphodiester bonds.[22] B represents nucleotide base moieties (A, U, G, or C) and M represents divalent metal (i.e., lead, copper, zinc, nickel). The end products of the reaction are 2′ and 3′-nucleoside monophosphates (NMP).

Statistical Analysis and Modeling. We performed all experiments with independently prepared duplicate samples with error bars representing the range calculated using Microsoft-Excel (version 2105) or GraphPad Prism 9.0. Metal solubilities for lead, copper, zinc, and nickel were modeled using Visual MINTEQ 3.1 (Table S2), with all solids selected. In the case of aluminum(III) solubility modeling, we included only amorphous aluminum hydroxide ($Al(OH)_3$(am)) as a precipitated solid. Similarly, only amorphous iron hydroxide ($Fe(OH)_3$(am)) solid was included for iron(III) solubility modeling.

RESULTS AND DISCUSSION

Mechanism of Metal-Catalyzed RNA Hydrolysis. To determine if metal-catalyzed hydrolysis would occur in solutions with circumneutral pH values under ambient temperatures, we first investigated ssRNA hydrolysis catalyzed by lead(II), which we identified as a combination that was likely to result in measurable hydrolysis. Specifically, ssRNA is more susceptible to metal-catalyzed hydrolysis than dsRNA[36,38] while lead is known to rapidly catalyze phosphodiester bond hydrolysis at circumneutral pH and temperatures equal to or higher than 37 °C.[21,29]

We first evaluated lead concentrations over 24 h at pH 7.0 and ambient temperature (~22 °C). We found that the lead concentrations remained constant over the experimental duration but were up to 66% lower than the expected concentrations, which were calculated based on the measured concentration of the lead stock solution accounting for dilution into the samples (Section S4.1). The difference between expected and measured lead concentrations is attributable to sorption to filter material[44−48] (Section S4.2). At circumneutral solution pH values, the sorption of metals to filter material (e.g., nylon[48,49]) is unavoidable because of the increased negative charges on the filters.[45] Furthermore, the extent of loss is highly dependent on solution volume and

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chemistry, specifically pH, cations, and metal concentrations.[45,47] Although the measured metal concentrations underestimate the actual concentrations in the experiments, we selected to report them because they are comparable with environmental dissolved lead concentrations that have been determined in filtered samples.[30,50−52] The expected and measured metal concentrations for data sets in the paper have been specified in the figure captions. Additional experiments excluded the contributions of undissolved metal (e.g., precipitated, adsorbed to tube walls) to ssRNA hydrolysis (Section S4.3).

Consistent with prior reports that lead catalyzes phosphodiester bond hydrolysis,[21,29] we observed that ssRNA degraded in the presence of lead as detected by gel electrophoresis (Figure 1A). At the highest lead concentration tested (46(±1) μM, Table S3), ssRNA concentration (initially 25 ng/μL) decreased from 11.3(±0.2) ng/μL at 0.3 h to below the lowest quantifiable standard (3.1 ng/μL) at 2.1 h, indicating ssRNA degradation was extremely rapid. In contrast, at the two lower lead concentrations tested (5.5(±0.2) and 21(±5) μM, Table S3), ssRNA concentrations remained quantifiable for at least 24.8 and 7.3 h, respectively.

At the lower lead concentrations tested, we were able to determine that the loss of measurable ssRNA followed first-order kinetics in the presence of lead (Figure S6), similar to ssRNA loss according to the first-order kinetics that we previously observed for base-catalyzed hydrolysis.[41] The ssRNA loss rate increased dramatically with lead concentration. When the lead concentration increased ∼4-fold from 5.5(±0.2) to 21(±5) μM, the corresponding rate constant for ssRNA loss increased 35-fold from 7.6(±2.7) × 10^{-3} to 2.7(±0.3) × 10^{-1} h^{-1}. Although we cannot verify that ssRNA loss followed the first-order kinetics when lead concentration was again doubled from 21(±5) to 46(±1) μM due to the rapid degradation of ssRNA, the rate constant in this case would have to be at least 10-fold higher (2.6(±0.5) h^{-1}), to reduce ssRNA concentration below the quantifiable limit within 2.1 h. The dramatic increase in ssRNA loss rates with increasing lead concentration differs from prior observations that hydrolysis rate constants increased proportionally with either the hydroxide concentration[41] or ammonia concentration[20] in solution. The hydrolysis rate constants also increased proportionally with the concentration of iron(II) and manganese(II) at pH 6.7, albeit at different reaction conditions (i.e., 250 mM MES buffer, 1 mM ethylenediaminetetraacetic acid (EDTA), 0−50 mM metals, 75 °C).[53] In a separate study, an 8-fold increase in the concentration of a zinc complex resulted in a 1.8-fold increase in hydrolysis rate constant for a diribonucleotide substrate at pH 7.6 and 64 °C.[22] In contrast to the prior results with other catalyzing species, our results suggest that hydrolysis rate constants increase greater than proportionally with the increase in lead concentration.

