

Is there a limit for high-pH life?

During the past decade, many novel alkaliphilic and alkalitolerant bacterial species have been described, mostly from saline alkaline habitats, such as soda lakes and soda deserts. Most of the papers have been published in this journal, and in many it is claimed that the novel bacteria are capable of growth at pH as high as 11–12.5. In most of the cases, this ‘potential’ is also reflected in the name of the organism. Despite this, it is surprising how little effort has been put into obtaining such evidence. Analysis of 19 recent publications (16 in *IJSEM*, 2 in *Extremophiles*, 1 in *Applied and Environmental Microbiology*) from 1999 to 2005 demonstrated that almost none of them contain solid proof of the potential of a novel organism to grow at pH above 10 (the media not buffered properly, final pH values not measured or indicated). We consider such a situation alarming and find such papers misleading for the scientific community and general public. When working with alkaliphiles, one should realize that a claim for growth limit as high as pH 12 is very serious, since at such an external pH the cytoplasmic pH is bound to be around 10. This would demand adaptation of all intracellular enzymes to highly alkaline conditions – a strategy not yet discovered in bacteria. Up to now, the highest pH limit for bacterial growth (*Bacillus firmus*, *Nitrosomonas halophila* ANs 5), solidly proved in the pH-controlled chemostat, is 11.3–11.4 (Sturr *et al.*, 1994; Sorokin *et al.*, 2001). At higher pH, a collapse of the pH gradient maintenance was observed.

There are several possible reasons for the described situation. Naturally, it is difficult to find a proper buffering system to maintain stable pH outside the buffering capacity of the $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$ system – the only natural high alkali buffer with maximum capacity between pH 9.5 and 10.3. Used at relatively high concentrations,

it allows accurate measurement of the ability to grow at pH 8.5–10.4 for salt-tolerant haloalkaliphiles living in soda lakes, but at pH above 10.4, even at high concentration, it can not guarantee stable pH because of the reaction with atmospheric or metabolic CO_2 . However, in most of the mentioned papers, where carbonate buffer was employed, it was used at very low concentrations (usually 10 g l^{-1} , 0.18 M alkalinity), which can not maintain pH in the hyperalkaline range. The situation is even more complicated for nonhalophilic organisms isolated from alkaline ‘cement’ springs, where extremely high pH is created by the presence of very small amounts (several mM) of $\text{Ca}(\text{OH})_2$. So it is becoming clear that there is a problem in obtaining realistic pH limits for growth of newly described organisms. Below we have tried to give some advice on how to obtain realistic data on pH dependence of organisms from alkaline habitats.

First of all, it is necessary to understand what compounds can be considered ‘pH buffers’, since this is a basis for designing proper mineral media for growth and activity tests. The essence is that the buffer resists change in pH when either acid or alkali is added to it. Therefore the buffer compounds are weak electrolytes, acids or bases, which do not ionize as easily and completely as strong acids and bases such as HCl and NaOH. Therefore, when one is trying just to adjust an unbuffered medium to a certain pH with a strong acid or base, the final medium will have no buffering capacity and will have a tendency to rapidly change its pH during incubation. The pH at which the buffer compound is half-dissociated is known as its $\text{p}K_a$ value, when the undissociated acid is at equilibrium with its conjugate base. Usually, the buffer has strongest buffering capacity at $\text{pH} \pm 0.5$ of its $\text{p}K_a$. Another parameter determining buffering capacity is, of course, the concentration of the buffer (Beynon & Easterby, 1996). Also, it is very important that the buffer is not toxic for the

organism in question. A useful internet site for understanding how pH buffers work can be found at <http://www.bi.umist.ac.uk/users/mjfrbn/Buffers/Bufintro.asp>

Our experience with haloalkaliphilic micro-organisms from soda lakes and soils showed that the sodium carbonate buffer system is most appropriate for pH profiling of growth and activity at a pH range between 8.5 (pure sodium bicarbonate) and 10.4 (70 % sodium carbonate/30 % sodium bicarbonate) at concentrations higher than 0.3 M total alkalinity. Still, it was necessary to control final pH values of the cultures, especially those which produced metabolic acids, such as sulfur-oxidizing bacteria. At lower pH, the best results for growth were obtained with a combination of sodium bicarbonate (20–50 mM), NaCl (to compensate for salinity) and 0–30 % CO_2 in the gas phase at liquid/gas ratio 1 : 10. This allowed maintenance of pH between 6.7 and 8.0. A combination of NaHCO_3 and KH_2PO_4 can also be used for this range. However, it was not possible to maintain stable pH in normal batch cultivation mode, even at 1 M alkalinity, at the level above pH 10.4, using any of these buffer systems. As already mentioned above, pH-controlled chemostat cultivation might solve this problem but this technique is not routine and therefore not widely available. In case this option is not available, we recommend measuring a pH profile for metabolic activity, such as the rate of substrate consumption/oxidation in a rapid test with washed cells. In the case of aerobic organisms, measurements of oxygen consumption rates provide a rapid pH response. In this case, pH change is minimal and the carbonate buffer works even at pH as high as 11.5. Of course the profile for activity would differ from the growth profile, since the activity is only a part of growth. However, the propensity for alkaliphily can be firmly evaluated by this test. According to our experience, the activity profile is usually 1 pH unit broader in both extremes than the growth profile

with nearly the same maximum. Our usual sets of buffers for activity tests include HEPES-NaCl-NaOH for pH 6–8 and NaHCO₃/Na₂CO₃ for pH 8·5–11·5. The total sodium content is from 0·5 to 4 M and total potassium is 50 mM. Another approach might be to use a very low substrate concentration in growth experiments to minimize the pH fluctuations.

Organic pH buffers, which are claimed to be compatible with biological systems, covering the pH range from 5·0 to 11·4 are available from Sigma-Aldrich (medicine.ucsf.edu/labs/brown/sigma_buffer_chart.pdf). However, we had a negative experience with the organic buffers in our work with soda lake haloalkaliphiles. For example, such popular organic alkaline buffers as Tris/HCl, CHES, CAPSO, CAPS, CABS and glycine-NaOH usually inhibited cell activity by 50–70 % compared with natural sodium carbonate buffers. One of the possible reasons might be too high a concentration used (50–100 mM). On the other hand, neutral buffers from the sulfonate family, such as MOPS and HEPES, were usually much less inhibitory even for growing cells.

The situation faced by researchers studying nonhalophilic alkaliphiles, such as bacteria living in 'freshwater cement springs' formed

during serpentinization of ultrabasic rocks (Pedersen *et al.*, 2004; Tiago *et al.*, 2004), is even more difficult than in the case of haloalkaliphiles, since such organisms can not grow at high osmotic pressure and therefore the growth medium can not be properly buffered. The extremely high pH in such habitats (up to 12·7) is created by the presence of Ca(OH)₂ soluble up to ~20 mM. However, as soon as it comes in contact with atmospheric or metabolic CO₂, the alkalinity is removed and neutral CaCO₃ is precipitated. The main strategy to grow bacteria from the alkaline spring at very high pH should be based on preventing interference from CO₂. For this purpose, sodium carbonate buffer at low concentrations not inhibitory for salt-sensitive microbes, natural Ca(OH)₂ or CABS (pK_a = 10·7) can be used. It is obligatory, however, to monitor the pH during growth and to evaluate the potential to grow at pH above 10 by taking into consideration pH changes in the cultures.

Overall, researchers working with alkaliphilic/alkalitolerant organisms should be more careful when evaluating the pH dependence of newly described species. Use of proper pH buffering systems and monitoring pH change during growth experiments is highly recommended.

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