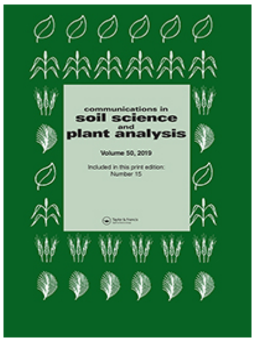

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Method for Preparation of Nutrient-depleted Soil for Determination of Plant Nutrient Requirements

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ABSTRACT
Research for nutrient optimization and crop nutrient use efficiency requires precise control on soil nutrient status. While nutrient-depleted soils are preferable to artificial soils or hydroponics, reliable and affordable methods for nutrient removal are lacking. We report the systematic standardization and validation of a simple method to wash soil with purified deionized water for the removal of nutrients such as nitrogen (N), phosphorus (P), potassium (K), calcium (Ca) and organic carbon. Sandy soil was washed with RO water (1:1, w/v) followed by several washes with Type 1 ultrapure water (2:1, w/v) and tested after each wash for the removal of organic carbon, N, P, K, and Ca. After seven washes, total dissolved solids (TDS) were reduced to 5 ppm, conductivity to 10 µS, organic carbon content was reduced by 78%, while N by 19.5%, P by 30%, K by 48% and Ca by 29%. Two genotypes of rice were grown for full life cycle under normal and low N fertilizer (urea) levels to demonstrate that soil depleted with nutrients by our method supports normal plant growth in the greenhouse and allows experiments impossible under field conditions. Precise control on the nutrient status of the soil by our method also helped demonstrate yield differences between genotypes and N regimes and also that higher grain yields can be obtained with low nitrogen (N) input. Thus, our method facilitates better design of experiments for precise determination of nutrient requirements for crop growth and nutrient use efficiency.

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Nutrient-depleted soil; nitrogen; potassium; phosphorus; calcium; organic carbon

Introduction

Nutrient use efficiency is an important goal of sustainable agriculture, due to the adverse environmental footprint of fertilizer production and consumption. The leakage of unused fertilizer nutrients such as nitrogen (N), phosphorus (P) and potassium (K) into the environment has been a growing cause for pollution of water, soil and air (Oliveira, Ludwick, and Beatty 1971; Sutton et al. 2013; Sutton et al. 2019). An important requirement for research in this field is the preparation of nutrient-free soil for precise control of the soil nutrients status. Hydroponic systems are a convenient way to have precise control on nutrients, but they need frequent replacement, fail to provide solid support and may cause hypoxia. Ideally, the physical properties of the soil should be retained with very low or no nutrients, which has been difficult or expensive. Artificial soils such as perlite, soil rite, vermiculite, etc., are neither nutrient-free nor are amenable for complete life cycle experiments. Therefore, methods for depleting natural soil of its nutrients remain an important requirement for research and development in this field.

The major targets of nutrient use efficiency research are macronutrients such as N, P and K and soils depleted of them are critical to determine their lowest dose requirements for the crop.

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Hydrogen peroxide (H_2O_2), sodium hypochlorite (NaOCl) and disodium peroxodisulfate ($Na_2S_2O_8$) have been reported for the reduction of soil organic matter and removal/destruction of nitrogen (N) (Anderson 1961; Jackson 1956; Meier and Menegatti 1997; Mikutta and Kaiser 2011; Mikutta et al. 2005; Sitaramam and Rao 1986; Zimmermann et al. 2007). Chloroform fumigation method was also in existence for the removal of nitrogen from soil (Brookes et al. 1985). But these methods raise concerns of occupational safety, due to the hazardous nature of the chemicals used in them. Further, acid or alkali treatment of soil can affect the properties of soil in terms of cation exchange capacity (Yiase, Adejo, and Adoga 2015), phosphate availability and solubility (Williams 1951), the chemical profile of soil (Brown 1987), and the soil variable charge (Jozefaciuk, Murányi, and Alekseeva 2002).