To investigate if the loss of ssRNA in the presence of metals led to products consistent with phosphodiester bond hydrolysis, we measured the formation of end products of the ssRNA hydrolysis reaction, namely, 2′ and 3′-nucleoside monophosphates (nucleotide monomers). We also expanded our experiments to include copper to compare product formation among different metals. Consequently, we quantified 2′ and 3′-adenosine monophosphate (AMP) concentrations at 24 and 48 h in ssRNA samples in the presence of 20−70 μM lead and copper at pH 7.0. These longer timeframes relative to other experiments were selected because several individual phosphodiester bonds must hydrolyze before the monomers are generated from the 1006 nt ssRNA molecule.[41] We did not detect AMP in the ssRNA samples incubated with copper (Figure 1B), which may result from insufficient degradation of ssRNA by copper to produce measurable monomers over the timescale tested. This finding is consistent with our observations, as discussed below, that ssRNA degradation mediated by copper was lower than that mediated by lead. In comparison, in samples containing 22(±9) μM lead, we observed a total AMP concentration of 0.9(±0.1) μM after 48 h. This concentration corresponds to ∼5% of the theoretical maximum AMP assuming complete conversion of the ssRNA to monomers. At 2-fold higher lead concentrations, a similar AMP concentration (0.9(±0.1) μM) was measured after only 24 h, which approximately doubled to 1.80(±0.02) μM after 48 h. Consequently, whereas loss of the ssRNA molecule detected by gel electrophoresis increased faster than proportionally to metal concentration, the product formation rate was proportional to metal concentration.

While the formation of the monomer product AMP indicates that hydrolysis occurs in our samples, further analysis of the ratio of the specific 2′- and 3′-nucleoside monophosphates can be employed to distinguish between specific hydrolysis mechanisms. Enzymatic phosphodiester bond hydrolysis results in the sole production of the 3′-nucleoside monophosphate,[54] whereas base-catalyzed hydrolysis leads to a 3′-AMP/2′-AMP concentration ratio of 1.2/1.[55] In contrast, for metal-catalyzed phosphodiester bond hydrolysis of a diribonucleotide, 3′-AMP/2′-AMP ratios of 2.3/1 and 3.0/1 have been previously reported for a macrocyclic zinc−amine complex and zinc nitrate, respectively.[22] Therefore, we hypothesized that metal-catalyzed hydrolysis of ssRNA, which is regioselective and leads to the preferential generation of 3′-AMP,[22] would lead to greater, but not exclusive, production of 3′-AMP relative to 2′-AMP. In the presence of 22(±9) μM lead, we found that ssRNA hydrolysis yielded products at a ratio of 6.8(±0.2) 3′-AMP/2′-AMP after 48 h (Figure 1C). Increasing the lead concentration to 55(±2) and 46(±1) μM yielded marginally lower product ratios of 5(±1) and 4.8(±0.1) after 24 and 48 h, respectively. The moderately preferential generation of 3′-AMP over 2′-AMP across all samples is consistent with lead-catalyzed hydrolysis acting as the dominant mechanism.

After confirming that the generated products were consistent with metal-catalyzed hydrolysis, we next investigated whether variations in ssRNA hydrolysis caused by different environmentally relevant metals was related to their Lewis acidity. The metal-catalyzed hydrolysis reaction is proposed to be initiated by the electrophilic activation of the phosphorus center (of the phosphodiester bond) by the metal ion, making it likely that metals with stronger Lewis acidity would cause stronger activation of the phosphorus atom.[22] In fact, the first-order rate constants for hydrolysis of a 2′,3′-cyclic monophosphate increased with increasing Lewis acidity of lanthanide metal catalysts (e.g., terbium(III), europium(III)).[29] Therefore, we expected that environmentally abundant metals with stronger Lewis acidities would also catalyze greater extents of ssRNA hydrolysis relative to other metals. To quantify the Lewis acidity of metal ions, we employed the pK_a values of the metal-bound water (eq 1).[56]

