Elsevier Editorial System(tm) for Materials Letters
Manuscript Draft

Manuscript Number: MLBLUE-D-15-00762R1

Title: Graphene-oxide modified polyvinyl-alcohol as microbial carrier to improve high salt wastewater treatment

Article Type: Letter

Keywords: hypersaline organic wastewater; immobilization; graphene oxide; polyvinyl alcohol (PVA); halotolerant bacteria; phospholipid fatty acids (PFLAs)

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Abstract: This work discussed the preparation and characterization of graphene oxide (GO) modified polyvinyl alcohol (PVA) for bacteria immobilization to enhance the biodegrdation efficiency of saline organic wastewater. GO-PVA material has lamellar structure with higher surface area to support bacterial growth and high salinity tolerance. It significantly stimulated the bacterial population by 0.9 times from 2.07×103 CFU/mL to 5.04×103 CFU/mL, and the microbial structure was also improved for salinity tolerance. Acinetobacter, Pseudomonas and Thermophilic hydrogen bacilli were enriched inside GO-PVA materials for glucose biodegradation. Compared to the CODCr removal efficiency with only PVA as the carrier (52.8%), GO-PVA material had better degradation performance (62.8%). It is proved as a good candidate for bioaugmentation to improve biodegradation efficiency in hypersaline organic wastewater.

COMMENTS FROM EDITORS AND REVIEWERS

Reviewer #1:

Comment on the manuscript entitled "Graphene-oxide modified polyvinyl-alcohol as microbial carrier to improve high salt wastewater Treatment" by Guizhong, Zhou, Zhao feng Wang, Wenqian Li, Qian Yao, Dayi Zhang

The present manuscript describes the synthesis and characterization of graphene oxide (GO) modified polyvinyl alcohol (PVA) for bacteria immobilization. The manuscript is written well and before publication author should consider following point

1. Author should check the language of the sentence "Bacteria immobilization is an appropriate technique to for wastewater treatment [13]."

   Answer: Thanks for the comments and the author has corrected sentence. The author has also asked native English speaker for the modification of the manuscript.

2. In experimental section author has mentioned "Discarding the supernatant, the pellet was washed by 5% hydrochloride three times and deionized water two times with centrifugation at 10,000 rpm for 30 minutes.", How can be pellet formation is possible in solution. Author should check this carefully.

   Answer: The author resuspended the pellets in the NaCl and water solution, and centrifuge at 10,000 rpm for 30 min. The residual reagents and salts will be therefore removed by discarding the supernatant. The author has corrected the sentence for a clearer description.

3. What is the concentration of Graphene solution which was mixed with PVA.

   Answer: From the measurement, the concentration of graphene solution was 20 g/L. Relative information has been added in the revised manuscript.

4. In figure caption of figure 1, author has mentioned that micrographs are taken on TEM instrument. However as mentioned in analysis section (JSM-6700F), the instrument seems to be FE-SEM. Please check this carefully. Also, if it is TEM, then details of sampling procedure to make TEM-Grids should be incorporated.

   Answer: Thanks for the careful check. Figure 1a and 1b are actually SEM images and the author made wrong description in the method and discussion section. The description has been corrected in the revised version.

5. Raman studies for the Pristine Graphene oxide and PVA-coated graphene oxide should be incorporated. Also TEM/FE-SEM of pristine Graphene oxide should be done.

   Answer: Thanks for the suggestion and the author has conducted the Raman spectrum analysis for the pristine graphene oxide, PVA, and PVA-GO materials, as well as some description in the main manuscript. The SEM image of graphene oxide was also added.

Reviewer #3: This work discussed the bacteria immobilization on the GO/PVA and tested the performance in biodegradation for hypersaline organic wastewater. The subject matter of this manuscript is suitable for the journal. However, in my opinion this work has some aspects that should be taking into account before acceptance.

1. The preparation method of GO/PVA was a routine work. The novelty of this work should be addressed.

   Answer: Thanks for the comments. Previously, the preparation of GO/PVA materials is to improve the mechanic properties, and our work is the first time to investigate its performance in bacterial immobilization. The preparation process was modified slightly to maintain the bacterial friendly and keep its suitability to support bacterial growth. The author has corrected the manuscript by highlighting the novelty of this work.

2. The Fig 1a and 1b were images of SEM rather than TEM.

   Answer: Figure 1a and 1b are SEM images and the author made wrong description in the method and discussion section. The description has been corrected in the revised version.

