Investigating the Use of Phosphate Removing Organisms in Bioremediation

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Abstract This study aims to investigate the use of phosphate removing organisms in bioremediation, testing the phosphate removal capabilities of 3 microorganisms, *Bacillus subtilis*, *Pseudomonas putida* and *Saccharomyces cerevisiae* under various conditions. To determine the concentration of residual phosphates, phosphate test kits were used along with a colorimeter. The findings revealed that all 3 microorganisms were capable of phosphate removal, although *S. cerevisiae* performed the best at 84% removal. In general, synergistic effects between the microorganisms were found to be present. Additionally, immobilisation of *S. cerevisiae* within calcium alginate beads was found to reduce phosphate removal capability, although *S. cerevisiae* still managed to achieve a significant percentage of phosphate removal. Immobilised *S. cerevisiae* cells were able to remove phosphates after being reused, albeit at a lower percentage. Dead *S. cerevisiae* cells were also found to be capable of removing phosphates. Our findings suggest that *S. cerevisiae* is the microorganism best suited for bioremediation, and that immobilisation can be a viable technique given the benefits it provides, such as allowing for cell reuse and protecting cells against hazardous conditions, as well as not disrupting the marine ecosystem by introducing *S. cerevisiae* cells directly into water.

Keywords Phosphates · Phosphate removal · Microorganisms · *S. cerevisiae* · Immobilisation

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1 Introduction

Eutrophication is a serious problem worldwide, threatening natural aquatic ecosystems, especially in suburban areas and developing countries [1]. Eutrophication induces hypoxia, depleting oxygen levels in water and causing marine life to die off, as well as reduces water quality [14]. Eutrophication is mainly caused by excess nutrient loading which promotes excessive algal growth [15]. This is especially so for phosphates, which are considered the limiting factor for the growth of algae [12]. Sources of excess nutrients include excessive use of fertilisers and pesticides in agriculture [11] and excessive soil erosion caused by unrestricted deforestation (DebRoy et al. [7].

Status quo methods to control eutrophication include chemical precipitation [10] and the use of powerful algaecides [6]. However, these methods have been found to be expensive, ineffective and to cause second-hand pollution. The use of bioremediation as an alternative has become increasingly prevalent as a cheaper and more effective alternative to control eutrophication. Bioremediation involves the use of phosphate removing organisms (PAO), a group of microorganisms that are capable of removing phosphates by accumulating phosphates within their cells as polyphosphates [13].

In particular, *Bacillus subtilis*, a gram-positive bacteria strain, has shown capability for phosphate removal. Anyako and Obot [2] found that *B. subtilis* was capable of removing up to 66% of phosphates present in iron ore, even considering that the iron ore had anti-microbial properties which caused the *B. subtilis* population to drop significantly over the course of the 7 week experiment. Similarly, *Pseudomonas putida*, a Gram-negative bacteria strain, has also demonstrated the ability to remove phosphates. Cai et al. [4] showed that *P. putida* was capable of quick and efficient phosphorus removal. They found that in one hour under anaerobic conditions, *P. putida* managed to remove 96% of phosphorus from activated sludge. The yeast *Saccharomyces cerevisiae* has also previously demonstrated the ability to remove phosphates. Breus et al. [3] reported that cells of *S. cerevisiae* removed 40% of phosphates from the media containing concentrations of phosphate and glucose, and this percentage increased up to 80% upon addition of 5 mM magnesium sulfate.

Immobilisation is a technique widely used in bioremediation, as it offers various advantages [16]. These include allowing for cell reuse, reducing the need for costly cell recovery and recycle, as well as providing resistance to extreme conditions such as extreme pH, temperature, presence of toxic chemicals and heavy metal ions, etc. Lau et al. [9] and Chevalier and De la Noue [5] reported that immobilisation does not inhibit the function of microorganisms in terms of bioremediation, but in fact catalyses it, due to numerous reasons including providing high flow rates, allowing high volumetric productivities, and providing suitable micro environmental conditions.

2 Objectives and Hypotheses

2.1 Objectives

Our objectives are to screen the effectiveness of different species of bacteria and yeast in the removal of phosphate, to investigate the effect of pH on the rate of removal of phosphates, investigate the possible synergistic effects of co-inoculating different combinations of bacteria on the amount of phosphates removed, to investigate the effectiveness of immobilised bacteria and yeast in phosphate removal, as well as to determine if living and non-living cells remove phosphates to the same extent.