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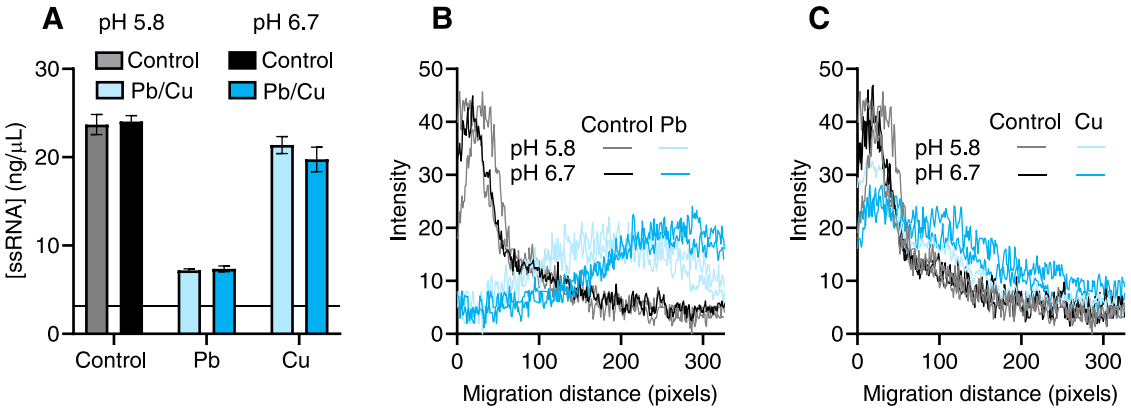

Figure 2. Effect of pH on metal-catalyzed RNA hydrolysis at 25 °C over a period of 24 h. Experiments were conducted with pH controlled either at 5.8 by 2.34 mM MES or at 6.7 by 2.34 mM MOPS buffers and with ionic strength controlled by 10 mM NaCl. Measured lead (Pb) concentrations were 4.9(±0.7) and 5.4(±0.7) μM at pH 5.8 and 6.7, respectively (expected concentration: 17 μM). Measured copper (Cu) concentrations were 8.1(±1.4) and 6.6(±0.2) μM at pH 5.8 and 6.7, respectively (expected concentration: 20 μM). (A) Error bars represent the range of two replicate experiments, and the black solid line indicates the lowest quantifiable standard (3.1 ng/μL). (B, C) Curves represent replicate intensity values over a range of migration distance for lead (Pb) and copper (Cu) samples.

$$pK_a = -\log K_a \text{ wherein } K_a = \frac{\{MOH^+\}\{H^+\}}{\{M^{2+}\}\{H_2O\}}$$
$$(M^{2+} + H_2O = MOH^+ + H^+) \quad (1)$$

To evaluate the effect of metal Lewis acidity on hydrolysis, we investigated the extent of ssRNA hydrolysis at pH 7 and ambient temperatures after 24 h. The metals lead(II), copper(II), zinc(II), and nickel(II) with metal-bound water pK_a values ranging from 7.5 to 9.9 were tested. Although iron(III) and aluminum(III) are stronger Lewis acids with pK_a values of 2.02−2.19[57,58] and 4.997,[57,58] respectively (Table S6), iron was excluded from our study because its modeled solubility at pH 7.0 was below 10^{-3} μM (Table S2). We also excluded aluminum because it is typically found under highly acidic conditions in the environment[59] and was not measurable at our experimental conditions (Table S2).

We found that ssRNA incubated with 8.3(±0.4) μM lead (pK_a 7.46−7.60,[57,58,60] Table S6) had a final concentration below the lowest quantifiable standard (3.1 ng/μL), indicating complete ssRNA degradation (Figure 1D). We further observed that ssRNA incubated with 13(±1) μM copper (pK_a 7.50−7.95,[57,58,61] Table S6) had a final concentration of 20.3(±0.2) ng/μL, showing that copper catalyzed ssRNA hydrolysis relative to the metal-free control, albeit to a lower extent than lead. In comparison, 13(±2) μM zinc (pK_a 8.96−9.00,[57,58,62] Table S6) and 7.9(±0.1) μM nickel (pK_a 9.897,[57,58] Table S6) did not hydrolyze ssRNA relative to the metal-free controls. Overall, we found that the ability of metal ions to catalyze ssRNA hydrolysis correlated inversely with their pK_a values, with lower pK_a values indicating stronger Lewis acidity. In addition to Lewis acidity, other factors (e.g., polarizability[59]) may influence metal-catalyzed hydrolysis, suggesting that lead's unique reactivity could be due to its large ionic radius and correspondingly high polarizability.[63]