In wetland systems, classical approaches such as ammonification, nitrification, denitrification, plant uptake, biomass assimilation, dissimilatory nitrate reduction and the physicochemical approaches such as ammonia volatilization and adsorption have been used for the removal of nitrogen from the soil (Saeed and Sun 2012). Some of the recent methods such as Babe (Bio-augmentation batch enhanced), Sharon (Single reactor for high activity ammonium removal over nitrite), Anammox (Anaerobic ammonium oxidation), Canon (Completely autotrophic nitrogen removal over nitrite), and Deammox (Denitrifying ammonium oxidation) have been demonstrated in conventional treatment processes for the removal of nitrogen (Bertino 2011; Haandel and Lubbe 2007).

For the removal of phosphorus, methods based on adsorption have been suggested (Arias, Del Bubba, and Brix 2001; De-Bashan and Bashan 2004; Quinton, Catt, and Hess 2001) whereas for the removal of potassium, acid-based methods are known (Hunter and Pratt 1957; Oliveira, Ludwick, and Beatty 1971). Nevertheless, these methods are either too lengthy or hazardous or raise effluent issues. Therefore, there is a strong necessity to develop simple, effective and sustainable methods to deplete nutrients from the soil, at least to the extent that they provide adequate control on nutrient status for research on crop nutrition. The objective of the current study was to develop a washing protocol for depletion of soil nutrients and to validate it in terms of its utility to support plant growth and to determine its minimal nutrient requirements.

Objects and methods

Soil was collected from Churu District, situated in the Rajasthan state of India. Located in the Thar desert, Churu is one of the driest places in India and thus contains arid sandy soils, which have low organic content and an alkaline pH (Kumar, Singh, and Sharma 2009). The soil was initially washed with equal volume (1 l/kg, w/v) of Type 3 filtered water, obtained by Reverse Osmosis (RO) using Bio-Age water purification unit (Mohali, India). The concentration of total dissolved solids (TDS) in the RO water was typically 50 ppm with a resistance of 5 MΩ and conductivity of 110 micro Siemens (μS). This was followed by repeated washing with half the volume (w/v) of Type I ultrapure water obtained from the same Bio-Age unit. It typically had undetectable TDS and conductivity, with a resistance of 18.2 MΩ. After each ultrapure wash, the washings were decanted and monitored for TDS and conductivity using Aquasol EC/TDS (New Delhi, India), and the soil samples were retrieved for testing, as summarized in Table 1. Soil samples were tested in triplicates at the soil testing laboratory of the Indian Agricultural Research Institute, New Delhi for quantification of organic carbon (% w/w) (Walkley and Black 1934), nitrogen (kg/ha, Kjeldahl method), phosphorus (kg/ha) (Olsen et al. 1954), potassium and calcium (kg/ha) (One normal ammonium acetate method). The Kg/ha values were converted using the formula: Concentration (mg/g) * density (1500 g/cm3) * depth (50 cm) * 100 = kg/ha.

Greenhouse and field studies

Two rice genotypes (*O. sativa* var. Indica, cv. Aditya and Triguna) were grown through their complete life-cycle in 2 L polyethylene beverage bottles filled with washed sand (prepared as described in the previous paragraph) in the greenhouse at 28°C with 12h/12h light/dark photoperiod. The bottles were cut open at the top and were perforated at the bottom to drain excess fluid.

Table 1. Soil samples and washing treatments. The Table summarizes the number of times the soil was washed with RO water and ultrapure water prior to testing the washed soil (S1–S7). Washing went on till S10, but only data up to S7 was shown, as later samples showed no further reduction in TDS and conductivity.

Soil sample	Washing treatments
S0	Unwashed
S1	1 RO + 1 Ultra Pure
S2	1 RO + 2 Ultra Pure
S3	1 RO + 3 Ultra Pure
S4	1 RO + 4 Ultra Pure
S5	1 RO + 5 Ultra Pure
S6	1 RO + 6 Ultra Pure
S7	1RO + 7 Ultra Pure

Seeds of rice genotypes were procured from IIRR, Hyderabad, India. Arnon Hoagland medium (Hoagland and Arnon 1950) with urea as a sole source of nitrogen was used to fertilize the plants as frequently as required with normal (15mM) and low (1.5 mM) dose of urea. The experiment was performed with 32 replicates for each of the 2 genotypes and N treatments and total grain yield was recorded at the end of life cycle.