2.2 Hypotheses

Our hypotheses are that different species of bacteria and yeast can remove phosphates to varying degrees, that bacteria show the highest rate of removal of phosphates at their optimal pH of growth, that co-inoculation of a mixture of bacteria demonstrates a synergistic effect in the removal of phosphates, higher than the summation of their individual phosphate removal effects, that immobilised bacteria and yeast are capable of removing phosphates from wastewater with efficiency similar to that of non-immobilised bacteria, and that living cells remove phosphate at a higher rate than non-living cells.

3 Methods and Materials

3.1 Experimental Variables

See Table 1.

Table 1 Experimental variables

Independent variables	Dependent variable	Controlled variables
Species of bacteria and yeast used	Final concentration of phosphates	Initial concentration of phosphates
pH value of phosphate medium		Absorbance of microorganism precultures at 600 nm
		Temperature of incubation

3.2 Procedure

3.2.1 Growth of Microorganism Precultures

Bacteria required (*Bacillus subtilis* ATCC19659 & *Pseudomonas putida* ATCC31800) were inoculated into 10 ml LB broth and grown overnight at 30 °C in a shaking incubator. The yeast (*Saccharomyces cerevisiae* Carolina) was inoculated into 10 ml potato dextrose broth and likewise grown overnight at 30 °C in a shaking incubator. The absorbance of each microorganism culture at 600 nm was then standardised at 0.800 using a UV–vis spectrophotometer.

3.2.2 Preparation of Phosphate Medium

Phosphate medium was prepared containing (per litre): 10 g glucose, 0.1 g KH_2PO_4, 0.5 g $(NH_4)_2SO_4$, 0.2 g NaCl, 0.1 g $MgSO_4.7H_2O$, 0.2 g KCl, 0.5 g yeast extract, 0.002 g $MnSO_4{\cdot}H_2O$ and 0.002 g $FeSO_4{\cdot}7H_2O$.

3.2.3 Phosphate Removal Test

In test setups, microorganism precultures were inoculated into phosphate medium at a final concentration of 20% (v/v). In the control setups, the same volumes of LB broth and potato dextrose broth were inoculated into phosphate medium. 3 replicates of each setup were prepared. Setups were then incubated at 30 °C for 1 day in a shaking incubator, and concentration of residual soluble phosphates were determined using the phosphate test kits (Hach) and a colorimeter. 0.1 ml of each setup mixture was added to 9.9 ml of deionized water to dilute phosphate concentration by a factor of 100. The contents of one sachet from the phosphate test kit (Hach) was then added to the diluted sample. The sample was then thoroughly shaken and left for 2 min, before concentration of phosphates was measured by a colourimeter (a part of the phosphate test kit).

3.2.4 Investigating Effects of pH on Removal of Phosphates

The pH value of the phosphate medium was then adjusted to 6 and 8 using the pH probe and sodium hydroxide/hydrochloric acid. Microorganism precultures were then added to phosphate medium of varying pH values as described above, with phosphate medium at pH 7 serving as control. 3 replicates of each setup were prepared. Then, the phosphate removal test as described above was carried out to determine the concentration of residual soluble phosphate.

3.2.5 Testing for Synergistic Effects in Phosphate Removal

In test setups, various combinations of microorganism precultures were inoculated into phosphate medium at a total final concentration of 20% (v/v). The following combinations were tested: equal volumes of *B. subtilis* and *P. putida* (10% each), equal volumes of *B. subtilis* + *S. cerevisiae* (10% each), equal volumes of *P. putida* + *S. cerevisiae* (10% each), and equal volumes of *B. subtilis* + *P. putida* + *S. cerevisiae* (6.67% each). In control setups, similar volumes of LB broth/potato dextrose broth were inoculated into phosphate medium with similar volumes as shown in the setups above. 5 replicates of each setup were prepared. The phosphate removal test as detailed earlier was again carried out to determine the concentration of residual soluble phosphate.