After identifying lead and copper as the metals with the greatest ability to catalyze ssRNA hydrolysis, we evaluated whether metals with stronger Lewis acidity would also catalyze dsRNA hydrolysis in addition to ssRNA hydrolysis. We found that dsRNA was not hydrolyzed compared to the metal-free control by any of the metal ions at ∼10 μM at pH 7.0 and ambient temperatures (Figure 1D). It is likely that dsRNA is recalcitrant to metal-catalyzed hydrolysis due to its secondary duplex structure,[37] which restricts phosphodiester bond cleavage.[36]

The observed trend of increasing ssRNA hydrolysis with increasing metal ion Lewis acid strength indicates that the Lewis acidity controls the ssRNA hydrolysis extent. Based on our results, we propose that environmentally abundant metals catalyze ssRNA hydrolysis by acting as Lewis acids.[29] The mechanism is initiated by activation of the phosphorus center by either the metal ion or the metal center of a metal−hydroxide complex (Figure 1E).[22] Next, the 2′-hydroxyl group is deprotonated either by the hydroxide ions in solution or by the metal−hydroxide complex. The deprotonated oxyanion then performs an intramolecular nucleophilic attack on the activated phosphorus center, leading to the formation of a 2′,3′-cyclic monophosphate. The end products of the metal-catalyzed RNA hydrolysis reaction are the 2′ and 3′-nucleoside monophosphates.[29] Similar to metal-catalyzed RNA hydrolysis, in base-catalyzed hydrolysis, the deprotonated 2′-hydroxyl group performs an intramolecular nucleophilic attack leading to phosphodiester bond cleavage.[41] In both catalyzed mechanisms, we observe that ssRNA hydrolyzes to a greater extent as compared to dsRNA.

Effects of pH and Ligands on Metal-Catalyzed ssRNA Hydrolysis. In addition to temperature and metal species, other factors (e.g., solution pH) may alter the extent of metal-catalyzed ssRNA hydrolysis. The environmentally relevant range of solution pH values spans acidic conditions (e.g., pH 3.85 in soil pore waters[64]) to alkaline conditions (e.g., pH 8.0−9.0[65,66] in some wastewater and surface waters). Increasing pH values will lead to concomitant concentration increases in both the metal−hydroxide complexes and hydroxide ions in the solution. Based on the proposed reaction mechanism (Figure 1E), increases in the concentration of either species would lead to increased metal-catalyzed hydrolysis. Previously, increasing pH from just 6.7 to 7.3 led to a ∼10-fold increase in the hydrolysis rate constant of a diribonucleotide catalyzed by a zinc complex at 64 °C.[22] Similarly, an increase in pH from 5.0 to 6.0 led to a ∼20-fold increase in the hydrolysis rate constant of a 2′,3′-cyclic monophosphate catalyzed by a macrocyclic zinc−amine complex at 90 °C.[29] Therefore, we hypothesized that metal-catalyzed RNA hydrolysis would be accelerated at

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alkaline conditions relative to acidic conditions within environmentally relevant ranges.

To select pH values to use to test this hypothesis, we established criteria to investigate ssRNA hydrolysis by copper and lead (the two metals tested that catalyzed hydrolysis, Figure 1D) to facilitate the interpretation of results. First, considering that RNA hydrolysis is catalyzed by both acidic and alkaline conditions,[19,41] we aimed to use pH values at which ssRNA would be stable in the absence of metals. Second, we selected pH values at which copper and lead were soluble at 1 μM concentrations or higher. Finally, we selected pH conditions that could be achieved using buffers that do not bind metal ions.