Field evaluation of the same two genotypes was performed at the farm of Indian Institute of Rice Research (IIRR), Hyderabad, India, for six seasons over 3 years (two seasons per year, Kharif and Rabi from 2010–11 to 14–15). Urea was used to fertilize the plants at 217 kg/ha or 100 kg N/ha (N100), split into three equal doses (1/3 at basal, 1/3 at tillering and 1/3 at panicle initiation stage). There was no externally added N in the control plot (N0). The soil was tested at IIRR and crop yield was measured using a sample of 1 m^2 from two different areas of each containing approximately 66 plants per sample.

Results and discussion

Repeated washing with ultrapure-deionized water rapidly reduces soil conductivity and TDS

Repeated washing with ultrapure water as summarized in Table 1 reduced the conductivity and TDS with every wash as shown in Figure 1. The TDS came down from 250 ppm after the first ultrapure wash to 5 ppm after the seventh ultrapure wash, achieving a total reduction of 98%. Similarly, conductivity was reduced from 490 µS after the first wash to 10 µS after the seventh wash. These reductions in TDS and conductivity were found significant at $p < .001$ (Figure1). There were no significant reductions in TDS and conductivity after the seventh wash, so washing was stopped after the tenth wash. Needless to say, the nature of soil and its prior nutrient composition determines the number of washes needed to achieve the desired level of nutrient depletion, but our data show that even sand requires up to seven washes to deplete its nutrients.

Washing significantly reduces levels of individual nutrients

The organic carbon content reduced sharply with each wash to 0.0267% (w/w, or 0.0266 g/100 g of soil) by the fourth wash (S4), achieving 78% reduction from unwashed sand but remained nearly constant thereafter (Figure 2(a)). The reduction in organic carbon content was significant at $p < .001$. The removal of organic carbon content by this method is far higher than by previous methods that rely on strong oxidizing agents such as hydrogen peroxide (H_2O_2) (Jackson 1956) or sodium hypochlorite (Anderson 1961; Menegatti, Frueh-Green, and Stille 1999; Jackson 1956; Meier and Menegatti 1997; Mikutta and Kaiser 2011; Mikutta et al. 2005; Soil Survey Staff 2004; Zimmermann et al. 2007). The inadequacy of these methods for effective removal of organic carbon has been reported earlier (Mitchell and Smith 1974). Even our method may require many more washes if soils

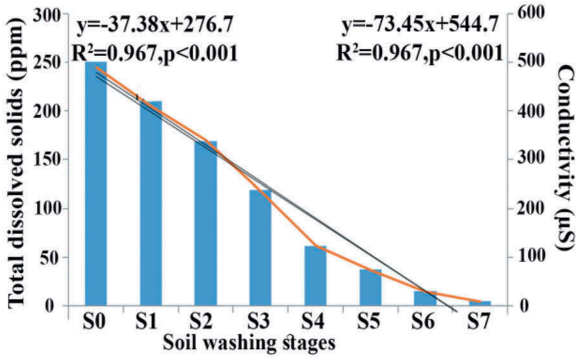

Figure 1. Total dissolved solids (TDS) and conductivity of soil at different stages of washing.

The TDS and conductivity of soil before (S0) and after different stages of washing (S1–S7 as detailed in Table 1) are shown, along with their correlation coefficients (R^2) and significance ($p < .001$).

