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Prebiotic gas flow environment enables isothermal nucleic acid replication

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Abstract

Nucleic acid replication is a central process at the origin of life. On early Earth, replication is challenged by the dilution of molecular building blocks and the difficulty of separating daughter from parent strands, a necessity for exponential replication. While thermal gradient systems have been shown to address these problems, elevated temperatures lead to degradation. Also, compared to constant temperature environments, such systems are rare. The isothermal system studied here models an abundant geological environment of the prebiotic Earth, in which water is continuously evaporated at the point of contact with the gas flows, inducing up-concentration and circular flow patterns at the gas-water interface through momentum transfer. We show experimentally that this setting drives a 30-fold accumulation of nucleic acids and their periodic separation by a 3-fold reduction in salt and product concentration. Fluid dynamic simulations agree with observations from tracking fluorescent beads. In this isothermal system, we were able to drive exponential DNA replication with Taq polymerase. The results provide a model for a ubiquitous non-equilibrium system to host early Darwinian molecular evolution at constant temperature.

eLife Assessment

This **important** work shows how a simple geophysical setting of gas flow over a narrow channel of water can create a physical environment that leads to the isothermal replication of nucleic acids. The work presents **convincing** evidence for an isothermal polymerase chain reaction in careful experiments involving evaporation and convective flows, complimented with fluid dynamics simulations. This work will be of interest to scientists working on the origin of life and more broadly, on nucleic acids and diagnostic applications.

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Introduction

The emergence of life on Earth is still an unsolved puzzle to contemporary research. It is estimated that this event dates back approximately 3.7 - 4.5 billion years, with fossil carbon isotope signatures being the oldest evidence for life around 3.7 billion years ago [1, 2]. In order to reconstruct how early molecular life began before this time, it is crucial to identify and understand plausible geological environments, which support early prebiotic reaction networks that could have lead to the life we know today [3].

The common theory is that the Darwinian evolution of informational polymers was at the core of the origin of life [3]. Among these, nucleic acids, like RNA, stand out for their capability to both store genetic information and catalyze their own replication through transient formation of double-stranded helices [4]. These abilities allow them to mutate and evolve, enabling them to adapt to diverse environments and eventually encode, build and utilize proteins as the catalysts used in modern life.

Dilution, however, poses a significant obstacle, since such prebiotic reactions require sufficiently high concentrations of their reagents to work [5]. Large reservoirs, such as the ocean, cannot compensate for diffusion, because they lack local sources of energy to drive reaction pathways out of equilibrium [6]. The resulting homogeneity renders these environments unlikely to have harbored early molecular life [7].

Local physical non-equilibria, however, have shown the ability to up-concentrate molecules, such as nucleic acids, in a variety of different geological settings [8]. Examples range from thermal gradients in rock pores, local evaporation, re-hydration cycles of warm ponds, adsorption to mineral surfaces, heated gas bubbles in porous rocks, foams, or the eutectic phase in freeze-thaw cycles [9–17].

However, the accumulation of salts and molecules comes at a cost. Single-stranded nucleic acids replicate into double-stranded forms. These strands must separate again to complete a full replication cycle. But strand separation becomes increasingly difficult after accumulation, because the melting temperature of oligonucleotides is strongly dependent on the local salt concentration [18]. Despite high Mg^{2+} concentrations being required for replication and catalytic activity [19], they can elevate the melting temperature of nucleic acid duplex structures to levels surpassing even the boiling point of water [20]. Oligonucleotides readily hydrolyze into nucleotide fragments under these conditions, rendering high temperature spikes as a primary strand separation mechanism more detrimental than beneficial [21].

Therefore, other mechanisms are required at the origin of life to separate nucleic acid strands with minimal thermal stress, and at best combined with an environment where supplied biomolecules are accumulated from the environment and trapped for long periods of time. Examples have used pH oscillations to drive nucleic acid strand separation, which can be caused either by differential thermophoresis of ionic species or by periodic freeze-thaw cycles [22–24]. Also, dew droplet cycles in a rock pore subjected to a temperature gradient can periodically melt strands by transiently lowering the salt concentration [25, 26]. Heated gas-water interfaces were also shown to promote many prebiotic synthesis reactions [14, 27, 28]. The above scenarios require temperature gradients or thermal cycling. This creates degradation stress for nucleic acids and limits the scenarios to geological settings with a thermal gradient. Here, we investigated a simple and ubiquitous scenario in which a water flux through a rock pore was dried by a gas flux at constant temperature (**Fig.1**). This can be found in the vicinity of underwater degassing events, where gases percolate through rocks to reach the surface, or in porous rocks at

the surface exposed to atmospheric winds [29, 30]. Such a setting would be very common on volcanic islands on early Earth which also offered the necessary dry conditions for RNA synthesis[31].

We created an experimental model of such an evaporation pore, shown in **Fig.1**, and studied how combined gas and water fluxes can lead to early replication of nucleic acids. We first analyzed accumulation flow speeds at the interface in **Fig.2**, then monitored cyclic strand separation dynamics in **Fig.3**, and finally showed how both drive DNA-based replication under isothermal conditions in **Fig.4**.

Results and Discussion

Molecule Accumulation at the Gas-Water Interface

We started off by constructing a laboratory model of the rock pore shown in **Fig. 1**. Here, we focused on the key properties of the system: An upward water flux evaporating at the intersection with the perpendicular gas flux. This leads to an accumulation of dissolved molecules at the interface since they cannot evaporate. Simultaneously, the momentum transfer of the gas flux induces circular currents in water, forcing molecules back into the bulk.

In the following, we analyse how these two effects act on dissolved nucleic acids. For simplicity, we used ambient air as the gas source, enabling us to focus solely on evaporation and the resulting currents. The velocity of the water flowing in was controlled by a syringe pump and chosen to match the velocity of the water evaporating in the given geometry. This ensured reliable and stable conditions in long lasting experiments. Fixed volumes of sample solutions (containing beads, labelled molecules, salts etc.) were always loaded ahead of an influx of pure water, simulating a continuous dilution scenario.

The micro scale gas-water evaporation interface consisted of a 1.5 mm wide and 250 µm thick channel that carried an upward pure water flow of 4 nl/s 10 µm/s perpendicular to an air flow of about 250 ml/min 10 m/s (Suppl. IV). The temperature of the chamber was controlled by a water bath at 45°C, while a self-built fluorescence microscope provided imaging (See Suppl. Fig.III.1). Two-dimensional finite element simulations were performed to model the diffusion of molecules in water, as well as the flow of water and gas.

First, using a particle tracking algorithm (See Suppl. Sec. V), we measured the flow velocities of individual fluorescent 0.5 µm beads to monitor the dynamics of the water flow as the air-flux streamed across the interface (**Fig.2(a)**). As expected, these velocities were dependent on their distance from the channel walls (see Suppl. Fig. VI.1(e)). The beads that were far from the interface were moving at water inflow velocities of 15 µm/s. Closer to the interface, the velocities increased to about 1 mm/s due to the momentum transfer of the gas flow (Supplementary Movie 1). This resulted in a circular flow pattern with the vortex center right below the interface. The flow lines in **Fig. 2a**) show how the upwards water flux reaches the interface on one side of the vortex, whereas on the opposite side, the beads are pushed back down into the bulk.

The extracted traces in **Fig. 2(a)** were compared with a finite element simulation. In a two-dimensional projection of the experimental geometry, we modeled laminar gas and water flow, diffusive nucleic acid mass transport in water, interfacial evaporation dynamics, and momentum transfer of gas flowing over the water surface (Suppl. Sec. VI). In agreement with the experimental results, the simulation showed a chamber-averaged water evaporation speed of 10.5 µm/s. The tangential velocity at the interface reached 0.9 mm/s. The modeled flow speed distributions agreed well with distribution of the experimental bead velocities, as shown in **Fig.2(a,c)**.