Using these criteria, we found that only a narrow pH range of 5.8−7.0 could be used to test the effect of pH on lead and copper-catalyzed ssRNA hydrolysis while being assured that metals would not precipitate. Specifically, to achieve solubilities of lead and copper greater than 1 μM, the pH values were limited to below 7.0 (Table S2). Additionally, acetate (pK_a 4.76)[67] and citrate (pK_a 4.76)[67] buffers were excluded because they bind to lead[68−71] and copper.[69,70,72] In contrast, MES (pK_a 6.10)[73] and MOPS (pK_a 7.14)[73] buffers do not bind lead[74] or copper.[75] In the absence of buffer, we observed that solutions prepared with RNA and lead or copper at pH 4.9 increased in pH values to 5.8−5.9 over the experiment duration, verifying that buffers are needed to control the pH. These requirements narrowed the applicable pH range for our experiments to 5.8−7.0, based on the optimum buffering range of MES and MOPS buffers. Over this pH range, ssRNA degradation in the absence of metal species was negligible (Figures S7 and 1D). In a prior study, ssRNA degradation rates were the slowest at pH 5.4 and 6.7 when tested across a pH range of 3.2−9.0 (250 mM buffer, 1 mM EDTA) at 75 °C.[53]

Based on these restrictions, we evaluated ssRNA (25 ng/μL) degradation in the presence of lead and copper via gel electrophoresis in solutions with pH values measured to be 5.8 and 6.7. In the presence of lead and copper, ssRNA concentrations were similar at both pH values after incubation at 25 °C for 24 h (Figure 2A). However, the hydrolysis products migrated to a greater distance at pH 6.7 as compared to pH 5.8, especially for lead (Figure 2B). A greater migration distance corresponds to smaller molecular weight (i.e., nucleotide length) hydrolysis products because smaller molecules migrate faster in the agarose gel. Although copper hydrolysis products also migrated to a greater distance at pH 6.7 as compared to pH 5.8 (Figure 2C), the difference was less pronounced than for the experiments with lead. Overall, ssRNA was hydrolyzed to a greater extent (corresponding to smaller hydrolysis products) at pH 6.7 as compared to pH 5.8 for both lead and copper incubations, in agreement with our hypothesis that increasing pH would lead to greater extents of hydrolysis.

In addition to solution pH, ligands may alter metal-catalyzed hydrolysis by binding metal ions (e.g., as shown in surface, soil pore, and wastewater systems[35,76,77]). Ligands in environmental systems can include both inorganic (e.g., sulfate, carbonate, bicarbonate, chloride) and organic (e.g., citrate) species.[35,76,77] In previous studies, ligands have been found to either slow down[24,26,29] or accelerate[27,34,78] the metal-catalyzed hydrolysis reaction depending on the ligand species. For example, imidazole accelerated the zinc-catalyzed hydrolysis of a diribonucleotide ∼3-fold at pH 7.0 and 80 °C, due to the concerted action of the Lewis acid (zinc ion) and base (imidazole).[34] In contrast, citrate slowed down the europium-catalyzed hydrolysis of a 2′,3′-cyclic monophosphate ∼370-fold at pH 5.2 and 90 °C, which was attributed to metal−ligand binding.[29] Therefore, we hypothesized that the extent of ssRNA hydrolysis would be influenced by the complexation between metal ions and ligands. We quantified the extent of metal−ligand binding via stability constant, β (eq 2).[56]

$$M^{2+} + L^{-} = ML^{+}; \quad \beta = \frac{\{ML^{+}\}}{\{M^{2+}\}\{L^{-}\}} \tag{2}$$

We assessed the effect of environmentally relevant ligands (i.e., chloride, carbonate, sulfate, citrate) on the extent of lead-catalyzed ssRNA hydrolysis at neutral pH. Although bicarbonate is present at concentrations ∼10^3-fold higher than carbonate at neutral pH, lead-carbonate is the dominant lead-bound ligand species at our experimental conditions due to carbonate's ∼10^4-fold higher stability constant[58] (Table S7). The selected ligands are typically present at concentrations up to 1 mM in surface waters (Table S7),[79] but we tested them at two distinct concentrations wherein either the free metal ion or the metal−ligand complex was dominant. At a ligand concentration of 50 μM, free metal ions are in molar excess of the metal−ligand complex for chloride, carbonate, and sulfate (Table S7). In contrast, for citrate, the metal−ligand complex is the dominant metal form in the solution (Table S7). At neutral pH, we found that ssRNA concentrations were below the lowest quantifiable standard (3.1 ng/μL) across all of the tested ligands, indicating that ligands at a concentration of 50 μM did not affect the extent of lead-catalyzed hydrolysis (Figure S8). However, compared to the ligand-free control, the migration distance decreased in the presence of sulfate (SO_4^{2-}), chloride (Cl^-), and carbonate (CO_3^{2-}), indicating that ligands impede metal-catalyzed hydrolysis (Figure S9). The hydrolysis products migrated the smallest distance in the presence of citrate ($citrate^{3-}$), which had the highest $\log(\beta \times [L])$ value. A high $\log(\beta \times [L])$ value indicates a higher concentration of the metal−ligand complex as compared to the free metal ion, implying that a strong binding of metal ions with ligands might limit their ability to mediate ssRNA hydrolysis. Additionally, citrate is a tridentate ligand,[80] and its multiple coordination sites may also contribute to a greater inhibition of lead-catalyzed ssRNA hydrolysis relative to bidentate or monodentate sulfate,[81] carbonate,[82] and chloride[81] ligands.

