RESEARCH ARTICLE

Correlation of extracellular polymeric substances and microbial communities in biofilms with phosphate

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The formation of early biofilm is an important node in the ecological dynamic process of biological fouling, and the levels of nutrients such as active phosphate could influence the formation and dynamic succession of biofilm. In order to clarify the influencing mechanism of active phosphate on biofilm formation and development, biofilms were developed under controlled conditions indoor, and the characteristics and dynamic succession of biofilm on the surface of attached plates at different phosphate levels were studied. The results showed that phosphate demonstrated significant effect on the formation of biofilm. The dry weight, dry weight without ash, chlorophyll-a, and extracellular polymeric substances (EPS) of biofilm all increased with the increase of phosphate level and reached the peak values when phosphate was at 40 μg/L, then stabilized and slightly decreased with the increase of phosphate concentrations. The proportion of polysaccharides in EPS of each layer in biofilm increased from outside to inside (from soluble EPS to loosely bound EPS to tightly bound EPS). In contrast, the proportion of protein showed the opposite. EPS of various types in biofilms enhanced with the increase of water temperature, while the amounts of protein and polysaccharide in EPS showed similar trend. When the phosphate concentrations were lower than 40 μg/L, the ratio of protein to polysaccharide (PN/PS) increased with the increase of phosphate level, and decreased somewhat when the phosphate was higher than 50 μg/L. The proportion of bacteria in microbial population of biofilm decreased with the increasing of phosphate level and immersion time of the attached material, while the proportion of diatoms increased gradually. In conclusion, 30-50 μg/L phosphate was more suitable for the biofilm formation. The study was helpful to understand the dialectical relationship between environmental factors and biological factors in the aquaculture environment and provided a theoretical basis for exploring the mechanism of marine biofilm formation.

Keywords: biofilm; phosphate; extracellular polymeric substances; microorganism community; aquaculture.

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Introduction

Aquaculture is recognized worldwide as the fastest growing food producing sector for human consumption [1]. China has the world's largest fishing industry, especially *Stichopus japonicus* cultivation [2]. The aquaculture ponds are mostly built according to the coastal terrain with large areas and little artificial interference [3]. As a semi-closed and semi-artificial ecosystem, the ecological composition in pond is relatively single and the self-regulation ability is poor. Therefore, a special ecological environment different from the surrounding environment has been formed [4].

In marine environments, biofilms are formed on submerged surfaces that are colonized by

multicellular sessile community of microorganisms that provide supporting structures of organic matter and cycle nutrients [5]. Due to the nocturnal habit of *S. japonicus*, artificial adherents are generally used to simulate its natural living environment in large-scale cultivation and provide an adhesion surface for biofilm at the same time, which could be used as feed for the sea cucumber [6]. The biofilms play important role in ecology, water quality control, aquaculture, and biological contamination control. Studies showed that the biofilm on the adherents in aquaculture ponds could improve the water quality, thus significantly increase the survival rate, growth rate, and biomass of cultured animals.

Biofilms are usually composed of less than 10% (w/w) embedded microbes and over 90% (w/w) extracellular polymeric substances (EPS) [7]. Biofilm has a three-dimensional structure composed of various EPS, which plays a crucial role in the adhesion and aggregation of microorganisms on solid surfaces [8]. The formation of biofilm is affected by the characteristics of adherence, and microbial quorum sensing. Environmental factors, such as nutrient level, pH, dissolved oxygen concentration, and temperature, also impact biofilm formation [9]. Through an ongoing ecological investigation carried out on the aquaculture pond of *S. japonicus* from 2020 to 2021, the main environmental factors influencing microorganism and microalgae communities were determined in water and sediment as temperature, salinity, suspended solids, dissolved oxygen, light intensity, and levels of nutrients. These factors are likely to influence the formation and dynamic succession of biofilm. However, the influencing mechanism needs further study. In this study, biofilms were developed under controlled conditions indoor, the characteristics and spatiotemporal change of biofilms were studied in order to clarify the influencing mechanism of phosphate on biofilm formation and development.

Materials and methods

Seawater

The seawater used for this study was taken from the pond farming *S. japonicus* (Shandong Oriental Ocean Sci-Tech Co., Ltd., Yantai, Shandong, China) and was filtered through a cellulose acetate filter (20 μm) with a salinity of 32‰ and pH 7.5～7.9.

Attachment plate

The plates of ground cage commonly used in the culture of *S. japonicus* were used as attachment plate. The plates were cut into 150 mm × 100 mm pieces, cleaned by immersing the plate in 75% ethanol for 3 mins, then, rinsed twice with ultra-pure water before sterilized at 121°C for 15 mins and dried naturally on a clean bench.

Develop of biofilm

The experiments were carried out in 36 aquariums of 400 mm × 300 mm × 240 mm with 200 mm water in depth. Seawater was renewed every day. 7 pieces of the treated attachment plate were hung uniformly in each tank, and the upper end was 50 mm below the water surface. Biofilms were developed on the surface of plate under the static. Six parallels were set for each treatment. According to the results of early field investigation, the levels of phosphate in this study were set as 5.0 μg/L, 10.0 μg/L, 20.0 μg/L, 30.0 μg/L, 40.0 μg/L, and 50.0 μg/L, respectively. One piece of attachment plate was taken out from each tank to analyze the biological and chemical characteristics of the biofilm at 1, 2, 4, 6, 8, 15, and 30 days, respectively.