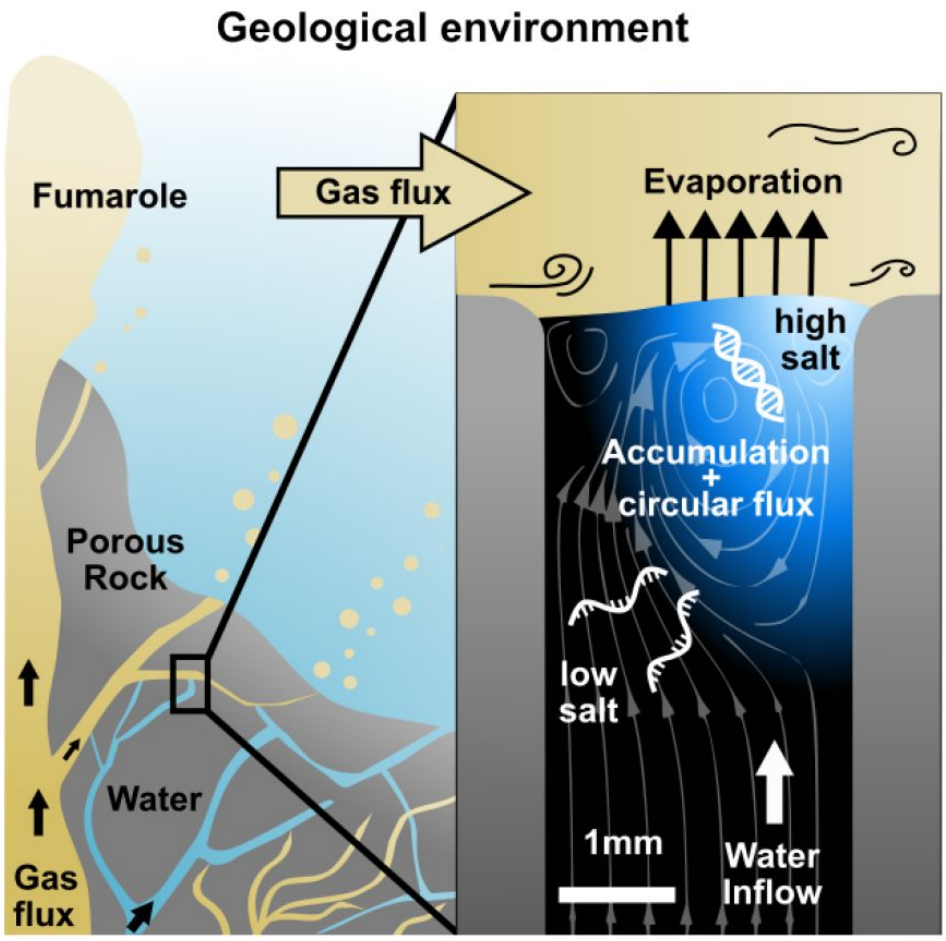

Figure 1.

Replication at the gas-water interface

We considered a geological scenario in which water, containing biomolecules, is evaporated by a gas flow at the scale of millimeters. In volcanic porous rock, many of such settings can be imagined. The gas flow induces convective water currents and causes it to evaporate. Dissolved nucleic acids and salts accumulate at the gas-water interface due to the interfacial currents, even if the influx from below is pure water. Through the induced vortex, nucleic acids pass through different concentrations of salt, promoting strand separation and allowing them to replicate exponentially. Our experiments replicate this environment on the microscale, subjecting a defined sample volume to a continuous influx of pure water with an airflux brushing across.

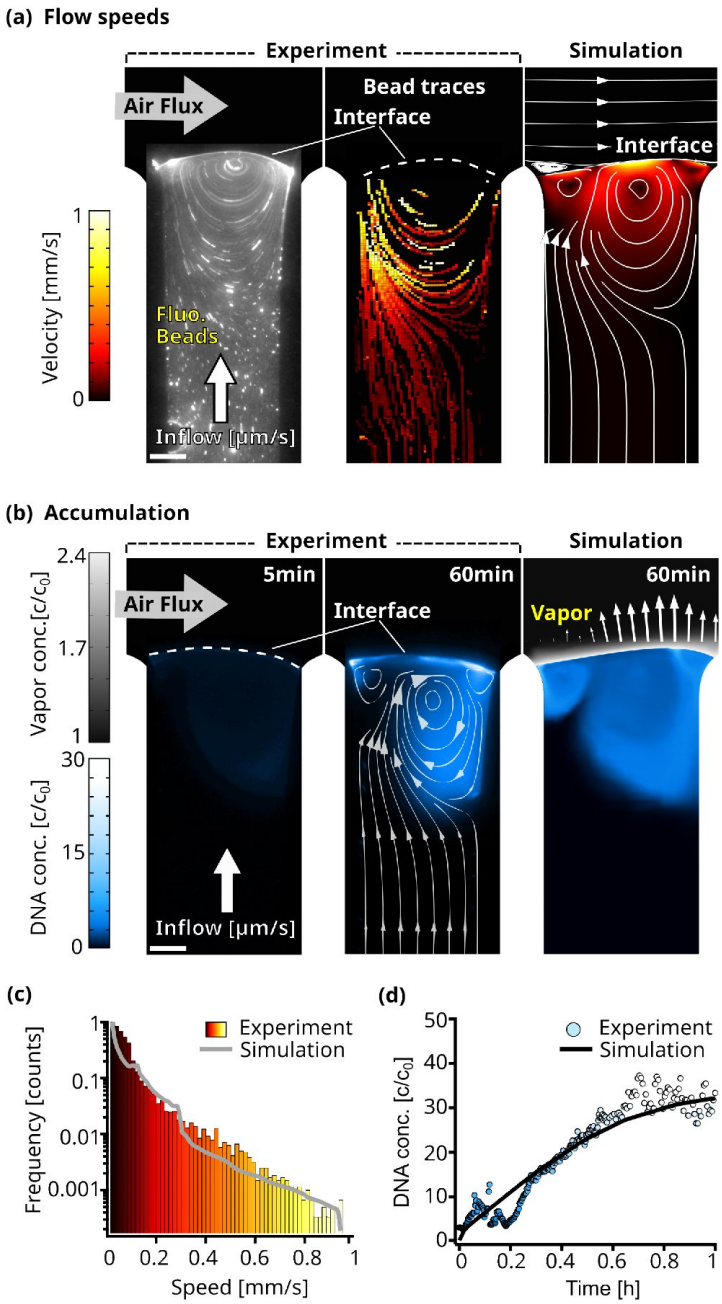

Figure 2.

Flow and accumulation dynamics

(a) Imaging of fluorescent beads (0.5 µm) reveals a flow vortex right below the air-water interface, induced by the air flux across the interface (left panel). The bead movements were traced (middle panel) and the measured velocities were confirmed by a detailed finite element simulation (right panel). The color scale is equal for both simulation and experiment and the scale bar = 500 µm. **(b)** The accumulation of fluorescently labeled 63mer DNA was imaged and confirmed our understanding of the environment based on a diffusion model. Concentration reaches up to 30 times relative to the start c0. The accumulation profile of the experiment (middle panel) and simulation (right panel) match well, showcased by overlaying the simulated flowlines. Blue colorscale represents DNA accumulation for experiment and simulation, while grey color scale shows the relative vapor concentration in the simulation. Arrows (right panel) proportionally show the evaporation speed along the interface. **(c)** The simulated and experimentally measured distribution of flow velocities of dissolved beads plotted in a histogram, showing a similar profile. Color scale is equal to (a). **(d)** The maximum relative concentration of DNA increased within an hour to ≈ 30 X the initial concentration, with values following the simulation

To further test our understanding of the dynamics of the system, we imaged fluorescently labeled nucleic acids. The expectation was that the continuous evaporation would lead to an accumulation of the strands at the interface, while the gas flow would induce a vortex analogous to the beads. Both are found to be present in the experiment and agree qualitatively with the finite element model. In our experiment, 10 µl of 5 µM FAM-labeled 63mer DNA were introduced into the system, followed by a continuous diluting pure water inflow. Temperature, water flow and air flow were unchanged from the previous experiment.

Water continuously evaporated at the interface, but nucleic acids remained in the aqueous phase and could only escape downward either by diffusion or by the vortex, thus accumulating near the interface. The evaporation rate at the interface was set to be proportional to the vapor concentration gradient and varied spatially along the interface between 5 and 10.5 µm/s (See Suppl. Fig. VI.1d)). As the gas flow continuously removed excess vapor, the evaporation rate remained constant. Thus, except for fluctuations, a stable interface shape was obtained. The vortex induced by the gas flowing across the interface pushed the molecules back deeper into the bulk (See the flow lines in **Fig.2(b)** taken from the simulation). 5 minutes after starting the experiment, the maximum DNA accumulation was 3-fold, while after one hour of evaporation, up to 30-fold accumulation was observed.