We next investigated the effect of ligands at a concentration of 50 mM on the extent of lead-catalyzed ssRNA (25 ng/μL) hydrolysis. At this ligand concentration, the metal−ligand complex will be the dominant metal form in solution for all ligands (Table S7). For the ligand-free control, evaluated at the same experimental conditions as the ligand samples, the concentration of ssRNA was below the lowest quantifiable standard (Figure 3). However, in the presence of chloride (Cl^-) and sulfate (SO_4^{2-}), the concentration of ssRNA increased to 13(±1) and 19.5(±0.3) ng/μL, respectively. The ssRNA concentration was measured to be 17.1(±0.3) and 22(±2) ng/μL in the presence of carbonate (CO_3^{2-}) and citrate ($citrate^{3-}$), respectively, showing that metal−ligand binding inhibits ssRNA hydrolysis (Figure 3). The reduced ability of the lead ions to catalyze hydrolysis upon complexation is likely due to their associated loss of electrophilicity,[24] which results in weaker electrophilic activation of the

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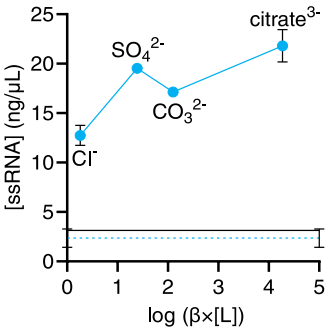

Figure 3. Effect of ligands on lead-catalyzed ssRNA hydrolysis at 24 °C over a period of 24 h. The ssRNA was incubated with ligands: citrate ($citrate^{3-}$, $\log\beta$ 5.67), carbonate (CO_3^{2-}, $\log\beta$ 6.53), sulfate (SO_4^{2-}, $\log\beta$ 2.69), and chloride (Cl^-, $\log\beta$ 1.56) at total concentrations of 50 mM in the presence of lead at measured concentrations close to 5 μM (measured and expected concentrations are provided in Table S10). Specifically, ligand concentration ([L]) was at 50 mM for all ligands except for $citrate^{3-}$ and CO_3^{2-} for which [L] was calculated using ligand acid−base speciation. Although bicarbonate (HCO_3^-) is present at higher concentrations in the solution at pH 7.0, CO_3^{2-} is the ligand of interest due to its stronger binding to lead (Table S7). Experiments were conducted with pH controlled at 7.0 by 2.34 mM MOPS buffer. To keep ligand concentration consistent, we did not control ionic strength. Error bars represent the range of two replicate experiments, and the black solid line indicates the lowest quantifiable standard (3.1 ng/μL). The ligand-free control is represented by a blue dashed line with a concentration below the lowest quantifiable standard.

phosphorus center of the phosphodiester bond. As a result, none of our tested ligands accelerated RNA hydrolysis, in contrast to imidazole.[34] It is likely that the combination of lower complexation ($\log\beta$ 1.1)[83] and high basicity (pK_b 7.028)[83] of imidazole resulted in hydrolysis rate acceleration due to concerted Lewis acid/base catalysis.[34] Overall, our results suggest that the optimum solution chemistry of metal-catalyzed RNA hydrolysis at ambient temperature and neutral pH is in the absence of ligands.