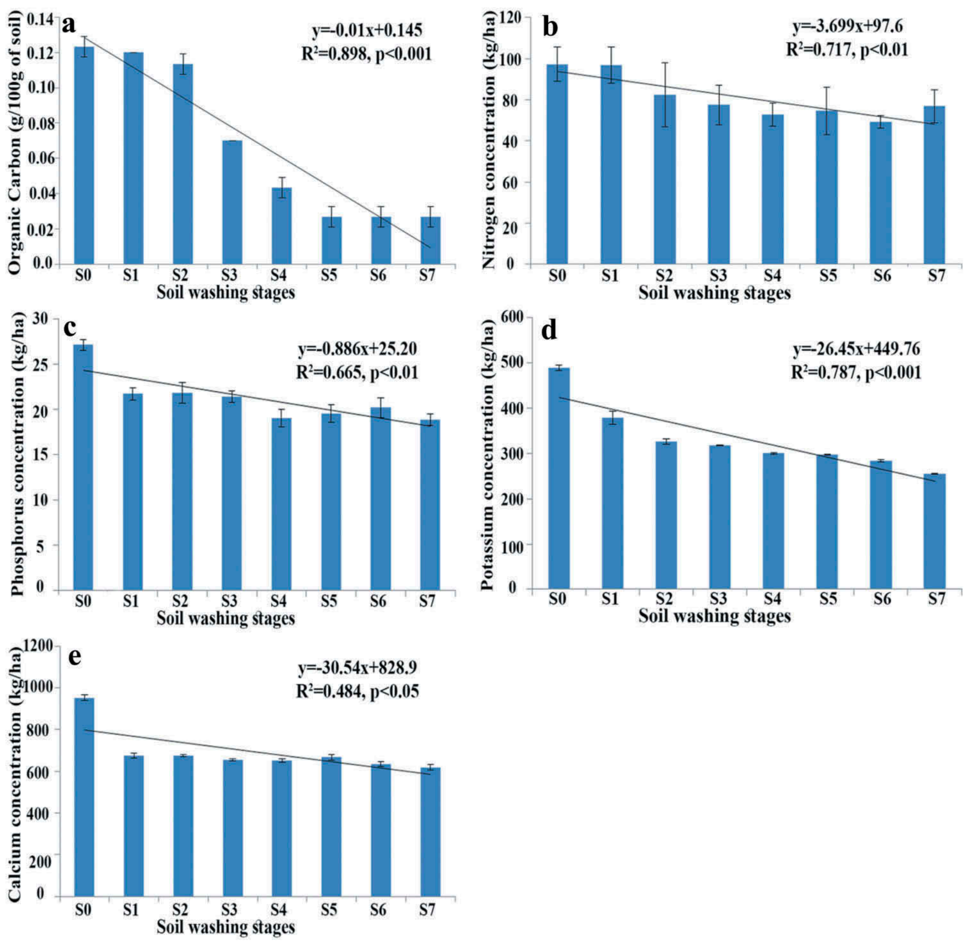

Figure 2. Effect of soil washing on nutrient depletion: The concentrations of soil nutrients after each stage of washing (as detailed in Table 1) have been plotted for: (a) organic carbon, (b) nitrogen, (c) phosphorus, (d) potassium, (e) calcium. The trend lines are shown with the respective coefficients of correlation (R^2) and significance ($P \leq .05$).

rich on organic content are to be depleted. Sandy soil is ideal for depletion of organic carbon with limited number of washes by our method, which also helps in minimizing external microbial populations in experiments that require exclusive focus on plant–soil interactions.

For the removal of nitrogen from the soil, various methods have been implemented such as chloroform fumigation method (Brookes et al. 1985), classical approaches involving ammonification, nitrification, and denitrification, physicochemical approaches such as ammonia volatilization or adsorption (Saeed and Sun 2012). More recent approaches include Bio-augmentation batch enhancement (Babe), Single reactor for high activity ammonium removal over nitrite (Sharon), Anaerobic ammonium oxidation (Anammox), completely autotrophic nitrogen removal over nitrite (Canon), and Denitrifying ammonium oxidation or Deammox (Bertino 2011; Haandel and Lubbe 2007; Orooj et al. 2014; Rashid and Seilsepour 2009; Huynh et al. 2019; Han et al. 2018). The chloroform fumigation method has a major disadvantage due to the hazardous nature of chloroform, whereas other methods are difficult to apply either due to the lack of knowledge on optimal operational conditions, or lengthy processes or environmental concerns. Our method, which is simple, non-hazardous and inexpensive, reduced the nitrogen level from an already low level in sandy soil (0.013 mg/kg or 1.3% w/w or 97.3 kg/h) to 0.0102 mg/kg (or 1.02% w/w or 76.83 kg/h) after seven washes, which is very significant ($p < .01$) (Figure 2(b)).