With the known diffusion coefficient of 95 $\mu m^2/s$ for the 63mer[9], a good agreement between the simulation and the experiment was achieved (**Fig.2(b)**, right panel). The DNA accumulation was located in the area where the flow induced by the gas flow created a vortex. In the simulation, the shape of the interface was kept constant, but in the experiment it fluctuated around a steady state shape, likely in response to small fluctuations in gas pressure and spatial variations in water surface tension (Supplementary Movie 2). The simulated maximum accumulation followed the experimental results and starts saturating after about one hour (**Fig.2(d)**).

Strand Separation Dynamics

As discussed earlier, strand separation is essential for the replication of nucleic acids. Only then can replication become exponential and compete with naturally exponential degradation kinetics. Usually, an elevation of temperature can separate strands but is accompanied with a higher risk for hydrolysis. The chosen isothermal setting requires changes in salt concentration for this process. More specifically, the circular fluid flow at the interface provided by the gas flux, together with Brownian motion, was expected to drive cyclic strand separation by forcing nucleic acid strands through areas of varying salt concentrations.

We used Förster resonance energy transfer (FRET) microscopy to optically measure the strand separation of DNA (**Fig.3**). A high FRET signal indicates that two DNA strands are bound, while a low FRET signal indicates that the strands are separated. In this way, FRET becomes an indirect measure of the salt concentration, since a low salt concentration will induce strand separation due to the reduced ionic shielding of the charged DNA or RNA backbones. Specifically, we chose a complementary 24mer DNA pair, with the FRET-pair fluorophores positioned centrally on opposite strands. 1 µL Sample (10 mM TRIS at pH 7, 50 µM $MgCl_2$, 3.9 mM NaCl, and 5 µM of each DNA strand) was injected into the chamber and flushed towards the interface by pure water with all other conditions equal to before.

Fig.3(a) shows micrographs of the recorded FRET values for each pixel (Supplementary Movie 3). Initially, the FRET signal increased near the interface (green), indicating areas where DNA is forming double stranded DNA. This area is localized around the vortex created by the gas flow across the interface. In the upward flow to the left of the vortex, DNA was found to be single-stranded (blue). During the course of the experiment, the low and high FRET regions remained stably separated (**Fig.3(b)**). This configuration suggests that the vortex could drive a cycle of replication and strand separation (see the scheme in **Fig.3(a)** - right panel).

To confirm this, we simulated the accumulation of Mg^{2+} ions in the chamber (Suppl. VII), since divalent ions have a large effect on the melting temperature of nucleic acids [18]. We then used a Monte Carlo random walk model (Suppl. VIII) to simulate individual 61mer DNA molecules following the vortex and undergoing Brownian motion. Such a path is shown in **Fig. 3(c)**, plotted over the simulated steady-state concentration of Mg^{2+} along with the simulated flow lines. Starting in a region of low Mg^{2+} concentration, the strand enters the vortex created by the gas flow. We have plotted the Mg^{2+}concentration along its path, showing significant salt oscillations of up to 3X the initial salt concentration, capable of inducing strand separation (**Fig. 3(d)**, and Supplementary Fig. VII.2). When plotting the simulated steady-state concentration of other dissolved – complementary – 61mer DNA molecules along its path, we observed even stronger oscillations of up to 4X the initial concentration. Together with significant drops in Mg^{2+} concentration, this suggests the possibility of exponential replication by strand separation cycles.

Isothermal Replication with PCR

We saw that nucleic acids and salts accumulated near the interface, but far from the interface, in the bulk below, the concentrations remained vanishingly low due to the diluting inflow of pure water. The air flux induced an accumulation pattern of vortices in which molecules were trapped. The salt and DNA concentration changed cyclically, resulting in periodic strand separation of nucleic acids. Motivated by the above results, we used a model system to test whether nucleic acid replication could actually be implemented in this environment.

We chose to use Taq DNA Polymerase because it does not have a protein-based strand separating mechanism. Starting with a 51mer template and two 30mer primer strands, each with a 5'-AAAAA overhang for detection, the reaction is expected to form a 61mer replicate (Suppl. IX), the same length as the DNA used in the random walk model in **Fig. 3(c)&(d)**. In contrast to standard PCR, which uses thermal cycling to separate the strands, we operated the experiment at isothermal conditions (68°C) and used 10 µl of the reaction mix (0.25 µM primers, 5 nM template, 200 µM dNTPs, 0.5 X PCR buffer,2.5 U Taq polymerase, 2 X SYBR Green I). This reaction mixture was then exposed to a constant pure water influx of 5 nl/s towards the gas-water interface, matching the rate of evaporation at the interface.

Through the oscillations in salts and DNA observed along the random walk, we expected the 61mer product strand to be able to separate from its respective template strand, enabling exponential replication. The progress was monitored using the intercalating dye SYBR Green I, which binds preferentially to double-stranded DNA [32]. **Fig.4(a)** shows fluorescence micrographs of the reaction in the chamber. Initially, minimal fluorescence is seen. This indicates that the replicated templates are below the detection limit of SYBR Green.

The SYBR Green fluorescence increased after two hours, recording the increase of replicated DNA forming duplex structures. In an identical setting when the gas- and water flux were switched off, no fluorescence increase was found. Replication was confirmed under flux with the 61mer product being visible in gel electrophoresis with depleted primers (**Fig.4(b)**). The fluorescence signal over time is shown in **Fig.4(c)**, recorded from a rectangular region of interest.

Fluorescence variations are caused by fluctuations in the position of the gas-water interface in addition to air bubbles caused by degassing of the liquid at this temperature (Supplementary Movie 4&5). With both gas flow and water influx turned off, no product band was found. We verified the replication reaction by repeating the experiments without the addition of the template, primer or DNA in the chamber as well as in a test tube (Suppl. Fig. IX.2). As expected, no product was found in any of these cases. We also compared the chamber experiment with a regular, temperature cycling based, PCR reaction in a test tube, revealing that in the chamber,