Effect of Metal Concentrations on Metal-Catalyzed RNA Hydrolysis. While metals at similar concentrations promote ssRNA hydrolysis according to their Lewis acidity (Figure 1D), metal species have different dissolved concentration ranges in the environment, which may influence their relative potential to catalyze RNA hydrolysis in these systems. Among the selected metals, zinc and nickel are the weakest Lewis acids (with metal-bound water pK_a values of 8.96−9.00 and 9.897,[57,58,62] respectively, Table S6) but are typically present in the environment at dissolved concentrations higher than lead, a much stronger Lewis acid. For example, concentrations of zinc have been reported up to 0.4 μM in rivers[30] and averaging 1.2(±0.4) μM in a municipal wastewater influent.[31] Similarly, nickel concentrations have been reported up to 0.2 μM in rivers[30] and averaging 0.14(±0.05) μM in municipal wastewater influent[31] (Table S2). However, even when included at concentrations at least 5-fold higher than their highest environmental concentrations, neither zinc (5−15 μM) nor nickel (2−8 μM) caused measurable RNA hydrolysis (Figure S10). Therefore, despite having environmental concentrations that are ∼10−300-fold higher than lead (Table S2), zinc and nickel are unlikely to cause RNA degradation at their environmental concentrations due to their low Lewis acidities.

Unlike zinc and nickel, we observed that copper and lead, which are stronger Lewis acids, catalyzed ssRNA hydrolysis at micromolar concentrations (Figure 4). In agreement with our

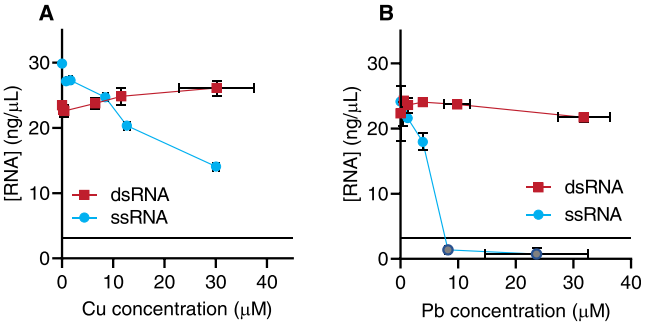

Figure 4. Effect of metal concentrations on the extent of metal-catalyzed RNA hydrolysis at 22−26 °C over a period of 24 h. (A, B) Experiments were conducted at specified measured metal concentrations (expected concentrations in Table S11) with pH controlled at 7.0 by 2.34 mM MOPS buffer and ionic strength controlled by 10 mM NaCl. Error bars represent the range of two replicate experiments, and the solid black line indicates the lowest quantifiable standard (3.1 ng/μL). Data points below the lowest quantifiable standard are marked in gray color.

prior results at a single ∼10 μM concentration (Figure 1D), dsRNA did not significantly degrade in the presence of copper or lead over concentrations ranging up to ∼30 μM (Figure 4), which can be attributed to its duplex structure that resists metal-catalyzed hydrolysis.[36−38] In contrast to dsRNA, the concentration of ssRNA remaining after 24 h decreased proportionally as the copper concentration was increased from 0.8(±0.1) to 30(±1) μM, resulting in a minimum residual ssRNA concentration of 14.1(±0.4) ng/μL at 30(±1) μM copper (Figure 4A). In comparison to copper-mediated hydrolysis, ssRNA concentrations remaining after 24 h decreased rapidly as lead concentrations increased from 1.3(±0.1) to 8.3(±0.4) μM and were below the lowest quantifiable standard (3.1 ng/μL) at all higher lead concentrations (Figure 4B).

While both lead and copper catalyzed ssRNA hydrolysis at the metal concentration ranges tested, the extent of ssRNA hydrolysis appears much more sensitive to the concentration of lead, the strongest Lewis acid (pK_a 7.46−7.60,[57,58,60] Table S6) included in our study. This finding agrees with our observation that ssRNA hydrolysis rates accelerate dramatically as lead concentration increases (Figure 1A). However, despite lead's remarkable ability to catalyze ssRNA hydrolysis rapidly at micromolar concentrations, it is unlikely to catalyze RNA hydrolysis in the environment due to its extremely low concentrations (e.g., up to 0.02 μM in rivers, 0.004(±0.001) μM in wastewater,[31] Table S2). For example, in the presence of 0.035(±0.005) μM lead, ssRNA (1 ng/μL) degradation was not observed by RT-qPCR (Section S5). Compared to lead, although copper did not result in the same dramatic increase in ssRNA hydrolysis extents, we anticipated that copper would be more likely to catalyze ssRNA hydrolysis in the environment due to its occurrence at relatively high concentrations (e.g., up to 0.06 μM in rivers, 0.3(±0.1) μM in wastewater,[31] Table S2). However, even in the presence of 0.67(±0.03) μM copper, we still did not observe ssRNA (1 ng/μL) degradation by RT-qPCR (Section S5). Consequently, none of the tested metal ions appear to catalyze ssRNA degradation at environmental

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concentrations, although copper remains the most likely to be relevant due to its occurrence at concentrations within an order of magnitude to those required to cause hydrolysis (Figure 4A).