3.2.6 Removal of Phosphates by Cells Immobilised in Calcium Alginate Beads

5 ml of broth culture of *Saccharomyces cerevisiae* was mixed with 2% sodium alginate solution in equal volumes. The mixture was then added dropwise into 0.1 M calcium chloride solution to produce calcium alginate beads containing entrapped cells. In test setups, beads were added into 4 ml phosphate medium. In control setups, beads containing entrapped potato dextrose broth, non-immobilised *S. cerevisiae* broth culture and non-immobilised potato dextrose broth were added into similar volume of phosphate medium as in the test setups. 5 replicates of each setup were prepared. Phosphate removal test as described earlier was then carried out to determine the concentration of residual soluble phosphate.

3.2.7 Removal of Phosphates by Living and Non-living Cells

Half the volume of the *S. cerevisiae* preculture was removed and immersed into a boiling water bath for 10 min. Boiled and unboiled precultures of *S. cerevisiae* were inoculated separately into phosphate medium at a final concentration of 50% (v/v) for test setups. In control setups, potato dextrose broth was added to phosphate medium at a final concentration of 50% (v/v). 5 replicates of each setup were prepared. Absorbance of each setup at 600 nm was measured. Phosphate removal test as described earlier was then carried out to determine the concentration of residual soluble phosphates.

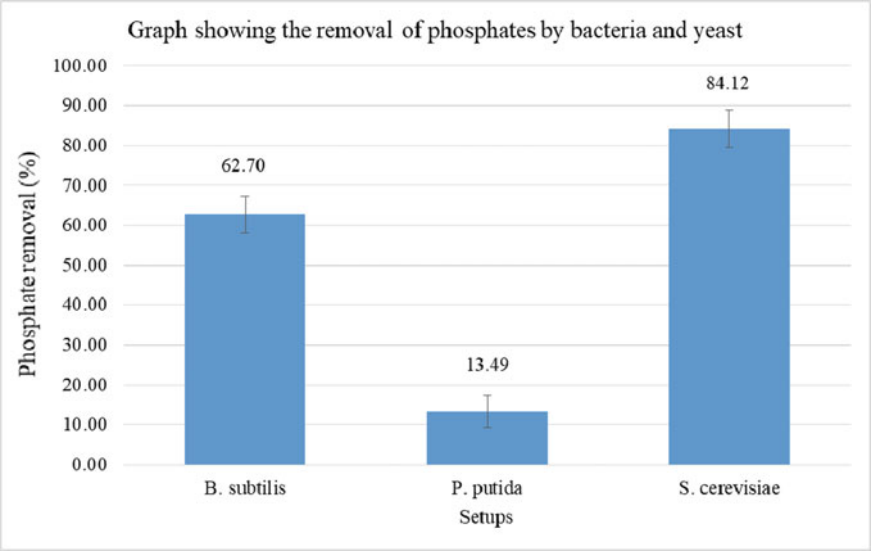

Fig. 1 Graph showing phosphate removal by various microorganisms

4 Results

4.1 Phosphate Removal Test

Figure 1 shows our results from the initial screening test for phosphate removal capability. Phosphate removal as a percentage was calculated by finding difference between phosphate concentration in the control setup (LB broth for bacteria, PDB broth for yeast) and test setup, divided by the phosphate concentration in the control setup.

B. subtilis and *P. putida* achieved 62.7% and 13.5% phosphate removal respectively in our initial screening tests. *S. cerevisiae* outperformed both the bacteria, achieving 84.1% phosphate removal. Thus, we decided to focus on *S. cerevisiae* in the tests for immobilisation and reusability, as well as the tests for phosphate removal by living and non-living cells.

4.2 Effect of pH on Phosphate Removal

Figure 2 and Table 2 illustrate the results of our phosphate removal tests carried out on the 3 microorganisms at various pH.