3. The author argued that the oxygen functional groups of grapheme oxide contributed to the high hydrophilia for bacterial growth. I don't think so. From the images of SEM, the microstructure of PVA/GO is different from PVA carrier. The lamellar structure can provide more sites for bacterial to immobilize and grow. The increased surface area of GO-PVA should contribute to the increased bacterial population and high removal efficiency of high salinity wastewater.

   Answer: Thanks for the comments. The author has made clearer discussion on the reason for high hydrophilia bacterial growth on GO/PVA materials, by considering the suggestions of the reviewer and the new image of graphene oxide's SEM image. The revised sentences and statements can be found in the revised edition (marked with yellow color).

Reviewer #4: The manuscript reports the preparation and characterization of composite from polyvinyl alcohol and graphene oxide for bacteria immobilization and the subsequent evaluation of this material for biodegradation of saline organic wastewater.

Interesting data are presented, but some indications should be taken into account.

1. Why do authors use the term "graphene oxide modified polyvinyl alcohol" for the material? Is there any chemical bond between both components?

   Answer: From many previously published papers, GO has the hydrophilic functional groups which can specifically interact with PVA, such as -C=O and C-OH groups. From further Raman spectrum analysis, the shifted Raman bands also proved the interaction between GO and PVA materials. All the references and description are further stated in the revised manuscript.

2. The images from Fig. 1-a and 1-b are not TEM micrographs.

   Answer: Figure 1a and 1b are SEM images and the author made wrong description in the method and discussion section. The description has been corrected in the revised version.

3. Figure 1-d (absorption spectrum) does not support any relevant information. It could be added to supplementary information. The same for Figure 2, from a to d.

   Answer: Thanks for the comments. The author has moved these figures into the supplementary materials.

4. The results of immobilization and growth of bacteria should be before those of the waste treatment efficiencies. Some comparison with literature results should be included in order to emphasize the contribution of present work.

   Answer: Thanks for the comments. The author has reorganized the manuscript structure in accordance with the comments. Due to the space limitation, some key discussions are also made by comparing our results with previous literature.

Highlight

1. New GO-PVA material achieves 90% more halotolerant bacteria immobilization.
2. The lamellar structure of GO-PVA material supports bacterial immobilization.
3. Immobilized microbial community was identified by PFLAs analysis.
4. 32% higher removal efficiency of high salinity wastewater by GO-PVA material.

*Manuscript

Graphene-oxide modified polyvinyl-alcohol as microbial carrier to improve high salt wastewater treatment

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Abstract: This work discussed the preparation and characterization of graphene oxide (GO) modified polyvinyl alcohol (PVA) for bacteria immobilization to enhance the biodegrdation efficiency of saline organic wastewater. GO-PVA material has lamellar structure with higher surface area to support bacterial growth and high salinity tolerance. It significantly stimulated the bacterial population by 0.9 times from 2.07×10^3 CFU/mL to 5.04×10^3 CFU/mL, and the microbial structure was also improved for salinity tolerance. *Acinetobacter*, *Pseudomonas* and *Thermophilic hydrogen bacilli* were enriched inside GO-PVA materials for glucose biodegradation. Compared to the COD_{Cr} removal efficiency with only PVA as the carrier (52.8%), GO-PVA material had better degradation performance (62.8%). It is proved as a good candidate for bioaugmentation to improve biodegradation efficiency in hypersaline organic wastewater.

Key words: hypersaline organic wastewater, immobilization, graphene oxide, polyvinyl alcohol (PVA), halotolerant bacteria, phospholipid fatty acids (PFLAs)

1. Introduction

As a novel functional nano-material, graphene has drawn many attentions recently [1-3], with a wide range of applications in modified electrodes [4], chemical power sources [5], solar batteries [6], catalysts [7], pharmaceutical carriers [8] and gas sensors [9]. Containing sufficient carboxyl, carbonyl, hydroxyl and epoxy groups, graphene oxide has excellent chemical and physical properties [10]. Therefore, together with the features of high hydrophily and reaction activity, graphene oxide is suitable as the carrier material for bacteria immobilization.

Hypersaline organic wastewater is one of the major problems in wastewater treatment industry. The high salinity inhibited the survival, growth and reproduction of microorganisms, consequently causing the difficulties in biodegradation for the saline wastewater [11, 12]. Bacteria immobilization is an appropriate technique for wastewater treatment [13]. It could also improve microbial tolerance of toxic or hazardous substances [14] during water treatment process [15], significantly increasing the amounts and activities of microbes.