Environmental Implications. This study investigated whether metal-catalyzed abiotic hydrolysis promotes the degradation of RNA in environmentally relevant aqueous systems (e.g., surface water, soil pore water, wastewater). Beyond prior studies that reported metal-catalyzed phosphodiester bond hydrolysis typically for smaller molecules and high temperatures,[21−25,27−29] we confirmed that metal-catalyzed hydrolysis also occurs for longer ssRNA molecules at ambient temperature. However, metal ions and complexes are unlikely to catalyze ssRNA degradation at environmental conditions for several reasons. First, the presence of ligands in environmental aqueous systems will impede the hydrolysis reaction. Second, the low dissolved metal concentrations (Table S2), particularly of the strong Lewis acid lead, in surface[30] and wastewater[31] systems are unlikely to cause significant ssRNA degradation at conditions relevant to these water systems. Our conclusion aligns with a prior report that other organophosphorus compounds degrade in the presence of 1 mM copper and lead, but this reaction may be limited due to the low dissolved metal concentrations in the environment.[59] Among the metals tested, we consider copper the most likely to hydrolyze ssRNA in the environment due to its moderate Lewis acidity and higher environmental concentrations compared to lead.

Although metal-catalyzed RNA hydrolysis is unlikely to occur in the environment, our results evaluating reaction mechanisms and the effects of solution chemistry may be relevant to future studies on mineral surface-catalyzed RNA hydrolysis. Metals in the form of minerals have high environmental abundances,[84] so metals present as minerals might play a greater role in RNA hydrolysis than in the dissolved phase. Metal oxides including goethite and anatase have been demonstrated to cause hydrolysis of organophosphorus compounds at neutral pH.[85,86]

Our results also show that metal ions or complexes are unlikely to catalyze dsRNA hydrolysis. This finding has important implications for the environmental fate of dsRNA, especially for the risk assessment of dsRNA biopesticides. In particular, our results further demonstrate that dsRNA, which had been assumed to be unstable regardless of its secondary structure,[87] is significantly more resistant to metal- as well as base-catalyzed hydrolysis.[41] Moreover, dsRNA dissipation in sterilized pond water[16] cannot be attributed to metal-catalyzed abiotic hydrolysis.

In wastewater matrices, metal ions have been previously suggested as an additional pathway for RNA degradation.[20] However, our results indicate that RNA is unlikely to be hydrolyzed by the tested metals at wastewater concentrations.[31] Nevertheless, at elevated metal concentrations, ssRNA would degrade to a greater extent than dsRNA, indicating the higher likelihood of viral dsRNA persistence compared to ssRNA viruses. For ssRNA viruses (e.g., SARS-COV-2), viral ssRNA degradation reported in sterilized wastewater matrices[18] does not appear to be attributable to metal-catalyzed hydrolysis. Although this prior study and ours considered ssRNA degradation in aqueous solutions, viruses have been found to be concentrated in the solid fraction of wastewater (i.e., sewage sludge[88,89]). Further investigation is required to assess if metals present in sewage sludge will contribute to viral RNA degradation. In addition, it is possible that other chemically catalyzed abiotic pathways contribute to RNA loss (e.g., ammonia-catalyzed RNA hydrolysis[20]) in wastewater systems.

Our results may also inform the "RNA world" hypothesis that prebiotic RNA abiotically synthesized from common carbon molecules led to the emergence of life.[90] However, RNA degradation by metals at prebiotic conditions including iron(II) and manganese(II) challenge this theory. Although iron(II) and manganese(II) are weaker Lewis acids (i.e., pK_a values of 9.6 and 10.6, respectively[91]) as compared to the metals tested in our study, they promoted ssRNA degradation likely due to the elevated millimolar concentrations and temperature applied in the study.[53] As we reported previously for alkaline RNA hydrolysis,[41] the stability of dsRNA against abiotic degradation reactions like metal-catalyzed hydrolysis may illuminate potential RNA structures that were viable in prebiotic environments.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.1c08468.

> Materials and supplies, RNA sequence information, methodological protocols, metal concentrations, solubilities, and binding with ligands, RT-qPCR analysis of ssRNA hydrolysis and supporting results (PDF)

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Notes

The authors declare no competing financial interest.

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