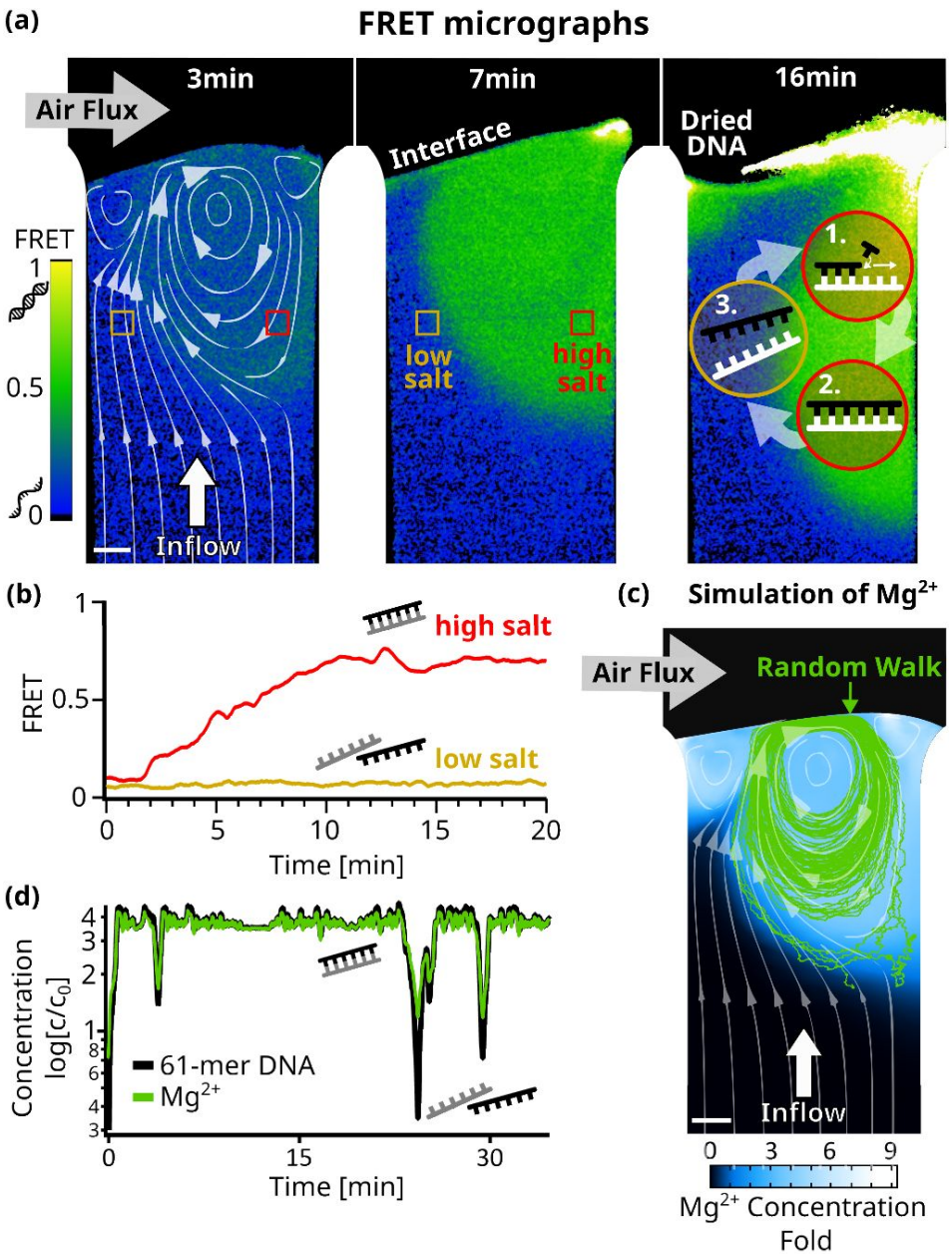

Figure 3.

Strand separation by salt cycling

Fluorescence resonance energy transfer measurements revealed cycles of strand separation. **(a)** Micrographs of 24bp DNA FRET pair in the chamber at 45°C. 1 µl sample (5 µM DNA, 10 mM TRIS pH7, 50 µM MgCl2, 3.9mM NaCl) was subjected to a 3 nl/s diluting upflow of pure water and a gas flow of 230 ml/min across. The induced vortex, shown by the simulated flow lines (left panel), overlays with regions of high FRET indicative of double-stranded DNA. The vortex flow was expected to enable replication reactions by (**1+2**) strand replication in the high salt region and (**3**) strand separation of template and replicate in the low salt region. **(b)** FRET signals confirmed strand separation in low salt regions and strand annealing in high salt regions in (a). After about 10 minutes, DNA and salt accumulated at the interface forming stable and clearly separated regions of low – where the influx from below reaches the interface – and high – located at the vortex – FRET signals. **(c)** Comsol simulation of Mg^{2+} ions (D = 705 $\mu m^2/s$ in the chamber agreed with the FRET signal and showed up to 9-fold salt accumulation at the interface. The path of a 61mer DNA molecule from a random walk model is shown by the green lines and the white flowlines are taken from the simulation. **(d)** Concentrations along the DNA molecule path in (c) show oscillations relative to the initial concentration of up to 3-fold for Mg^{2+} and 4-fold for 61mer DNA.This could enable replication cycles, as the vortex provides high salt concentrations for replication, while drops in salt and template concentrations regularly trigger strand separation.

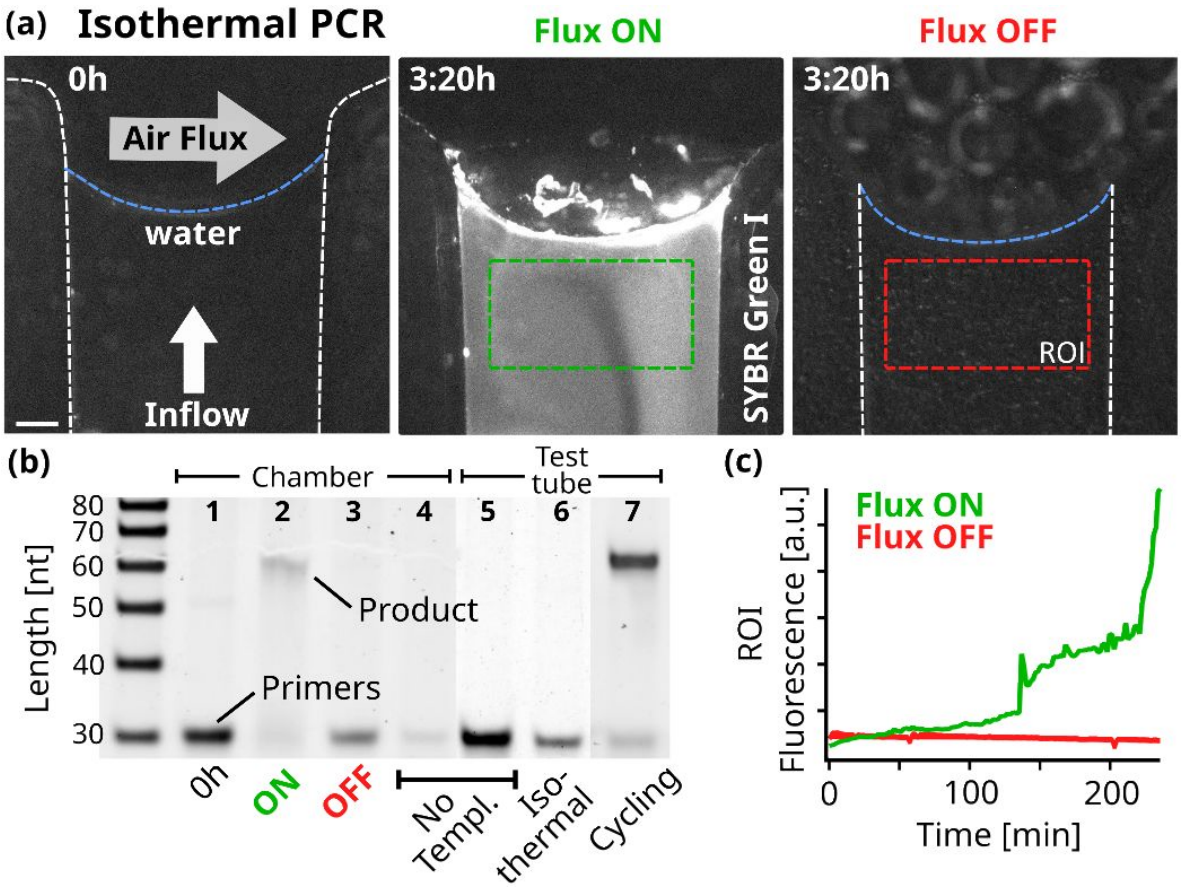

Figure 4.

Replication

(a) Fluorescence micrographs of the PCR reaction in the chamber. At isothermal 68°C, 10 µl of reaction sample was subjected to a constant 5 nl/s pure water flow towards the interface where a 250ml/min gas flowed perpendicularly. The initial state on the left shows the background fluorescence. Fluorescence increased under flux (middle, after 3:20h), while without flux the fluorescence signal remained minimal (right). The reaction sample consisted of 0.25 µM primers, 5 nM template, 200 µM dNTPs, 0.5 X PCR buffer,2.5 U Taq polymerase, 2 X SYBR Green I. Scale bar is 250 µm. **(b)** 15% Polyacrylamide Gel Electrophoresis of the reactions and neg. controls. After 4 hours in the reaction chamber with air- and water-flux ON, the 61mer product was formed under primer consumption **(2)**, unlike in the equivalent experiment with the fluxes turned OFF **(3)**. At the beginning of the experiment **(1)** or in the absence of template **(4)**, no replicated DNA was detected. The reaction mixture was tested by thermal cycling in a test tube **(5-7)**. As expected, replicated DNA was detected only with the addition of template: **(7)** shows the sample after 11 replication cycles. The sample was also incubated for 4 hours at the chamber temperature (68°C) yielding no product **(6)**. Primer band intensity variations are caused by material loss during extraction from the microfluidic chamber. **(c)** SYBR Green I fluorescence increased when gas and water flow were turned on, but remained at background levels without flow. Fluorescence was averaged over time from the green and red regions of interest shown in (a). SYBR Green I fluorescence indicates replication, as formed products are able to hybridize.

about 10-11 cycles of PCR were finished after the 4 hours of experiment (Suppl. IX). The findings above confirm that the gas flow at the simulated rock opening was necessary for nucleic acid replication.

