Response to comments:

Reviewer:

This manuscript presents an investigation into the effects of microbial activities on sediment flocculation in estuarine environments using a single bacterial strain and microstructural characterization. While the dataset is extensive and the interdisciplinary effort is evident, several key issues, including severely limited environmental relevance, critical logical flaws, methodological inadequacies, and overstatement of conclusions, render the current version unsuitable for publication. Thus, I recommend rejecting the manuscript.

Major comments:

The experimental design uses only one bacterial strain (Stutzerimonas decontaminans) and a fixed shear rate of 65 s^{-1} without justification or variation, yet the study extrapolates to complex natural estuarine processes involving diverse microbial communities and highly variable hydrodynamic regimes. Moreover, the maximum experimental duration of 72 h is insufficient for biofilm development, which typically requires weeks to months, and this temporal mismatch is not adequately acknowledged.

Response: We thank the reviewer for this important comment. As described in the first paragraph of Section 2.2, we stated: "Bio-flocculation processes were investigated in MSD systems containing montmorillonite clay and *Stutzerimonas decontaminans* (hereafter *S. decontaminans*), a dominant bacterium in the Pearl River Estuary known for high EPS production (Li et al., 2018)."

We acknowledge that only a single EPS-secreting bacterial strain was used in this study. To address this point, we have added the following statement at the end of the first paragraph in Section 4 (Conclusions): "In addition, the composition and quantity of EPS secreted by microorganisms differ among species (Flemming & Wingender, 2010), which in turn modulate flocculation efficiency. Although this study were conducted using a single bacterial strain, which helps clarify the role of model strains in estuarine bioflocculation, future research is still needed to thoroughly investigate the effect of multiple microorganisms on the estuarine bioflocculation".

As described in the fourth paragraph of section 2.2, we have mentioned the detail of the information of the fixed shear rate of 65 s^{-1}: "Continuous turbulent mixing (G = 65 s^{-1}) minimized aggregation-driven settling," and we further confirm it, "which was representative of the high-shear hydrodynamic conditions typical of estuarine environments".

While biofilm formation in eukaryotes like algae may take weeks to months (e.g., 30 days in Ho et al.), prokaryotic biofilms typically develop within several days (Zhang et al., 2023; Xu et al., 2001). AFM observations have also shown abundant biofilm formation by *Pseudomonas putida* on montmorillonite within 48 h (Huang et al., 2015). Our preliminary 120-hour experiment (see Figure S1) showed floc size increase from 0-60 h and decrease from 72-120 h. Thus, rather than the exact timing of biofilm formation, this study focuses on the phase of microbial-driven floc development. We have added this clarification in the end of first paragraph of Section 2.2, "Preliminary tests showed that the D_m of flocs in MSD increased from 0 to 60 h but decreased from 72 to 120 h (Fig S1). Accordingly, the 12 - 48 h period, representing the phase of microbial-driven floc development, was selected for subsequent bioflocculation experiments". We have added the preliminary experimental results to the supplementary material (Figure S1).

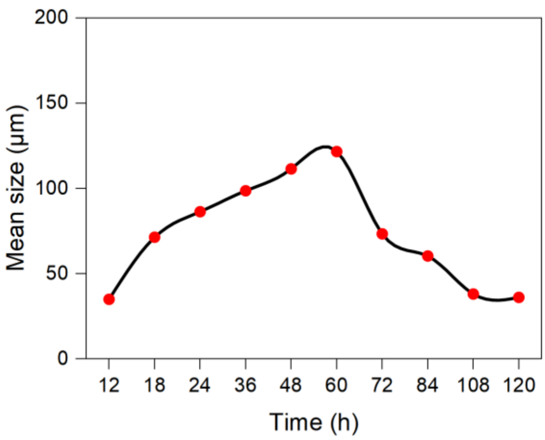


Figure S1. Mean size of flocs in MSD during 120 h

The following references support our response:

Ho, Q. N., Fettweis, M., Hur, J., Desmit, X., Kim, J. I., Jung, D. W., ... & Lee, B. J. (2022). Flocculation kinetics and mechanisms of microalgae-and clay-containing suspensions in different microalgal growth phases. Water Research, 226, 119300.

