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Peer Review

Review of: "The Limits of Life at Extremely Low Water Activity: Lithium-Concentration Ponds in a Solar Saltern (Salar de Atacama, Chile)"

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Review of

The Limits of Life at Extremely Low Water Activity: Lithium-Concentration Ponds in a Solar Saltern

Demergasso et al.

Conducting research in any type of extreme environment is a difficult endeavor. It is particularly hard in an environment such as a lithium evaporation facility, where there are multiple changing conditions and multiple inputs, each with a different chemistry and impact. That difficulty is always multiplied when it is in an environment that has not been examined before. Having worked in many extreme conditions (some for the first time as well), I was very interested in this work and the results, some of which were rather surprising and certainly raise some questions.

Before I get to those questions, I do want to say that I thought the paper was well-written, with everything clearly stated. I was also impressed by the authors recognizing and articulating the problems they faced. All too often, those of us working in these types of systems aren't able to make those admissions; in other words, the issues are rather glossed over once we lay out our procedures. Still, I have some questions and concerns.

In the field sampling, the authors described using 20 L jerry cans for sampling and how they A) washed the cans with distilled water, ethanol, distilled water, and brine before sampling; B) sampled using stainless steel dippers; and C) showed an image of two people in boots obtaining the samples. They state that "because of this, contamination is highly unlikely." I disagree with that statement. Anyone wanting to criticize this work could easily attack that, especially given the results they mention. For instance, were

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new jerry cans used? Was the distilled water sterilized? Ethanol is overrated and is actually a very poor sterilant. It not only needs long contact times, but the dilution used is critical to even provide some level of disinfection. Pure ethanol (95% or even 100%) is actually worse than 70%. The brine wash itself will certainly kill non-extremophilic (non-sporulating) organisms, but again, it needs time to work, and that time was not specified. There is also no mention of cleaning the dipper used or the waders used by the men going out into the ponds. None of this was provided here, so it is impossible to sort out the results. I only say that from years of experience in these areas and recognizing the difficulties of a study like this. In this instance, as in many, the authors seemed to feel that so-called "standard" techniques were appropriate. They are not. My team always concentrated on the methods used to obtain these initial samples since this forms the basis of the entire study. If those original samples can be questioned, everything else is questionable. Sometimes, in remote areas like this (or when one is 2500 ft. underground), this can be very difficult, but we must try hard, and we must give specific details or state why the techniques yielded high-quality samples. In this case, I did not feel that the samples (while they were usable) were truly high quality. If I were designing this sampling, I would have used new jerry cans and a stronger liquid sterilant (not Chlorox) or, if they were metal, sterilized them first. The cans should have been opened facing upstream and allowed to fill slower, with the personnel standing downstream, so what they carried in is washed away from the sample.

I certainly do accept the reality of the in-lab difficulty of filtering a concentrated brine through small filters, but I would never resort to the dialysis system using demineralized, distilled water with brines of this sort. That system would definitely kill most of the fully adapted indigenous microbes – particularly the halophilic organisms that must be present in this system at least up to the final lithium crystallizers. I can accept using dialysis solely for nucleic acid work but not for cell counts since most of the really salt-adapted organisms would lyse or become pleomorphic blobs (again, I have seen that and speak from experience). Given that the authors used centrifugation later in the work, I was surprised they did not attempt it right from the start. Dialysis in a situation like this is almost impossible to use and certainly does not maintain the osmotic conditions these organisms are used to having. The comment that the water outside the dialysis membrane does not contact the sample is not correct since dialysis cannot stop water movement. This would result in a very difficult direct estimate of cell numbers in a sample. Plus, DAPI does not work all that well in high salt, but if it was used on the dialyzed material, organisms like *Halomonadaceae* would survive (although they might be more oval-shaped than normal). Consequently, I must also question the data on "cell abundance."

In looking at the primers used for this work, I noted that they are primarily attuned to bacterial variable regions. The primers used to target the 16S of Archaea are generally 304F and 1000R, or for halophiles, primers like AR333F, UNI1533R; AR344F GC; or AR915R would have been a better addition to this study (at least if I were doing the work). These should have at least been included as separate primers in the PCR work.

In regard to the culture work, LB is a great medium to support the growth of non-salt-adapted cultures. I don't recall ever seeing it used for work in extreme conditions, largely due to the tryptone, which can have some inhibitory aspects, and because LB does not have some additional additives normal for these systems. The problem one runs into in an evaporative system such as this is that the chemistry is constantly changing; some nutrient needs precipitate away or get bound in the interstitial brines. The authors did recognize that their medium had increased A_W, which was good, so why didn't they compensate for that, as the focus of the study was life at low A_W limits? Plus, their incubation time was too short for the growth of any extreme-adapted microbes. Multiple transfers into fresh "brine" (that was not defined well to me, at least) did nothing more than select for the fastest-growing organisms, which will always trend toward transients blown into these lagoons by wind or carried by birds or other systems. It would have been better to use a medium made directly from the brine (or enrich the brine itself) using a bit of phosphate salt and either amino acids (or better yet, glycerol and pyruvate or glycerol and acetate). These are common carbon sources in environments like this, and the Archaea all seem to grow well on it, while other organisms aren't as good. Lastly, incubate for longer times to give the slower-growing adapted organisms more time.

When I looked at the supplementary data, I also became really confused about why the authors used the techniques they did. In Fig 1S, they clearly show that these brines have a lot of dissolved ions, and they knew the concentrations, so I was puzzled about why they did not compensate better. Perhaps they did, and I missed (or did not exactly follow) it given the extensive number of procedures they discuss. At the same time, I am really puzzled by the chemistry data they show for these ponds. The Boreholes clearly contain very concentrated brines, yet many of the ponds are extremely dilute. So where did all of that extra water come from? Perhaps in the next revisions, the authors should include a sort of flow diagram to show how the brine moves. Were these ponds actually numbered in reverse so that Pond 1 is at the end of the process? That is what I get from supplementary table 1. I also don't see what proof is provided by Supplementary material C-3 "fixation by Ethanol?" The extraction fluid (high KCl) bears no resemblance

to the brine, and since you are extracting nucleic acids, Ethanol is well known as the precipitant, so this is proof of a standard.

I feel that I have gone on long enough at this point. As I stated above, the methods of sampling and analysis must perforce impact the data collected. So since I have questioned that, I don't think it is fair for me to then question the discussion. So I will summarize below.

In summary, I do applaud the authors for tackling a difficult environment that has not been well studied. At the same time, I think they should have spent more time designing their overall plans and techniques. I don't question the data obtained from any study. Data cannot lie; it comes out of the experimental design, and all data is useful. As this is a first study of this system, I did examine it with interest. I think it is useful, but I must also state that I think the general profiles of species, genera, and families of huge numbers of soil (non-extremophilic) groups arose largely from the procedures I have pointed to above. While I think a study like this should occur, in the end, I must say that I think it is an underestimate of what is actually present (i.e., living in and adapted to this unique environment) and could even be characterized as a misdirection. One problem with Science in general is that we don't have a good mechanism to identify research such as this as "being a first attempt and therefore not the best" while using it to stimulate more studies. This research needs to be repeated, but I am torn because I truly do not believe it provides a clear insight into the microbial ecology of this fascinating environment and all of its implications.

I wish I could have come to a better conclusion.

Declarations

Potential competing interests: No potential competing interests to declare.