For the removal of soil phosphorus, a few methods based on adsorption are known (Arias, Del Bubba, and Brix 2001; De-Bashan and Bashan 2004; Quinton, Catt, and Hess 2001) but they are time-consuming and expensive. Even though sandy soils are already very low in P content, our method could further reduce it by a sharp 31% in the first four washes (S4) to 0.00251 mg/kg or 18.86 kg/h before remaining constant thereafter. This reduction of P was highly significant at $p < .01$ (Figure 2(c)). Nevertheless, adsorption based methods may be more relevant for nutrient recovery from wastewater and other polluted sources, especially in countries like India where the soils are generally P-deficient and cause import dependence.

Potassium removal by acid hydrolysis method is known to be effective (Hunter and Pratt 1957; Huynh et al. 2019) but undesirable due to the hazardous nature of the acids involved. Our washing method is a completely non-hazardous way to reduce K levels to 0.0339 mg/kg (or 3.39% w/w or 254.66 kg/h). Most of the reduction happened in the first two washes and tapered off after two more washes, achieving 39% reduction in potassium from S0-S4. Further washings produced only a reduction of an additional 9%. Our method's overall reduction of K in sandy soil by 48% is highly significant at $p < .001$ (Figure 2(d)). Similarly, calcium content too dropped in the first four washes to 0.0825 mg/kg (or 8.25% w/w or 619 kg/h) in S4, though no reduction was observed in subsequent washes (Figure 2(e)). This may be typical of sandy soil, while other soils may need more washes for depletion. Thus, our method reduced the already low calcium content in sandy soil by a further 29%, which is significant at $p < .05$.

It must be emphasized that from the point of view of plant nutrition, depletion of the availability of nutrients from the soil is adequate and absolute depletion may not be necessary as long as the residual nutrients are immobile or unavailable to the plant. Yet, our method of washing sandy soil produced nutrient levels lesser than those reported by Sigma's certificate of analysis for 'clean sand-4' (Lot LRAB2926), which mentions nutrients as undetected for nitrogen (below minimum detection limit or MDL of 0.01%), phosphorus (<MDL 0.01%) and potassium (<MDL 0.008 mg/kg), whereas the levels of calcium was 0.01 mg/kg and total carbon was 240 mg/kg. The correlation of nutrient concentrations at different stages of washing with conductivity and TDS further validates our approach (Figure 3). This clearly indicates that monitoring TDS and conductivity after every washing till they reach the minimal stable value is a good enough proxy to monitoring the concentration of each nutrient in washed soil.

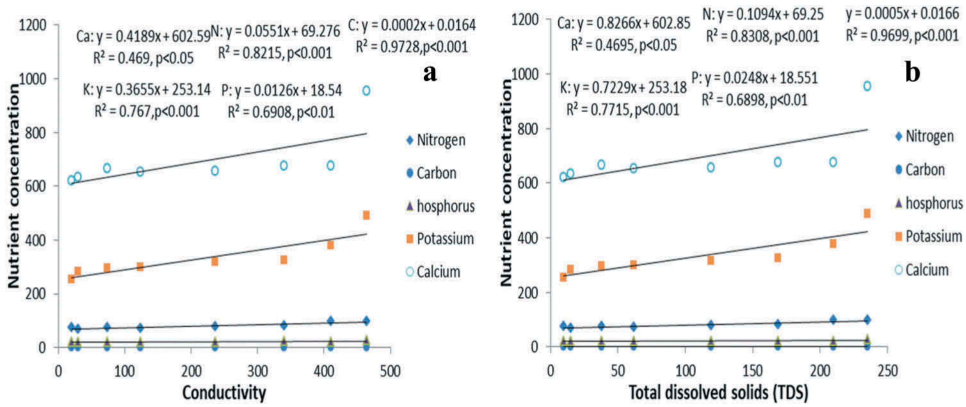

Figure 3. Correlation of nutrient depletion with conductivity and TDS.
The mean nutrient levels in the soil after each wash have been plotted against mean conductivity (a) and TDS (b). The trend lines are shown for each nutrient with the respective coefficients of correlation (R^2) and significance ($P \leq .05$).

Washed sand supports full cycle plant growth

In order to demonstrate the utility of the nutrient-depleted soil generated by our method to grow plants, two genotypes of rice were grown in pots under controlled conditions in the greenhouse as detailed in Objects and Methods. Field performance of the same two genotypes was monitored separately in farm soil. The composition of the farm soil and nutrient-depleted soil (washed sand) is provided in Table 2.