B. subtilis achieved optimal phosphate removal at pH 7, and was significantly affected by any change in pH. *P. putida* achieved optimal phosphate removal at pH 8,

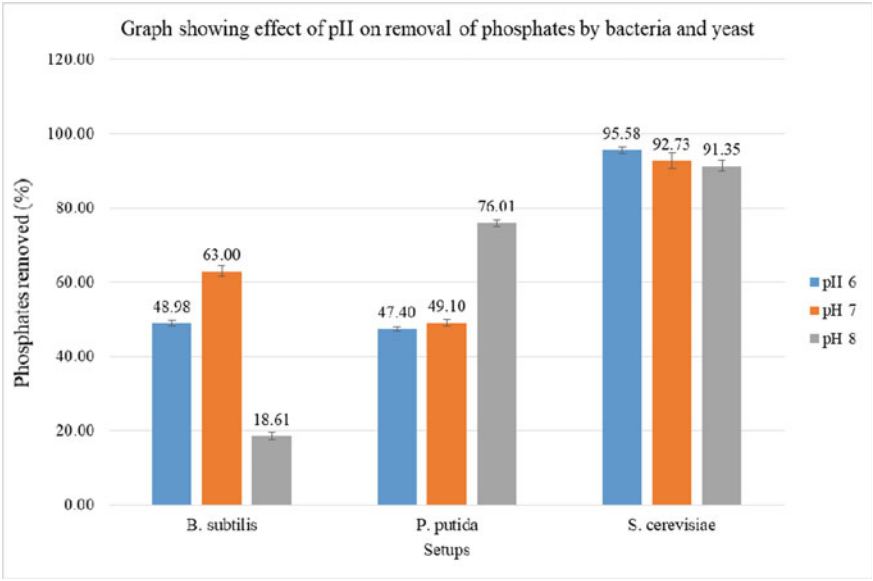

Fig. 2 Graph showing effect of pH on phosphate removal by various microorganisms

Table 2 Effect of pH on phosphate removal

Microorganism	Removal at pH 6/%	Removal at pH 7/%	Removal at pH 8/%
B. subtilis	47.6	63.0	18.6
P. putida	45.9	49.1	76.0
S. cerevisiae	95.6	92.7	91.7

and was similarly affected by a drop in pH. *S. cerevisiae* achieved optimal phosphate removal at pH 6, and was not significantly affected by an increase in pH, remaining consistent at around above 90% removal, indicating that *S. cerevisiae* is resistant to pH variations.

4.3 Synergistic Effects in Phosphate Removal

Table 3 demonstrates how we compared the different setups to show if there was a synergistic effect.

To determine if synergistic effect was present, we calculated the average removals of setups with individual microorganisms (expected removal), and compared it to the actual phosphate removal by the setup with a mixture. As can be seen from the table, the actual removal by the combined setup exceeded average removal by individual setups, showing a synergistic effect. As the Kruskal–Wallis p value was below 0.05

Table 3 Synergistic effects in phosphate removal

Combination	Removal by combined setup/%	Average removal of individual setups	Kruskal–Wallis p value
B. subtilis + *P. putida*	9.8	6.8	0.01729
B. subtilis + *S. cerevisiae*	87.9	52.3	0.00192
P. putida + *S. cerevisiae*	81.6	50.6	
B. subtilis + *P. putida* + *S. cerevisiae*	69.5	36.6	

for all mixtures, it indicates a significant difference in percentage phosphate removal, demonstrating a clear synergistic effect.

4.4 Phosphate Removal by Immobilised S. cerevisiae *Cells*

Figure 3 shows the phosphate removal by both immobilised and non-immobilised yeast cells.

Compared to the non-immobilised yeast cells, the immobilised yeast cells achieved 73.4% phosphate removal capability. The Mann–Whitney U test p value was 0.011, showing that there is a significant difference between percentage removal of immobilised and non-immobilised yeast. However, most of the phosphate removal