This study, for the first time, developed the bacteria immobilization on graphene oxide and tested the performance in biodegradation for hypersaline organic wastewater. The microbes from the activated sludge could tolerate high salinity and achieve high organic pollutants removal. From further phospholipid fatty acids (PFLAs) analysis, the dynamics of microbial community structure revealed the dominancy of halotolerant bacteria and their functions in the graphene oxide carriers.

2. Experimental section

Graphene oxide synthesis: Compared to previous research on surface modification of polyvinyl alcohol (PVA) by graphene oxide (GO) to improve the mechanic properties, this research addressed its bacterial friendly and immobilization, and the GO synthesis method was therefore modified accordance with Hummer's protocol [16]. Briefly, 5.0 g potassium peroxymonosulfate and 5.0 g phosphorus pentoxide were added into 25 mL sulphuric acid at 90^oC with constant mixing. The 6.2 g graphite was slowly added and kept reaction at 80^oC for 4.5 hours. By adding water to the volume of 1.0 L, the mixture was kept standing overnight and treated with suction filtration. The particles on the film were collected and dried at room temperature for 24 hours. The preliminary graphene oxide was then added into 240 mL iced sulphuric acid with 30.0 g $KMnO_4$. After stirring for 20 minutes, the mixture was further stirred at 35^oC for 2 hour, followed by adding 460 mL deionized water with further reaction for another 2 hours. Twenty-five milliliter of 30% H_2O_2 solution was subsequently added into the mixture and kept standing overnight. Discarding the supernatant, the pellet was resuspended in 500 mL 5% hydrochloride and centrifuged at 10,000 rpm for 30 minutes to remove the supernatant with residual reagents. The washing step was repeated three times, followed by another three times washing by deionized water to remove the salts.

Microbial carrier synthesis and immobilization: Halotolerant bacteria were embedded with two different types of microbial carriers for wastewater treatment, as PVA and GO/PVA. The PVA carrier solution was prepared by dissolving 22.5 g PVA in 122.5 g deionized water. Seventy-five milliliter graphene oxide stock solution (20 g/L) was added into the 150 mL PVA carrier solution to make the GO-PVA carrier solution. After 1 hour water bath agitation at 90^oC and cooled down to 30^oC, the 75 mL enriched halotolerant bacteria were mixed with material solution and kept stirring for 1 hour. The carrier gel was further frozen at -18^{o}C for 24 hours, followed by natural thawing at room temperature and crushed into granules.

Bacteria cultivation and wastewater treatment: The halotolerant bacteria were enriched from the wastewater biosludge of Village Li Waste Water Treatment Plant (120°21.11'E, 36°9.32'N). The high salinity wastewater was prepared by adding 0.72 g glucose and 0.62 g NH_4Cl in 1.0 L deionized water. Ten grams of PVA or GO-PVA with halotolerant bacteria were added into each 1.0 L wastewater respectively, and another treatment was carried out with only halotolerant bacteria. The water and PVA/GO-PVA materials were sampled at 0, 4, 6, 8, 10, 12 and 24 hours for analysis.

Analysis: Images of scanning electron microscopy (SEM) were obtained by JSM-6700F at 8.0 kV (JEOL, UK). The infrared (IR) spectroscopy analysis followed the KBr pellet method (Supplementary Material), and the Raman spectrum was obtained by inVia Raman Microscope (Renishaw, UK) with 633 nm laser. The determination of chemical oxygen demand (COD_{Cr}) followed the fast digestion-spectrophotometric method (Supplementary Material). Phospholipid fatty acids (PLFAs) were extracted KOH-CH_3OH (0.2 M) solution and analyzed by Sherlock Microbial Identification System (MIDI Inc., USA, version 6.2) (Supplementary Material).

3. Results and discussion

SEM images of GO, PVA and GO-PVA were illustrated in Fig. 1a-c. The pristine GO has no significant reticulate structure, whereas it is only observed in PVA and improved in GO-PVA material. Similar to previous research [17, 18], the pure PVA polymer composite has uniform grain distribution with small holes (2-20 μM) randomly distributed on the top surface. This structure is not suitable for bacteria attachment and proliferation. On the contrast, GO-PVA material has significant lamellar structure, which is formed by graphene oxide and can support the immobilization and growth of bacteria. The reinforcing PVA effectively suppresses the salt transportation and maintain biocompatible environment for bacteria [19]. It is therefore suggested that GO-PVA materials have appropriate microstructure with higher surface area for bacteria to immobilize and provide suitable condition to help bacteria keep high biodegradation efficiency by tolerating high salinity wastewater.