Prebiotic chemical reactions such as polymerization of imidazole[20] or 2',3'-cyclic phosphate-activated nucleotides[28, 33] will benefit from the reduced RNA hydrolysis in the gentle isothermal replication environment. Most importantly, the combination of actively generated high concentrations by evaporation, dry-wet cycles at the interface caused by interface fluctuations, shielding from harmful UV, and the possibility of constant feeding by water influx makes the environment a compelling candidate for implementing the geophysical boundary condition of the early RNA world stage of emergent life.

Conclusion

In this work we investigated a prebiotically plausible and abundant geological environment to trigger replication of early Life. We considered an isothermal setting of gas flowing over an open rock pore filled with water. Previously, thermal gradients have been used to separate the strands of nucleic acids, risking its degradation. Now, the combined gas and water flow at an open pore trigger salt oscillations. We found that this condition supports oligonucleotide replication. We began by probing the system with fluorescent bead and DNA measurements, finding our results to agree with fluid dynamics theory using finite elements simulations. While DNA accumulates at the vortex close to the interface, oscillations in nucleic acid and salt concentration are created by a combination of molecular accumulation and interfacial flow, periodically separating nucleic acid strands under chemically gentle conditions. Due to the limitations of RNA-based replication, we probed the environment with protein-driven DNA replication and found isothermal replication in this common geological micro-environment, showing that it provides a setting for early nucleic acid replication chemistry. Physical non-equilibria, such as steep temperature gradients, pose many boundary conditions, decreasing the likelihood of readily finding such a setting. This isothermal environment, however, greatly extends the repertoire of prebiotic settings that enable replication on early planets.

Methods

A microfluidic chamber was created between two sapphire plates (0.5mm thick on the front and 1mm thick on the back), sandwiching a 0.25mm thin Teflon sheet that defined the geometry created by a computer-controlled cutter. The plates were held together by a steel frame bolted to an aluminum back to ensure gas tightness. The back was connected to a water bath (Julabo) to control the temperature. Samples were injected into the chamber using syringe pumps (Nemesys) with tubings inserted into holes in the back sapphire. Gas flow was generated under pressure control using the AF1 dual pump system (Elveflow). Temperature was measured during the experiments with a thermal sensor attached to the back sapphire. A more detailed schematic of the microfluidic chamber can be found in Suppl. IV.

DNA fluorescence measurements were performed in a self-built tilted epi-fluorescence microscope setup using two M490L4 and M625L3 light-emitting diodes (Thorlabs), a 470/622 H dual-band excitation filter (AHF), a 497/655 H dual-band dichroic mirror, and a 537/694 H dual-band emission filter. A more detailed schematic of the setup can be found in Suppl. III. DNA strands were ordered from biomers.net including purification by high-performance liquid chromatography (Suppl. II.1). The strands used for fluorescence quantification of accumulation are (5'-3')-24bp DNA: *CY5*CGTAGTAAATATCTAGCTAAAGTG, 63bp DNA: *FAM*CCAGCCTCCAGTGCCTCGTATCATTGTG-

CCAAAAGGCACAATGATACGAGGCACTGGAGGCTG diluted to 5 µM in Nuclease free water. Images were captured using a Stingray F145B camera (Allied Vision). Bead experiments used 0.5 µM fluorescent microspheres (Invitrogen) diluted 1/2000 in water (Suppl. Sec. V).

2D finite element simulations were performed using COMSOL Multiphysics 5.4. Fluid dynamics were simulated by solving the Navier-Stokes equation in two dimensions. Parameters used are available in table VI.1 in the supplementary Information. The complete description of the model can be found in Suppl. VI.

FRET imaging was performed using a second custom-built fluorescence microscopy setup consisting of light-emitting diodes (M470L2, M590L2; Thorlabs) combined by a dichroic mirror on the excitation side, while an Optoplit II with a ratiometric filter set (DC 600LP, BP536/40, BP630/50) and a Stingray-F145B ASG camera (Allied Vision Technologies) through a 1X objective (AC254 100-A-ML Achromatic Doublet; Thorlabs) detected and superimposed both fluorescence emission channels (Suppl. III). The DNA sequences used for FRET experiments were: strand 1 5'-CGTAGTAAATAT*FAM*CTAGCTAAAGTG-3', strand 2 5'-CACTTTAGCTAGAT*ROX*ATTTACTACG-3'. The two labeled complementary strands were diluted from stock solution (100 µM in nuclease-free water) and mixed together to a final concentration of 5 µM in buffer (10 mM TRIS, 50 µM $MgCl_2$, 3.9 mM NaCl, pH7). To promote annealing of the two complementary strands, the solution was heated and slowly cooled from 80°C to 4°C (ramp rate of -1°C per 5 s) in a standard thermocycler (Bio-Rad CFX96 Real-Time System) prior to each experiment.

Polymerase chain reaction (PCR) was performed using an AllTaq PCR Core Kit (QUIAGEN). Samples were mixed with 0.5 X AllTaq PCR Buffer, 5 nM template strand, 0.25 µM primers, 200 µM of each dNTP, 2 X SYBR Green I and AllTaq polymerase at 2.5 U/reaction. The reaction in the ther-mocycler was performed using a temperature protocol of 95°C for 2 minutes for heat activation of the enzyme, then annealing the primers to 52°C for 10 seconds, then 68°C for 10 seconds, and finally 10 seconds at 95°C. This cyclewas repeated 40 times (See Suppl. Fig. IX.2b)). The reaction in the chamber was performed with 10 µl of the above mixture at 68°C. The solution was also heat activated at 95°C for 2 min followed by an annealing step to 52°Cbefore loading into the chamber. The DNA sequences for the reaction were as follows Template (5'-3')-51bp DNA: TTAGCAGAGCGAGGTATGTAGGCGGGACGCTCAGTGGAACGAAAACTCACG, Reverse primer (5'-3')-30bp DNA: AAAAACGTGAGTTTTCGTTCCACTGAGCGT, forward primer (5'-3')-30bp DNA: AAAAATTAGCAGAGCGAGGTATGTAG-GCGG.

For PAGE and gel imaging, a 15% denaturing (50% urea) polyacrylamide gel with an acrylamide:bis ratio of 29:1 was solidified with TEMED (tetram-ethylethylenediamine) and ammonium persulfate. 2 µl of sample was mixed with 7 µl of 2X loading buffer (Orange G, formamide, EDTA), of which 5 µl were loaded onto the gel. Staining was performed with 2X SYBR Gold in 1X TBE buffer for 5 minutes and the gel was imaged using the ChemiDOC MP imaging station (Bio-Rad).

Data availability

Supplementary data beyond the supplementary material will be given upon request.

Author contributions

Project conception: P.S., D.B., Research design: P.S., D.B., Methodology development: P.S., Experiments: P.S., E.E. Data analysis: P.S., Programming: P.S., Manuscript writing: P.S., Manuscript reviewing: P.S., D.B., Supervision: D.B. Funding acquisition: D.B.

Competing interests

The authors declare no competing interests.

Additional information

The online version contains supplementary material available at https://...

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Editors

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Reviewer #1 (Public review):

This manuscript from Schwintek and coworkers describes a system in which gas flow across a small channel (10^-4-10^-3 m scale) enables the accumulation of reactants and convective flow. The authors go on to show that this can be used to perform PCR as a model of prebiotic replication.

Strengths:

The manuscript nicely extends the authors' prior work in thermophoresis and convection to gas flows. The demonstration of nucleic acid replication is an exciting one, and an enzyme-catalyzed proof-of-concept is a great first step towards a novel geochemical scenario for prebiotic replication reactions and other prebiotic chemistry.

The manuscript nicely combines theory and experiment, which generally agree well with one another, and it convincingly shows that accumulation can be achieved with gas flows and that it can also be utilized in the same system for what one hopes is a precursor to a model prebiotic reaction. This continues efforts from Braun and Mast over the last 10-15 years extending a phenomenon that was appreciated by physicists and perhaps underappreciated in prebiotic chemistry to increasingly chemically relevant systems and, here, a pilot experiment with a simple biochemical system as a prebiotic model.

I think this is exciting work and will be of broad interest to the prebiotic chemistry community.