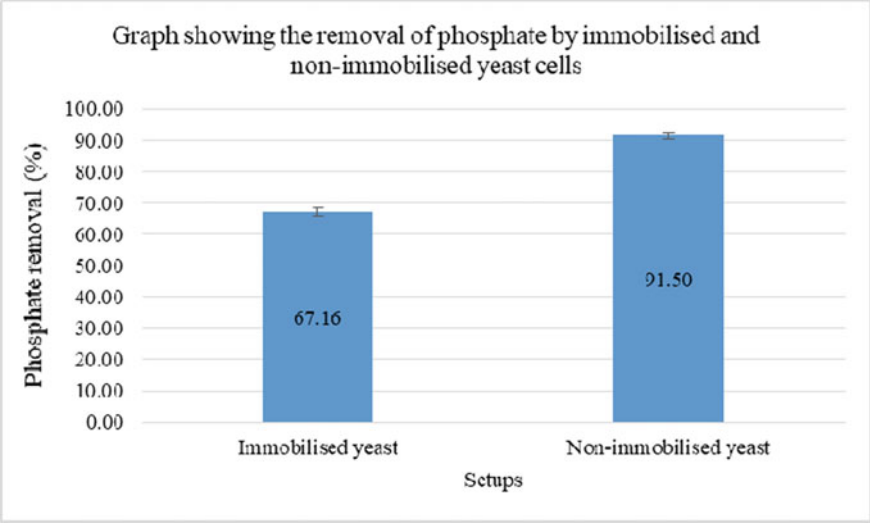

Fig. 3 Graph showing effect of immobilisation on phosphate removal by *S. cerevisiae*

capability is preserved when *S. cerevisiae* is immobilised, and immobilisation is still a viable technique for phosphate removal.

4.5 Phosphate Removal by Living/Non-living Cells

Phosphate removal values were divided by absorbance at 600 nm to account for cell division in the unboiled culture, as shown in Table 4. The adjusted phosphate removal values are also shown in Fig. 4.

After adjusting for absorbance, it can be seen that both the boiled and unboiled cultures achieved relatively similar phosphate removal, suggesting that even boiled

Table 4 Removal of phosphates by boiled and unboiled cultures, adjusted for absorbance

	Boiled culture	Unboiled culture
Average phosphate removal (%)	30.2	56.8
Average absorbance at 600 nm	0.942	1.538
Adjusted phosphate removal (%)	32.1	36.9

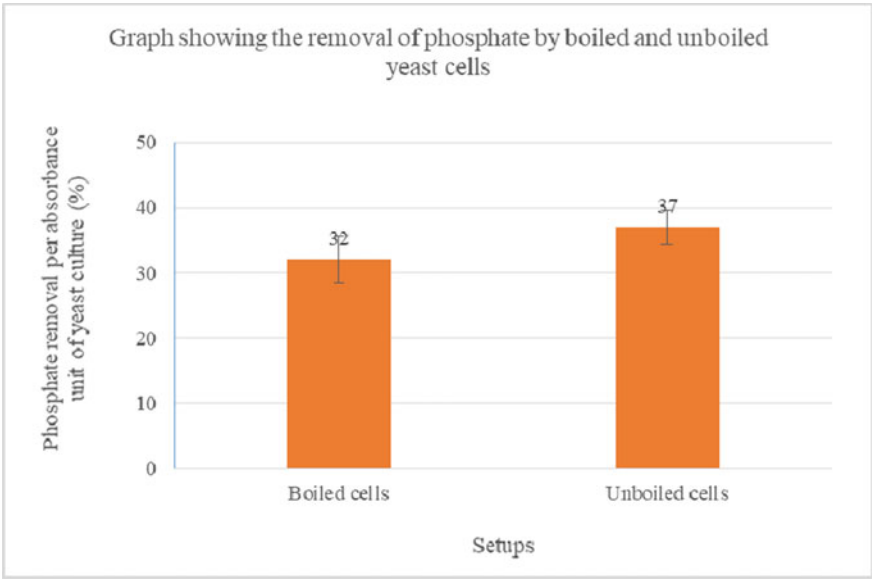

Fig. 4 Graph showing phosphate removal by boiled and unboiled *S. cerevisiae* cells

S. cerevisiae was capable of significant phosphate removal. The Mann–Whitney U-test p value was 0.4009, demonstrating that there was no significant difference in phosphate removal capability of living and non-living *S. cerevisiae* cells.

4.6 Reusability of Immobilised S. Cerevisiae

Figure 5 shows the results of phosphate removal by reused immobilised yeast cells and fresh immobilised yeast cells.

Compared to fresh immobilised yeast, the reused immobilised yeast achieved 80.4% phosphate removal capability. The Mann–Whitney U test *p* value was 0.209, showing that there was no significant difference in percentage phosphate removal of fresh and reused immobilised yeast cells. However, given that phosphate removal capability only decreased by about 20% with each reuse of the immobilised yeast cells, immobilisation of yeast cells could allow reuse for a few cycles, showing the potential benefit of immobilisation.