Zhang, Y., Zhang, T., Qiu, Y., Zhang, M., Lu, X., Yang, W., ... & Lu, R. (2023). Transcriptomic profiles of *Vibrio parahaemolyticus* during biofilm formation. Current microbiology, 80(12), 371.

Xu, K. D., Franklin, M. J., Park, C. H., McFeters, G. A., & Stewart, P. S. (2001). Gene expression and protein levels of the stationary phase sigma factor, *RpoS*, in continuously-fed *Pseudomonas aeruginosa* biofilms. FEMS microbiology letters, 199(1), 67-71.

Huang, Q., Wu, H., Cai, P., Fein, J. B., & Chen, W. (2015). Atomic force microscopy measurements of bacterial adhesion and biofilm formation onto clay-sized particles. Sci Rep 5: 16857.

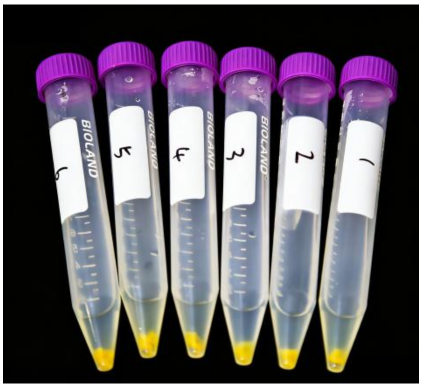
The causal interpretation that upregulation of flagellar genes drives initial attachment is not supported, as transcriptomic data only show temporal sequences and both processes could be independently triggered by unknown signals such as quorum sensing. Additionally, the lack of direct comparison between the mixed system and the pure SD at identical time points, together with unreported initial bacterial size and floc size for the SD group, prevents isolating the effect of mineral-induced flocculation from intrinsic growth dynamics.

Response: We thank the reviewer for this insightful comment. We agree that the regulation of flagellar genes and EPS synthesis may be independently triggered by unknown signals such as quorum sensing. In our study, the global regulatory signals we examined are quorum-sensing-related signals involved in biofilm formation (e.g., *Rcs, RpoS*). We have revised the text to clarify that our causal interpretation is based on the path analysis in the part of section 3.5, while acknowledging that the underlying regulatory signals may involve quorum-sensing mechanisms. The following sentence has been added at the end of the fourth paragraph of Section 4: "Path analysis results supported that global regulatory factors activated the flagellar system, and the flagellar genes drove initial attachment, thereby promoting floc development (Fig.11). However, more regulatory signals (e.g., quorum sensing) that trigger these processes independently remain to be further investigated".

We agree that the intrinsic growth dynamics of bacteria should be an important factor for the microbe-induced flocculation. A direct time-point-by-time- point comparison between the MSD and the pure SD was not performed, because the bacterial cells were too small (0.5 - 5 μm, shown in Figure 4.c) to be effectively resolved by the high-speed camera for such comparative analysis. Moreover, this comparison was beyond the scope of the present study, which focused on the metabolic role of bacteria in floc development (0 - 48 h) rather than on the absolute differences between biotic and abiotic flocculation at each time point.

We have added a sentence at the end of the second paragraph of Section 2.2. "Bacterial strain and initial inoculum used in the SD are showed in the Fig S2". In addition, the images of the bacterial strain and initial bacterial inoculum used in the experiment have been added to the Appendix (see Fig. S2).

(a)



(b)

Figure S2. (a) Bacterial strain and (b) initial inoculum used in the SD

The study contains internal inconsistencies (total EPS, polysaccharide, and protein contents did not change significantly), yet transcriptomic data show late activation of polysaccharide biosynthesis pathways and a significant increase in POC, and this contradiction is not resolved. The mineral control group was run for only 160 min while the biomineral group ran for 48 h, and the potential impact of long-term shear on mineral fragmentation cannot be ruled out despite claims of early equilibrium.