Weaknesses:

The manuscript states: "The micro scale gas-water evaporation interface consisted of a 1.5 mm wide and 250 µm thick channel that carried an upward pure water flow of 4 nl/s ≈ 10 µm/s perpendicular to an air flow of about 250 ml/min ≈ 10 m/s." This was a bit confusing on first read because Figure 2 appears to show a larger channel - based on the scale bar, it appears to be about 2 mm across on the short axis and 5 mm across on the long axis. From reading the methods, one understands the thickness is associated with the Teflon, but the 1.5 mm dimension is still a bit confusing (and what is the dimension in the long axis?) It is a little hard to tell which portion (perhaps all?) of the image is the channel. This is because discontinuities are present on the left and right sides of the experimental panels (consistent with the image showing material beyond the channel), but not the simulated panels. Based on the authors' description of the apparatus (sapphire/CNC machined Teflon/sapphire) it sounds like the geometry is well-known to them. Clarifying what is going on here (and perhaps supplying the source images for the machined Teflon) would be helpful.

The data shown in Figure 2d nicely shows nonrandom residuals (for experimental values vs. simulated) that are most pronounced at t~12 m and t~40-60m. It seems like this is (1) because some symmetry-breaking occurs that isn't accounted for by the model, and perhaps (2) because of the fact that these data are n=1. I think discussing what's going on with (1) would greatly improve the paper, and performing additional replicates to address (2) would be very informative and enhance the paper. Perhaps the negative and positive residuals would change sign in some, but not all, additional replicates?

The authors will most likely be familiar with the work of Victor Ugaz and colleagues, in which they demonstrated Rayleigh-Bénard-driven PCR in convection cells (10.1126/science.298.5594.793, 10.1002/anie.200700306). Not including some discussion of this work is an unfortunate oversight, and addressing it would significantly improve the manuscript and provide some valuable context to readers. Something of particular interest would be their observation that wide circular cells gave chaotic temperature profiles relative to narrow ones and that these improved PCR amplification (10.1002/anie.201004217). I think contextualizing the results shown here in light of this paper would be helpful. Again, it appears n=1 is shown for Figure 4a-c - the source of the title claim of the paper - and showing some replicates and perhaps discussing them in the context of prior work would enhance the manuscript.

I think some caution is warranted in interpreting the PCR results because a primer-dimer would be of essentially the same length as the product. It appears as though the experiment has worked as described, but it's very difficult to be certain of this given this limitation. Doing the PCR with a significantly longer amplicon would be ideal, or alternately discussing this possible limitation would be helpful to the readers in managing expectations.

https://doi.org/10.7554/eLife.100152.1.sa2

Reviewer #2 (Public review):

Schwintek et al. investigated whether a geological setting of a rock pore with water inflow on one end and gas passing over the opening of the pore on the other end could create a non-equilibrium system that sustains nucleic acid reactions under mild conditions. The evaporation of water as the gas passes over it concentrates the solutes at the boundary of evaporation, while the gas flux induces momentum transfer that creates currents in the water that push the concentrated molecules back into the bulk solution. This leads to the creation of steady-state regions of differential salt and macromolecule concentrations that can be used to manipulate nucleic acids. First, the authors showed that fluorescent bead behavior in this system closely matched their fluid dynamic simulations. With that validation in hand, the authors next showed that fluorescently labeled DNA behaved according to their theory as well. Using these insights, the authors performed a FRET experiment that clearly demonstrated the hybridization of two DNA strands as they passed through the high Mg++ concentration zone, and, conversely, the dissociation of the strands as they passed through the low Mg++ concentration zone. This isothermal hybridization and dissociation of DNA strands allowed the authors to perform an isothermal DNA amplification using a DNA polymerase enzyme. Crucially, the isothermal DNA amplification required the presence of the gas flux and could not be recapitulated using a system that was at equilibrium. These experiments advance our understanding of the geological settings that could support nucleic acid reactions that were key to the origin of life.

The presented data compellingly supports the conclusions made by the authors. To increase the relevance of the work for the origin of life field, the following experiments are suggested:

(1) While the central premise of this work is that RNA degradation presents a risk for strand separation strategies relying on elevated temperatures, all of the work is performed using DNA as the nucleic acid model. I understand the convenience of using DNA, especially in the latter replication experiment, but I think that at least the FRET experiments could be performed using RNA instead of DNA.

(2) Additionally, showing that RNA does not degrade under the conditions employed by the authors (I am particularly worried about the high Mg++ zones created by the flux) would further strengthen the already very strong and compelling work.

(3) Finally, I am curious whether the authors have considered designing a simulation or experiment that uses the imidazole- or 2',3'-cyclic phosphate-activated ribonucleotides. For instance, a fully paired RNA duplex and a fluorescently-labeled primer could be incubated in the presence of activated ribonucleotides +/- flux and subsequently analyzed by gel electrophoresis to determine how much primer extension has occurred. The reason for this suggestion is that, due to the slow kinetics of chemical primer extension, the reannealing of the fully complementary strands as they pass through the high Mg++ zone, which is required for primer extension, may outcompete the primer extension reaction. In the case of the DNA polymerase, the enzymatic catalysis likely outcompetes the reannealing, but this may not recapitulate the uncatalyzed chemical reaction.

https://doi.org/10.7554/eLife.100152.1.sa1

Author response:

> ***Reviewer #1 (Public review):***
>
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> *confusing on first read because Figure 2 appears to show a larger channel - based on the scale bar, it appears to be about 2 mm across on the short axis and 5 mm across on the long axis. From reading the methods, one understands the thickness is associated with the Teflon, but the 1.5 mm dimension is still a bit confusing (and what is the dimension in the long axis?) It is a little hard to tell which portion (perhaps all?) of the image is the channel. This is because discontinuities are present on the left and right sides of the experimental panels (consistent with the image showing material beyond the channel), but not the simulated panels. Based on the authors' description of the apparatus (sapphire/CNC machined Teflon/sapphire) it sounds like the geometry is well-known to them. Clarifying what is going on here (and perhaps supplying the source images for the machined Teflon) would be helpful.*

We understand. We will update the figures to better show dimensions of the experimental chamber. We will also add a more complete Figure in the supplementary information. Part of the complexity of the chamber however stems from the fact that the same chamber design has also been used to create defined temperature gradients which are not necessary and thus the chamber is much more complex than necessary.

> *The data shown in Figure 2d nicely shows nonrandom residuals (for experimental values vs. simulated) that are most pronounced at t~12 m and t~40-60m. It seems like this is (1) because some symmetry-breaking occurs that isn't accounted for by the model, and perhaps (2) because of the fact that these data are n=1. I think discussing what's going on with (1) would greatly improve the paper, and performing additional replicates to address (2) would be very informative and enhance the paper. Perhaps the negative and positive residuals would change sign in some, but not all, additional replicates?*

To address this, we will show two more replicates of the experiment and include them in Figure 2.

We are seeing two effects when we compare fluorescence measurements of the experiments.

Firstly, degassing of water causes the formation of air-bubbles, which are then transported upwards to the interface, disrupting fluorescence measurements. This, however, mostly occurs in experiments with elevated temperatures for PCR reactions, such as displayed in Figure 4.

Secondly, due to the high surface tension of water, the interface is quite flexible. As the inflow and evaporation work to balance each other, the shape of the interface adjusts, leading to alterations in the circular flow fields below.

Thus the conditions, while overall being in steady state, show some fluctuations. The strong dependence on interface shape is also seen in the simulation. However, modeling a dynamic interface shape is not so easy to accomplish, so we had to stick to one geometry setting. Again here, the added movies of two more experiments should clarify this issue.

> *The authors will most likely be familiar with the work of Victor Ugaz and colleagues, in which they demonstrated Rayleigh-Bénard-driven PCR in convection cells (10.1126/science.298.5594.793, 10.1002/anie.200700306). Not including some discussion of this work is an unfortunate oversight, and addressing it would significantly improve the manuscript and provide some valuable context to readers. Something of particular interest would be their observation that wide circular cells gave chaotic temperature profiles relative to narrow ones and that these improved PCR amplification (10.1002/anie.201004217). I think contextualizing the results shown here in light of this paper would be helpful.*

Thanks for pointing this out and reminding us. We apologize. We agree that the chaotic trajectories within Rayleigh-Bénard convection cells lead to temperature oscillations similar to the salt variations in our gas-flux system. Although the convection-driven PCR in Rayleigh-Bénard is not isothermal like our system, it provides a useful point of comparison and context for understanding environments that can support full replication cycles. We will add a section comparing approaches and giving some comparison into the history of convective PCR and how these relate to the new isothermal implementation.