Both genotypes of rice grew normally on nutrient-depleted soil fertilized only with inorganic nutrients throughout the life cycle and set seeds. However, the grain yields were very low in washed sand as compared to the field conditions (Table 3). This could be a reflection of the generally known yield differences between the greenhouse and open fields, apart from differences in nutrient retention between washed sand and farm soil. Moreover, the microbial population in nutrient-depleted soil is expected to be very low as compared to farm soil. This could be particularly true for heterotrophs involved in nutrient cycles such as urease-producers and nitrifying microbes, which are considered critical to grow crops with urea as the sole N source. Our method demonstrates the capacity of the rice plant to utilize urea directly for its growth and grain production, and also that such capacity is limited in the absence of conditions that promote the growth of soil microbial communities. The fact that the yield drop from field soil to nutrient-depleted sand varied drastically between the two genotypes (Table 3) indicates the possible differences in their endogenous urease activity as we reported elsewhere (Sharma et al. 2018) or the differential role of endophytes. Indeed, it is possible to eliminate the role of external microorganisms in the study of soil-nutrient-plant interaction in our method by using autoclaved soil.

Table 2. Nutrient composition of nutrient-depleted soil (washed sand) and farm soil used for plant growth. The concentrations of nutrients in washed sand (after one wash with RO water and seven washes with ultrapure water) are compared with those in unwashed farm soil.

Soil parameter	Washed sand	Farm soil
Organic Carbon	0.0267%	0.70%
Available Nitrogen	75 kg/ha	215 kg/ha
Available Potassium	297 kg/ha	442 kg/ha
Available Phosphorus	20 kg/ha	46 kg/ha
Available Calcium	0.008%	5.01%

Table 3. Grain yield of two rice genotypes grown in washed sand and farm soil under different N regimes. Nutrient-depleted washed sand was obtained after one wash with RO water and seven washes with ultrapure water and fertilized with Arnon Hoagland medium containing urea as the sole source of N. Rice grain yields in it are compared with those in unwashed farm soil.

	Grain yield in normal N		Grain yield in low N (1.5 mM urea in pot, or no added N in field)	
Rice genotypes	**Yield in washed sand** (with 15 mM urea)	**Yield in farm soil** (with 100 kgN/ha urea)	**Yield in washed sand** (with 1.5 mM urea)	**Yield in farm soil** (with no added N)
Aditya	0.39 t/ha	4.44 t/ha	0.67 t/ha	2.93 t/ha
Triguna	1.08t/ha	4.53 t/ha	1.37 t/ha	3.56 t/ha

An interesting revelation from Table 3 is the higher yield obtained at lower level of added N in washed sand (1.5 mM urea, one-tenth of normal) as compared to normal level (15 mM). This trend is exactly opposite to the normal trend of lower yields in the farm soil when grown only on low or residual N. In part, this could be an effect of more frequent fertilization in the pots as compared to the three splits of urea used in the field. This could also be due to inhibition of plant growth at higher doses of N in the pots, which may have been partly offset by soil microbial action on urea. In any case, there are ample reports of better yield with lesser N input even in the field conditions (Huang et al. 2017, 2019; Li et al. 2018; Tingyu et al. 2018). Our method of nutrient depletion allows more precise determination of soil-nutrient-plant interactions and their optimization.

Conclusions

We have developed a simple, affordable, non-hazardous and effective washing method for nutrient depletion of soil. The washed soil was tested for organic carbon, N, P, K and Ca to confirm their depletion to a significant level. We also showed that TDS and conductivity are good enough proxies to measure nutrient depletion by up to 98%. The suitability of the nutrient-depleted soil generated by our method to support plant growth was confirmed by growing two genotypes of rice for a complete life cycle and demonstrating the yield differences between genotypes and N regimes. Our method can be tuned in accordance with the type of soil and allows precise control on nutrient status. It is also amenable to excluding microbial contribution to study soil-plant-nutrient dynamics in a manner that cannot be done easily in any other normal or artificial soil.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Authors’ Contribution

NS performed the greenhouse experiments, data analysis, and wrote the first draft with VJS. SK contributed the field experimental data. NR helped in the planning, mentoring and supervision of the experiments, data interpretation, and manuscript editing and finalization.

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