Response: We thank the reviewer for identifying this interesting point. We acknowledge the apparent contradiction between the EPS measurements and the transcriptomic/POC data, and we provide the following explanation. As described in the part of section 2.5, the EPS quantification method used in this study primarily measures dissolved EPS (i.e., soluble EPS), with the sum of polysaccharide and protein contents representing the total dissolved EPS. In contrast, the polysaccharides synthesized on bacterial cell surfaces exist as bound EPS (Laspidou et al., 2002). As bacterial abundance increased, the upregulation of polysaccharide biosynthesis pathways led to increased synthesis of bound EPS on cell surfaces, which is consistent with the observed significant increase in POC.

To address this issue, we have added the following paragraph at the third paragraph of Section 4 (Discussion): "In this study, although the total contents of EPS, polysaccharides, and proteins did not change significantly, the increase in exopolysaccharide synthesis was found to be consistent with the significant increase in POC (Fig7.c, Fig10.b). These results may be explained by the different forms of EPS present in the aqueous environment. Generally, soluble EPS is released from the cell surface through dissolution, hydrolysis, or shearing, whereas bound EPS is associated with biomass synthesis (Laspidou et al., 2002). Transcriptional upregulation of polysaccharide synthesis-related genes on the bacterial surface may facilitate an increase in bound EPS. Collectively, these findings support that bacterial activity and bound EPS are the primary factors governing bioflocculation."

Please note that this reference was already cited in the original manuscript:

Laspidou, C. S., & Rittmann, B. E. (2002). A unified theory for extracellular polymeric substances, soluble microbial products, and active and inert biomass. Water research, 36(11), 2711-2720.

We thank the reviewer for raising this important point regarding the difference in experimental durations between MM and MSD. Under steady shear conditions, mineral flocs tend to become more compact over time, rather than being susceptible to fragmentation. A number of studies have shown that once flocculation equilibrium is reached, no further changes occur in inorganic flocculation systems (Ye et al., 2023; Zhao et al., 2021), unless the shear rate is altered (Nan et al., 2016). Therefore, the 160-minute duration used for the mineral control group in this study is both reasonable and sufficient to achieve a stable state. The following references support our response:

Ye, L., Wu, J., Huang, M., & Yan, J. (2023). The role of suspended extracellular polymeric substance (EPS) on equilibrium flocculation of clay minerals in high salinity water. Water Research, 244, 120451.

Zhao K, Pomes F, Vowinckel B, Hsu T-J, Bai B, Meiburg E. (2021).Flocculation of suspended cohesive particles in homogeneous isotropic turbulence. Journal of Fluid Mechanics. 921:A17. doi:10.1017/jfm.2021.487

Nan, J., Wang, Z., Yao, M., Yang, Y., & Zhang, X. (2016). Characterization of re-grown floc size and structure: effect of mixing conditions during floc growth, breakage and re-growth process. Environmental Science and Pollution Research, 23(23), 23750-23757.

The microscopic characterization using air-dried SEM and AFM samples is inappropriate for highly hydrated EPS-rich flocs, as air-drying causes polymer network collapse and alters morphology and roughness, meaning the observed structures do not represent in-situ conditions. Alternative approaches such as cryogenic preparation or ESEM should have been used.

Response: We thank the reviewer for this valuable comment. In the current study, air-dried samples were used for SEM analysis primarily to observe the distribution of bacteria and EPS on mineral surfaces. Although dehydration is inevitable during air-drying and may have some impact on the structure of floc, it does not affect the final measurement results of surface potential for either the minerals or the bacterial cells. Therefore, AFM was used to measure the surface potentials of these components. We recognize that air-drying may lead to the collapse of the hydrated EPS network and affect floc morphology. In future work, we plan to use cryogenic electron microscopy or environmental SEM (ESEM) to better preserve the native hydrated structure of flocs and enable more accurate in-situ characterization.

The discussion is too brief and largely descriptive, failing to adequately explain biological mechanisms, and the manuscript over-extrapolates from an idealized laboratory system to complex estuarine processes without a dedicated “Limitations” section, while several interpretative statements appear in the Results section rather than the Discussion.

We thank the reviewer for these constructive comments. In response, we have made the following revisions:

1. Biological mechanisms: The biological mechanism explanation originally located in the first paragraph of the Discussion (Section 4) has been moved to the seventh paragraph of the same section to better integrate with the relevant context.

2. Comparison with field studies: A comparison between our laboratory-based bioflocculation results and field estuarine processes has been added to the first paragraph of the Discussion (Section 4).