> *Again, it appears n=1 is shown for Figure 4a-c - the source of the title claim of the paper - and showing some replicates and perhaps discussing them in the context of prior work would enhance the manuscript.*

We appreciate the reviewer for bringing this to our attention. We will now include the two additional repeats for the data shown in Figure 4c, while the repeats of the PAGE measurements are already displayed in Supplementary Fig. IX.2. Initially, we chose not to show the repeats in Figure 4c due to the dynamic and variable nature of the system. These variations are primarily caused by differences at the water-air interface, attributed to the high surface tension of water. Additionally, the stochastic formation of air bubbles in the inflow—despite our best efforts to avoid them—led to fluctuations in the fluorescence measurements across experiments. These bubbles cause a significant drop in fluorescence in a region of interest (ROI) until the area is refilled with the sample.

Unlike our RNA-focused experiments, PCR requires high temperatures and degassing a PCR master mix effectively is challenging in this context. While we believe our chamber design is sufficiently gas-tight to prevent air from diffusing in, the high surface-to-volume ratio in microfluidics makes degassing highly effective, particularly at elevated temperatures. We anticipate that switching to RNA experiments at lower temperatures will mitigate this issue, which is also relevant in a prebiotic context.

The reviewer’s comments are valid and prompt us to fully display these aspects of the system. We will now include these repeats in Figure 4c to give readers a deeper understanding of the experiment's dynamics. Additionally, we will provide videos of all three repeats, allowing readers to better grasp the nature of the fluctuations in SYBR Green fluorescence depicted in Figure 4c.

> *I think some caution is warranted in interpreting the PCR results because a primer-dimer would be of essentially the same length as the product. It appears as though the experiment has worked as described, but it's very difficult to be certain of this given this limitation. Doing the PCR with a significantly longer amplicon would be ideal, or alternately discussing this possible limitation would be helpful to the readers in managing expectations.*

This is a good point and should be discussed more in the manuscript. Our gel electrophoresis is capable of distinguishing between replicate and primer dimers. We know this since we were optimizing the primers and template sequences to minimize primer dimers, making it distinguishable from the desired 61mer product. That said, all of the experiments performed without a template strand added did not show any band in the vicinity of the product band after 4h of reaction, in contrast to the experiments with template, presenting a strong argument against the presence of primer dimers.

> ***Reviewer #2 (Public review):***
>
> *Schwintek et al. investigated whether a geological setting of a rock pore with water inflow on one end and gas passing over the opening of the pore on the other end could*

> *create a non-equilibrium system that sustains nucleic acid reactions under mild conditions. The evaporation of water as the gas passes over it concentrates the solutes at the boundary of evaporation, while the gas flux induces momentum transfer that creates currents in the water that push the concentrated molecules back into the bulk solution. This leads to the creation of steady-state regions of differential salt and macromolecule concentrations that can be used to manipulate nucleic acids. First, the authors showed that fluorescent bead behavior in this system closely matched their fluid dynamic simulations. With that validation in hand, the authors next showed that fluorescently labeled DNA behaved according to their theory as well. Using these insights, the authors performed a FRET experiment that clearly demonstrated the hybridization of two DNA strands as they passed through the high Mg++ concentration zone, and, conversely, the dissociation of the strands as they passed through the low Mg++ concentration zone. This isothermal hybridization and dissociation of DNA strands allowed the authors to perform an isothermal DNA amplification using a DNA polymerase enzyme. Crucially, the isothermal DNA amplification required the presence of the gas flux and could not be recapitulated using a system that was at equilibrium. These experiments advance our understanding of the geological settings that could support nucleic acid reactions that were key to the origin of life.*
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> *The presented data compellingly supports the conclusions made by the authors. To increase the relevance of the work for the origin of life field, the following experiments are suggested:*
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> *(1) While the central premise of this work is that RNA degradation presents a risk for strand separation strategies relying on elevated temperatures, all of the work is performed using DNA as the nucleic acid model. I understand the convenience of using DNA, especially in the latter replication experiment, but I think that at least the FRET experiments could be performed using RNA instead of DNA.*

We understand the request only partially. The modification brought about by the two dye molecules in the FRET probe to be able to probe salt concentrations by melting is of course much larger than the change of the backbone from RNA to DNA. This was the reason why we rather used the much more stable DNA construct which is also manufactured at a lower cost and in much higher purity also with the modifications. But we think the melting temperature characteristics of RNA and DNA in this range is enough known that we can use DNA instead of RNA for probing the salt concentration in our flow cycling.

Only at extreme conditions of pH and salt, RNA degradation through transesterification, especially under alkaline conditions is at least several orders of magnitude faster than spontaneous degradative mechanisms acting upon DNA [*Li, Y., & Breaker, R. R. (1999). Kinetics of RNA degradation by specific base catalysis of transesterification involving the 2 ‘-hydroxyl group. Journal of the American Chemical Society, 121(23), 5364-5372.*]. The work presented in this article is however focussed on hybridization dynamics of nucleic acids. Here, RNA and DNA share similar properties regarding the formation of double strands and their respective melting temperatures. While RNA has been shown to form more stable duplex structures exhibiting higher melting temperatures compared to DNA [*Dimitrov, R. A., & Zuker, M. (2004). Prediction of hybridization and melting for double-stranded nucleic acids. Biophysical Journal, 87(1), 215-226.*], the general impact of changes in salt, temperature and pH [*Mariani, A., Bonfio, C., Johnson, C. M., & Sutherland, J. D. (2018). pH-Driven RNA strand separation under prebiotically plausible conditions. Biochemistry, 57(45), 6382-6386.*] on respective melting temperatures follows the same trend for both nucleic acid types. Also the diffusive properties of RNA and DNA are very similar [*Baaske, P., Weinert, F. M., Duhr, S., Lemke, K. H., Russell, M. J., & Braun, D. (2007). Extreme accumulation of nucleotides in simulated hydrothermal pore systems. Proceedings of the National Academy of Sciences, 104(22), 9346-9351.*].

Since this work is a proof of principle for the discussed environment being able to host nucleic acid replication, we aimed to avoid second order effects such as degradation by hydrolysis by using DNA as a proxy polymer. This enabled us to focus on the physical effects of the environment on local salt and nucleic acid concentration. The experiments performed with FRET are used to visualize local salt concentration changes and their impact on the melting temperature of dissolved nucleic acids. While performing these experiments with RNA would without doubt cover a broader application within the field of origin of life, we aimed at a step-by-step / proof of principle approach, especially since the environmental phenomena studied here have not been previously investigated in the OOL context. Incorporating RNA-related complexity into this system should however be addressed in future studies. This will likely require modifications to the experimental boundary conditions, such as adjusting pH, temperature, and salt concentration, to account for the greater duplex stability of RNA. For instance, lowering the pH would reduce the RNA melting temperature [*Ianeselli, A., Atienza, M., Kudella, P. W., Gerland, U., Mast, C. B., & Braun, D. (2022). Water cycles in a Hadean CO2 atmosphere drive the evolution of long DNA. Nature Physics, 18(5), 579-585.*].

> *(2) Additionally, showing that RNA does not degrade under the conditions employed by the authors (I am particularly worried about the high Mg++ zones created by the flux) would further strengthen the already very strong and compelling work.*

Based on literature values for hydrolysis rates of RNA *[Li, Y., & Breaker, R. R. (1999). Kinetics of RNA degradation by specific base catalysis of transesterification involving the 2 '-hydroxyl group. Journal of the American Chemical Society, 121(23), 5364-5372.*], we estimate RNA to have a halflife of multiple months under the deployed conditions in the FRET experiment (High concentration zones contain <1mM of Mg2+). Additionally, dsRNA is multiple orders of magnitude more stable than ssRNA with regards to degradation through hydrolysis *[Zhang, K., Hodge, J., Chatterjee, A., Moon, T. S., & Parker, K. M. (2021). Duplex structure of double-stranded RNA provides stability against hydrolysis relative to single-stranded RNA. Environmental Science & Technology, 55(12), 8045-8053.*], improving RNA stability especially in zones of high FRET signal. Furthermore, at the neutral pH deployed in this work, RNA does not readily degrade. In previous work from our lab [*Salditt, A., Karr, L., Salibi, E., Le Vay, K., Braun, D., & Mutschler, H. (2023). Ribozyme-mediated RNA synthesis and replication in a model Hadean microenvironment. Nature Communications, 14(1), 1495.*], we showed that the lifetime of RNA under conditions reaching 40mM Mg2+ at the air-water interface at 45°C was sufficient to support ribozymatically mediated ligation reactions in experiments lasting multiple hours.