Fig. 1(d) shows the infrared spectra of graphene oxide, showing the strong absorption peak at 3240 cm^{-1} referring to the stretching vibration of -OH. The characteristic absorption peak of graphene oxide is also observed at 228 nm in UV-vis spectrum (Fig. S2). The wide absorption IR peaks ranging from 2500 cm^{-1} to 3700 cm^{-1} represent the stretching vibration of H_2O absorbed by graphene oxide. Absorption peak at 1654 cm^{-1} refers to the stretching vibration of sp^2 C=C. Absorption peaks at 1722 cm^{-1}, 1394 cm^{-1}, 1221 cm^{-1} and 1032 cm^{-1} represent the vibration of -C=O, O-H, C-OH, C-O. Similarly results are found in Raman spectrum in Fig. 1(e). PVA has strong peaks of υ(C-O-C) at 805 cm^{-1}, υ(C-O-C) at 1032 cm^{-1} and 1100 cm^{-1}, δ(CH_3) asym at 1450 cm^{-1} and υ(C=O) at 1760 cm^{-1}. The G-bang of GO material at 1590 cm^{-1} shifts to 1560 cm^{-1} in GO-PVA material, and the 1350 cm^{-1} D-band shifts to 1370 cm^{-1} [20]. The evidence further suggests the strong interaction between hydrophilic functional groups of GO material and PVA matrix [21], and such structure supports bacterial immobilization for bioaugmentation.

The chromatogram of phospholipids fatty acids (PLFAs, Fig. S3 and Table S1-S4 in Supplementary Material) shows the microbial biodiversity in different materials by specific responsive peaks. The 17:0 cyclo-fatty acids are the specific biomarkers of anaerobic bacteria, and their increasing dominancy with the salinity demonstrated their salinity tolerance [22]. Branched-chain fatty acids of the iso- and anteiso- series (14:0 iso, 15:0 iso, 15:0 anteiso, 16:0 iso, 16:0 anteiso, 17:0 iso and 17:0 anteiso) are mainly identified in Gram-positive bacteria, or Gram-negative sulfate-reducing bacteria, *Cytophaga* and *Flavobacterium* [22]. They vary in different salinity treatments with no significant difference.

Microbial community (Fig. 2a) illustrated that *Acinetobacter*, *Pseudomonas* and *Thermophilic hydrogen bacilli* were the dominant species for glucose degradation. In 5% salinity treatment, their relative abundance was 24.55%, 44.66% and 2.45%, respectively (Table S5). *Pseudomonas* was the most dominant bacteria and their abundance increased with the salinity, showing their tolerance to the high salty. The proportion of aerobic bacteria decreased from 82% in 0% salinity to 77% in 5% salinity treatment (Fig. 2b), whereas anaerobic bacteria increased. The results suggested that GO-PVA carrier specifically stimulate the anaerobic bacteria with high capacities of tolerating salt and metabolizing glucose.

The COD_{Cr} removal efficiency of three materials was illustrated in Fig. 3a. The COD_{Cr} removal efficiency of GO-PVA material (62.8%) was significantly higher than that of PVA carrier (52.8%) and no carrier (30.8%). GO/PVA carrier had better bioaugmentation performance due to the high specific surface area and functional groups of graphene oxide to support and stimulate bacterial growth. The amount of cultivable halotolerant bacteria was 5.04×10^3 and 4.44×10^3 CFU/mL for the immobilization on GO/PVA and PVA materials (Fig. 3b). Compared to the treatment with no carrier (2.07×10^3 CFU/mL), GO-PVA carrier significantly encouraged the growth of the microorganisms responding to the glucose degradation, showing their excellent performance as bioaugmentation materials. Comparing to other high salinity wastewater biodegration process, this GO-PVA immobilization methods can significantly improve microbial diversity, the amounts of degraders and COD_{Cr} removal efficiency [23-25], showing great potential in engineering application. Though graphene oxide had antibacterial effects [26], surface modification could solve this problem and improve the bacterial growth [27]. In this work, the manufacturing process of GO/PVA carrier was viewed as the surface modification to mitigate graphene oxide's toxicity to bacteria. The reaction between graphene oxide's carboxyl and PVA's hydroxyl forms the coacervate, offering the places for bacteria to attach and growth.