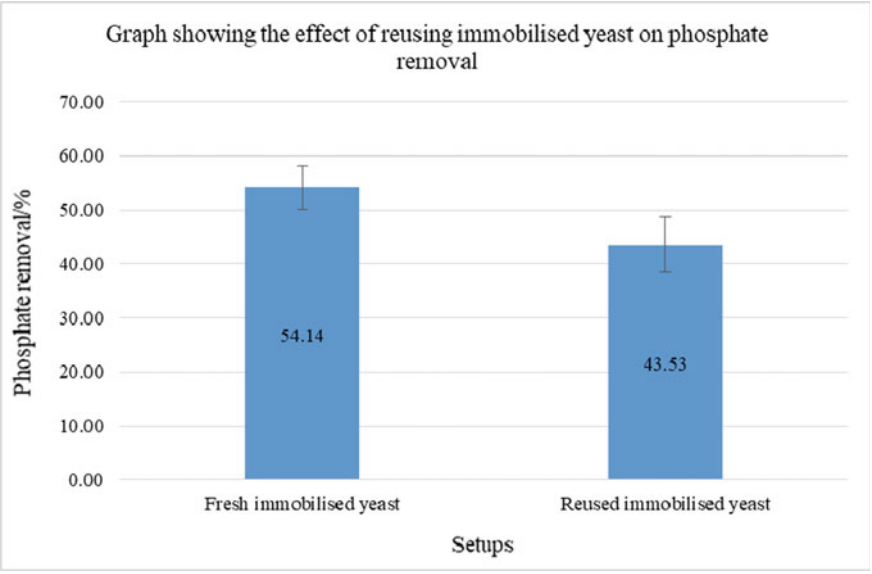

Fig. 5 Graph showing phosphate removal by freshly immobilised yeast cells and reused immobilised yeast cells

5 Conclusion and Discussion

Our project discovered that *S. cerevisiae* was the most efficient microorganism for phosphate removal, and that it also demonstrated a resistance to pH changes. We also found that immobilisation of *S. cerevisiae*, although impacting phosphate removal capability, remained a viable option for bioremediation, and that dead *S. cerevisiae* cells were still capable of phosphate removal. Lastly, we found that *S. cerevisiae* demonstrated a synergistic effect in phosphate removal when co-inoculated with *B. subtilis* and/or *P. putida.*

Other researchers have found that phosphate transport and signaling in *S. cerevisiae*, specifically by the PHO84 and PHO87 transporters, does not require ATP or metabolism to be activated, only a presence of glucose [8]. This would allow dead *S. cerevisiae* cells to remove phosphates in the presence of glucose and phosphates in phosphate medium by transporting them into the cell to be stored as polyphosphates, which supports our findings.

In general, although this was not supported by our findings, other researchers have found that immobilisation could in fact promote the removal of phosphates. Nakamura et al. (1995) found that bacterium strain *Microlunatus phosphovorus* NM-1, when immobilised in polyacrylamide gel, rapidly took up phosphates present in the medium under aerobic conditions, with a phosphate take-up rate of about 10–20 mg-P/g-cell·h. Similarly, Swe Cheng et al. (2017) found that *Scenedesmus bijugatus* when immobilised still achieved a rapid phosphate removal rate of 0.25 mg L^{-1} d^{-1}.

Even though immobilisation was shown to negatively impact phosphate removal, the effect of immobilisation on phosphate removal was shown to not be very severe. Immobilisation confers numerous benefits onto the bioremediation process, such as protecting microorganisms from toxic pollutants and heavy metal ions, as well as granting increased resistance to temperature and pH changes. It also improves efficacy of bioremediation by allowing for cell reuse in multiple batches, and also ensures that microorganisms do not contaminate the final treated product. As such, the tradeoffs of bioremediation indicated by our project appear to be worth it in real-life application of bioremediation.

Some limitations of our research include that the cell counts of bacteria/yeast may differ between setups and experiments due to differing growth rates, which would result in varying degrees of phosphate removal both within experiments and between experiments. Immobilised and non-immobilised yeast cells may also have reproduced at different rates, affecting final cell count which could not be accounted for using absorbance (unlike our experiment involving boiled and unboiled cells).

For further work, more investigation into the optimal conditions (temperature and concentration of nutrients) for *S. cerevisiae* to remove phosphates is needed, along with investigation into other potential synergistic effects in phosphate removal following co-immobilisation of *S. cerevisiae* with other microorganisms.

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