3. Limitations section: A dedicated “6. Future Perspectives” section has been added as Section 6, with the following content: "Our findings emphasize that sediment transport models and estuarine management strategies must explicitly account for microbial processes if they are to predict sediment and carbon fluxes

under future environmental change. Given that the bioflocculation observed in laboratory settings may not fully capture those occurring in natural environments, future studies should aim to bridge this gap through in situ validation experiments or by incorporating more complex, multi-species communities into mesocosm designs. Specifically, combining controlled experiments with field measurements of TEP/EPS concentrations, floc size distributions, and settling velocities across estuarine gradients would help validate the conceptual framework proposed here".

4. Removal of interpretative statements from Results: All interpretative statements that previously appeared in the Results section have been removed, specifically from Section 3.1, 3.3 and 3.4.

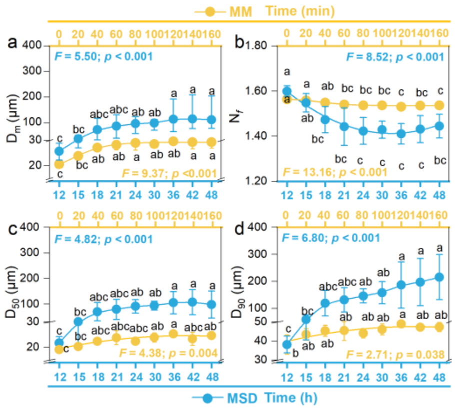
Minor comments:

In Section 3.1 (line 242), “As shown in Fig. 1e” should be revised to “As shown in Fig. 3e”.

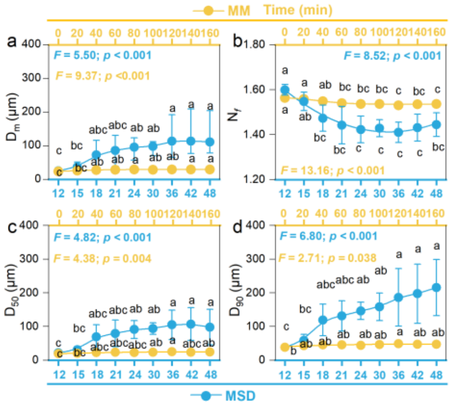
Response: Thanks, and done.

In Figures 3(a) and 3(c), the y-axis scale should be refined with finer increments in the range of 30 µm to 100 µm to better reflect the size distribution of D_m and D_50 after 15 hours, and in Figures 3 and 7 the meanings of labels such as “a”, “ab”, “abc” are unclear and should be explained.

Response: We thank the reviewer for the suggestion regarding the y-axis scales in Figures 3(a) and 3(c). In these figures, we intentionally applied a break on the y-axis to better highlight the size evolution characteristics of D_m and D_{50} during inorganic and bio-flocculation processes. We compared the differences with and without the axis break in the following two figures (a,b) and found that the version with the break more clearly reveals the evolution of floc D_m and D_{50} after 15 h. Therefore, we prefer to retain the original version with the axis break. We hope the reviewer will find this explanation acceptable.



(a) result figure with axis break



(b) result figure without axis break

To improve the readability of the captions for Figures 3 and 7, we have revised the original statement from "Different lowercase letters represented significant differences between treatments (*P* < 0.05) by HSD test" to "Different lowercase letters (e.g., a, ab, abc) represented significant differences between treatments (*P* < 0.05) by HSD test."

Abbreviations and notations are inconsistent (e.g., "fig. 9" vs. "Figure 10") and should be unified throughout the manuscript.

Response: Thanks, we have fixed the error.

The title emphasizes estuarine environment and hydrodynamics, but the manuscript focuses extensively on biological changes with minimal discussion of hydrodynamics. Thus, either the title or the content should be revised to resolve this discrepancy.

Response: We thank the reviewer for this valuable comment. In response, we have revised the title to better reflect the manuscript's focus on flocculation rather than hydrodynamics. The original title "Suspended sediment transport modulated by microbial activities in estuarine waters: Insights from molecular and structural perspectives" has been changed to "Suspended sediment flocculation modulated by microbial activities in estuarine waters: Insights from molecular and structural perspectives."