4. Conclusions

This work introduced graphene oxide to modify polyvinyl alcohol as bacterial carrier for high salinity wastewater treatment. PVA modification significantly reduced the toxicity of graphene oxide on bacteria growth and GO-PVA carrier effectively improved the growth of the functional microorganisms. The wastewater treatment performance indicated that GO/PVA carrier was suitable bioaugmentation materials for biodegradation in hypersaline organic wastewater.

5. References

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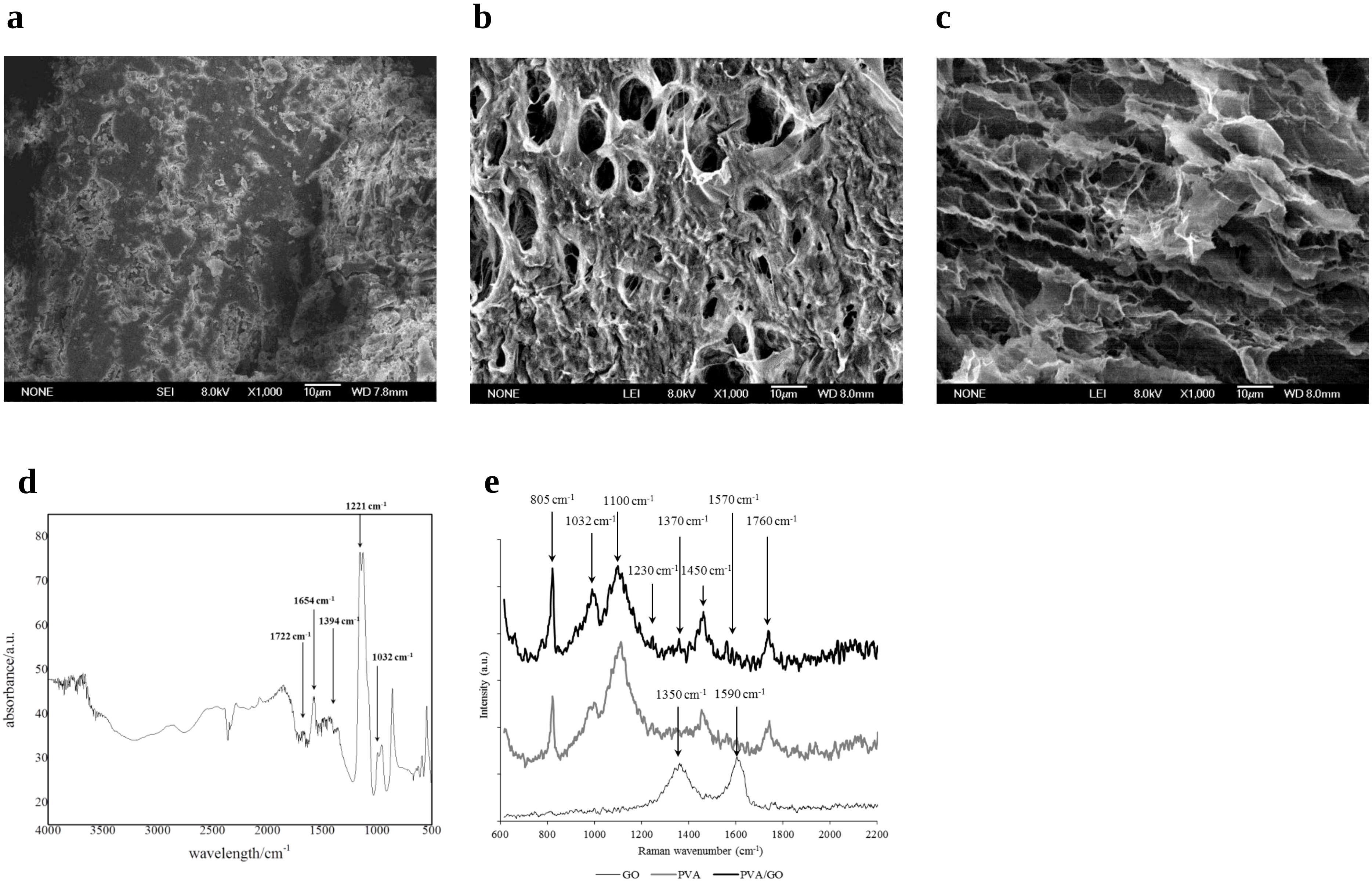
6. Figure Caption

Fig. 1. SEM images of GO (a), PVA (b) and GO-PVA (c) carrier. The infrared spectrogram (d) and Raman spectrum (e) of GO-PVA materials.