With that in mind, gaining insight into the median Mg2+ concentration across multiple averaged nucleic acid trajectories in our system (see Fig. 3c&d) and numerically convoluting this with hydrolysis dynamics from literature would be highly valuable. We anticipate that longer residence times in trajectories distant from the interface will improve RNA stability compared to a system with uniformly high Mg2+ concentrations.

> *(3) Finally, I am curious whether the authors have considered designing a simulation or experiment that uses the imidazole- or 2',3'-cyclic phosphate-activated ribonucleotides. For instance, a fully paired RNA duplex and a fluorescently-labeled primer could be incubated in the presence of activated ribonucleotides +/- flux and subsequently analyzed by gel electrophoresis to determine how much primer extension has occurred. The reason for this suggestion is that, due to the slow kinetics of chemical primer extension, the reannealing of the fully complementary strands as they pass through the high Mg++ zone, which is required for primer extension, may outcompete the primer extension reaction. In the case of the DNA polymerase, the enzymatic catalysis likely*

> *outcompetes the reannealing, but this may not recapitulate the uncatalyzed chemical reaction.*

This is certainly on our to-do list. Our current focus is on templated ligation rather than templated polymerization and we are working hard to implement RNA-only enzyme-free ligation chain reaction, based on more optimized parameters for the templated ligation from 2'3'-cyclic phosphate activation that was just published [*High-Fidelity RNA Copying via 2',3'-Cyclic Phosphate Ligation, Adriana C. Serrão, Sreekar Wunnava, Avinash V. Dass, Lennard Ufer, Philipp Schwintek, Christof B. Mast, and Dieter Braun, JACS doi.org/10.1021/jacs.3c10813 (2024)*]. But we first would try this at an air-water interface which was shown to work with RNA in a temperature gradient [*Ribozyme-mediated RNA synthesis and replication in a model Hadean microenvironment, Annalena Salditt, Leonie Karr, Elia Salibi, Kristian Le Vay, Dieter Braun & Hannes Mutschler, Nature Communications doi.org/10.1038/s41467-023-37206-4 (2023)*] before making the jump to the isothermal setting we describe here. So we can understand the question, but it was good practice also in the past to first get to know the setting with PCR, then jump to RNA.

> ***Reviewer #2 (Recommendations for the authors):***
>
> *(1) Could the authors comment on the likelihood of the geological environments where the water inflow velocity equals the evaporation velocity?*

This is an important point to mention in the manuscript, thank you for pointing that out. To produce a defined experiment, we were pushing the water out with a syringe pump, but regulated in a way that the evaporation was matching our flow rate. We imagine that a real system will self-regulate the inflow of the water column on the one hand side by a more complex geometry of the gas flow, matching the evaporation with the reflow of water automatically. The interface would either recede or move closer to the gas flux, depending on whether the inflow exceeds or falls short of the evaporation rate. As the interface moves closer, evaporation speeds up, while moving away slows it down. This dynamic process stabilizes the system, with surface tension ultimately fixing the interface in place.

We have seen a bit of this dynamic already in the experiments, could however so far not yet find a good geometry within our 2-dimensional constant thickness geometry to make it work for a longer time. Very likely having a 3-dimensional reservoir of water with less frictional forces would be able to do this, but this would require a full redesign of a multi-thickness microfluidics. The more we think about it, the more we envisage to make the next implementation of the experiment with a real porous volcanic rock inside a humidity chamber that simulates a full 6h prebiotic day. But then we would lose the whole reproducibility of the experiment, but likely gain a way that recondensation of water by dew in a cold morning is refilling the water reservoirs in the rocks again. Sorry that I am regressing towards experiments in the future.

> *(2) Could the authors speculate on using gases other than ambient air to provide the flux and possibly even chemical energy? For example, using carbonyl sulfide or vaporized methyl isocyanide could drive amino acid and nucleotide activation, respectively, at the gas-water interface.*

This is an interesting prospect for future work with this system. We thought also about introducing ammonia for pH control and possible reactions. We were amazed in the past that having CO2 instead of air had a profound impact on the replication and the strand separation [*Water cycles in a Hadean CO2 atmosphere drive the evolution of long DNA, Alan Ianeselli, Miguel Atienza, Patrick Kudella, Ulrich Gerland, Christof Mast & Dieter Braun, Nature Physics doi.org/10.1038/s41567-022-01516-z (2022)*]. So going more in this direction absolutely makes sense and as it acts mostly on the length-selectively accumulated molecules at the interface,

only the selected molecules will be affected, which adds to the selection pressure of early evolutionary scenarios.

Of course, in the manuscript, we use ambient air as a proxy for any gas, focusing primarily on the energy introduced through momentum transfer and evaporation. We speculate that soluble gasses could establish chemical gradients, such as pH or redox potential, from the bulk solution to the interface, similar to the Mg2+ accumulation shown in Figure 3c. The nature of these gradients would depend on each gas's solubility and diffusivity. We have already observed such effects in thermal gradients [*Keil, L. M., Möller, F. M., Kieß, M., Kudella, P. W., & Mast, C. B. (2017). Proton gradients and pH oscillations emerge from heat flow at the microscale. Nature communications, 8(1), 1897*.] and finding similar behavior in an isothermal environment would be a significant discovery.

> *(3) Line 162: Instead of "risk," I suggest using "rate".*

Oh well - thanks for pointing this out! Will be changed.

> *(4) Using FRET of a DNA duplex as an indicator of salt concentration is a decent proxy, but a more direct measurement of salt concentration would provide further merit to the explicit statement that it is the salt concentration that is changing in the system and not another hidden parameter.*

Directly observing salt concentration using microscopy is a difficult task. While there are dyes that change their fluorescence depending on the local Na+ or Mg2+ concentration, they are not operating differentially, i.e. by making a ratio between two color channels. Only then we are not running into artifacts from the dye molecules being accumulated by the non-equilibrium settings. We were able to do this for pH in the past, but did not find comparable optical salt sensors. This is the reason we ended up with a FRET pair, with the advantage that we actually probe the strand separation that we are interested in anyhow. Using such a dye in future work would however without a doubt enhance the understanding of not only this system, but also our thermal gradient environments.

> *(5) Figure 3a: Could the authors add information on "Dried DNA" to the caption? I am assuming this is the DNA that dried off on the sides of the vessel but cannot be sure.*

Thanks to the reviewer for pointing this out. This is correct and we will describe this better in the revised manuscript.

> *(6) Figure 4b and c: How reproducible is this data? Have the authors performed this reaction multiple independent times? If so, this data should be added to the manuscript.*

The data from the gel electrophoresis was performed in triplicates and is shown in full in supplementary information. The data in c is hard to reproduce, as the interface is not static and thus ROI measurements are difficult to perform as an average of repeats. Including the data from the independent repeats will however give the reader insight into some of the experimental difficulties, such as air bubbles, which form from degassing as the liquid heats up, that travel upwards to the interface, disrupting the ongoing fluorescence measurements.

> *(7) Line 256: "shielding from harmful UV" statement only applies to RNA oligomers as UV light may actually be beneficial for earlier steps during ribonucleoside synthesis. I suggest rephrasing to "shielding nucleic acid oligomers from UV damage.".*

Will be adjusted as mentioned.

> *(8) The final paragraph in the Results and Discussion section would flow better if placed in the Conclusion section.*

This is a good point and we will merge results and discussion closer together.

> *(9) Line 262, "...of early Life" is slightly overstating the conclusions of the study. I suggest rephrasing to "...of nucleic acids that could have supported early life."*

This is a fair comment. We thank the reviewer for his detailed analysis of the manuscript!

> *(10) In references, some of the journal names are in sentence case while others are in title case (see references 23 and 26 for example).*

Thanks - this will be fixed.

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