Fig. 2 The microbial community structure change (a) illustrated the dominancy of *Pseudomonas* under high salinity conditions. The aerobic and anaerobic community analysis (b) suggested the relative abundance of aerobic bacteria decreased with the increasing salinity.

Fig. 3. Wastewater treatment efficiency (a) and bacterial count (b) with none, PVA and GO-PVA carriers.

Figure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d
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absorbance/a.u.
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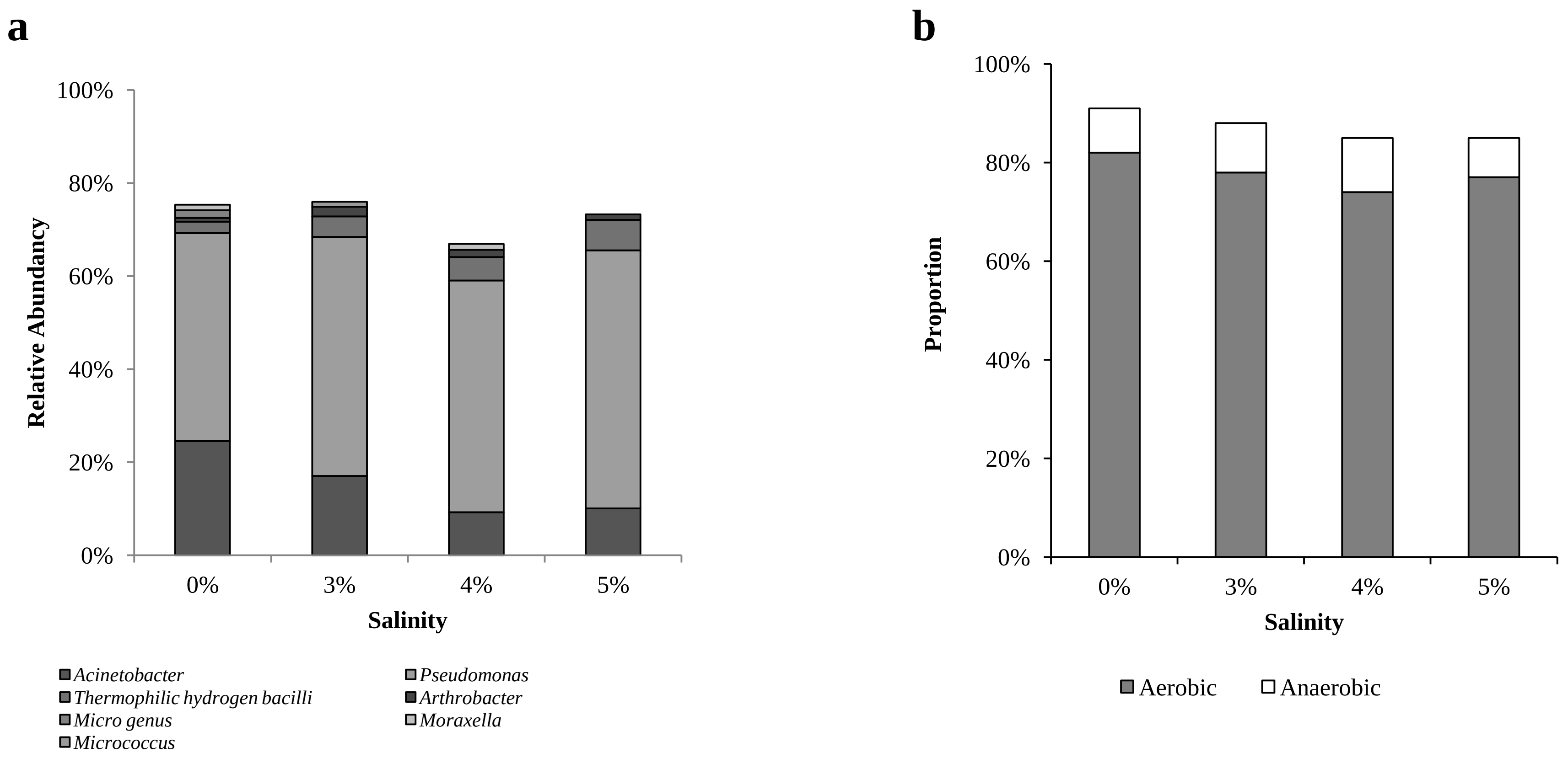
PVA/GO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Salinity
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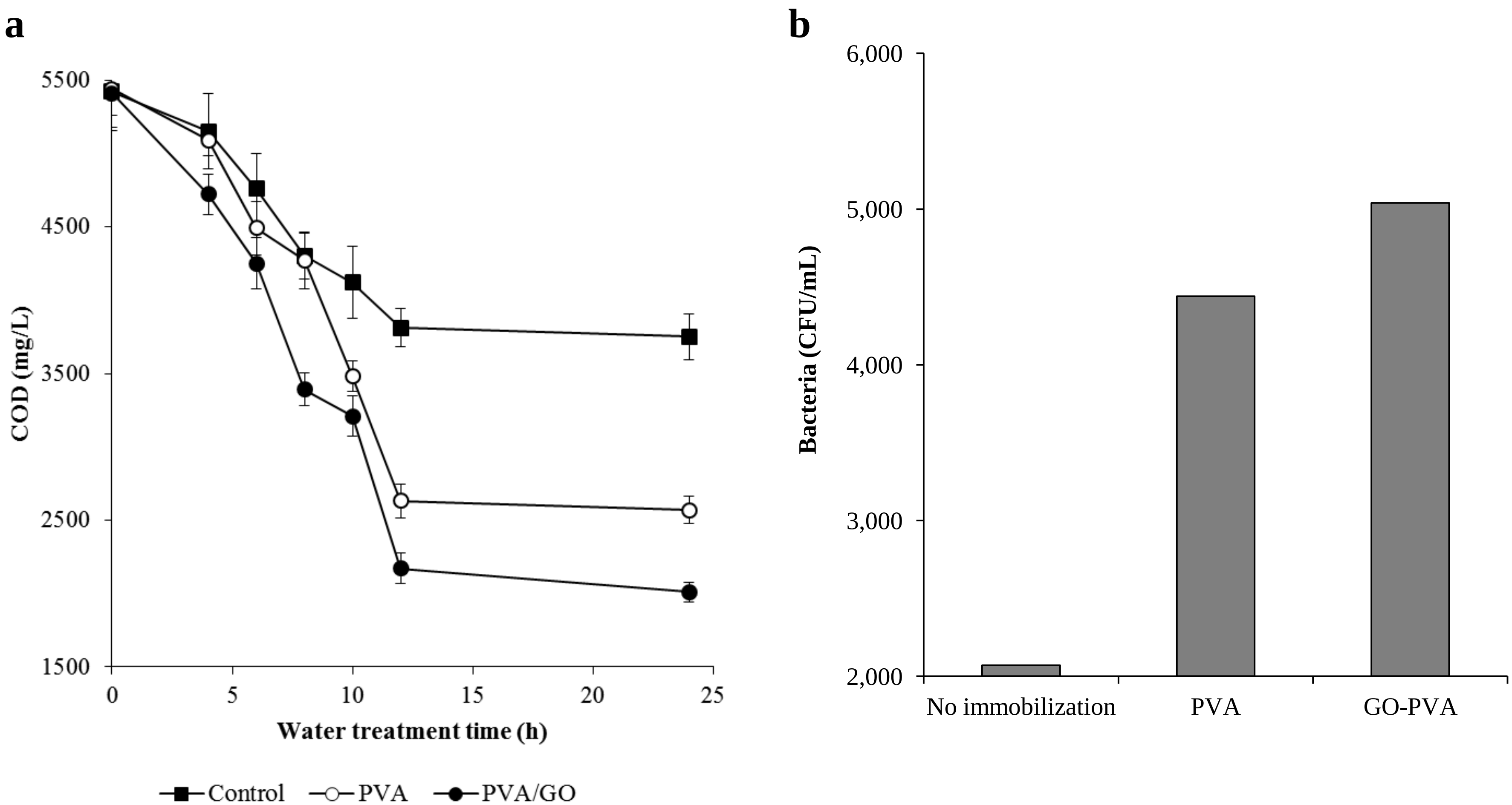
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2,000
Bacteria (CFU/mL)
No immobilization
PVA
GO-PVA