Biofilm collection

The attachment plates were carefully rinsed with 0.9% sterile saline to remove unattached microorganisms. Then, each plate was transferred to a polyethylene bottle containing sterilized 500 mL phosphate buffer (PBS) (50 mmol/L, pH 7.4). The samples were processed by using an ultrasonic instrument (150 W, 40 kHz) (Shenzhen Chaojie Industrial Co., Ltd, Shenzhen, Guangdong, China) for 3 mins with several sterile glass beads (Chemical Plant of Nankai University,

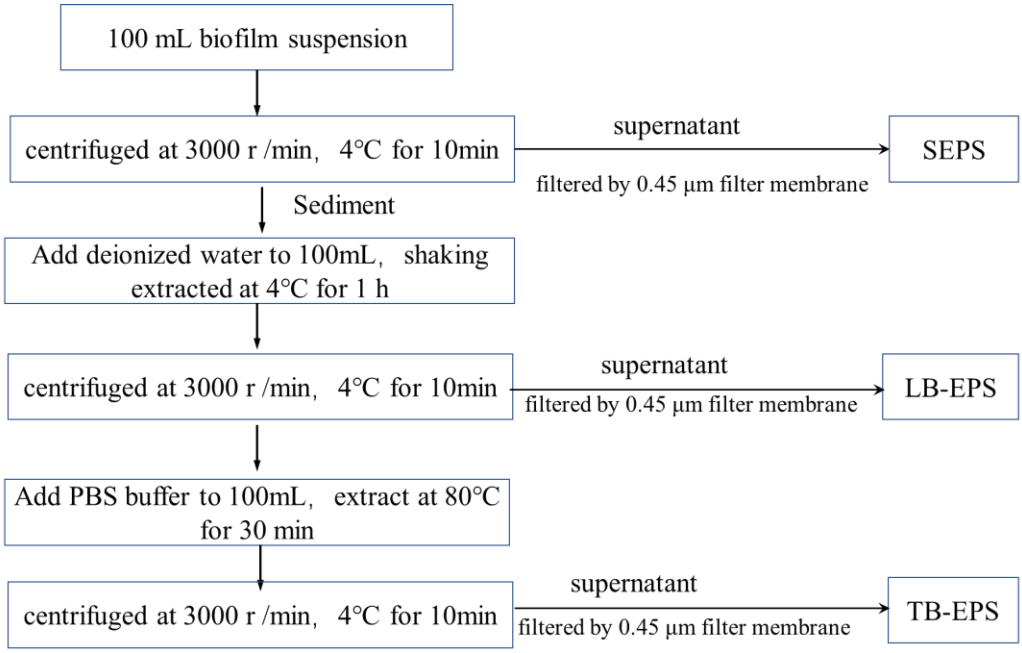

Figure 1. Extraction steps of EPS.

Tianjin, China). The samples then were extracted by using an oscillatory extraction instrument (Shanghai Ranhui Industrial Co., LTD, Shanghai, China) at 225 rpm, 4°C for 30 mins. The extracts were filtered by using a 50 μm sieve (Macklin, Shanghai, China) to obtain bacterial suspension of biofilm. The suspension was centrifuged at 8,000 rpm for 10 mins. The sediment was transferred to a 2 mL centrifuge tube and stored at -80℃ for future experiments.

Determination of dry weight of biofilm

The dry weight of biofilm was determined by the gravimetric method. The centrifugal sediment of the biofilm was dried at 105℃ for 24 h. The obtained mass was the dry weight of the biofilm (DW) (mg/cm^2). The dried biofilm was further burned at 450℃ for 6 h. The obtained mass was the content of inorganic matter in the biofilm, and the lost mass was the ash free dry weight (AFDW) (mg/cm^2) which was the content of organic matter in the biofilm.

Determination of chlorophyll-a (Chl-a) of biofilm

The biofilm suspension was shaking extracted by 10 mL of 90% acetone at 4℃ for 1 h in a plug centrifuge tube. After centrifuged at 3,000 rpm for 10 mins, the absorbances of the supernatant were determined by spectrophotometer (Lengguang Technology, Shanghai, China) at 750, 664, 647, and 630 nm, respectively, with 90% acetone as blank control. The content of Chl-a (μg/g) was calculated according to the following formula: ·

$$Content\ of\ chl\ a = \frac{12.12\times(OD_{664}-OD_{750})-1.58\times(OD_{647}-OD_{750})-0.08\times(OD_{630}-OD_{750})\times V\times DW}{A}$$

where, *V* was the volume of the extracted liquid (L), *OD* was the absorbance, *DW* was the dry weight of the biofilm (g), *A* was the optical path of the cuvette (cm).

Determination of extracellular polymeric substances (EPS) in biofilm

100 mL of biofilm suspension was heated with water bath at 80℃ for 30 mins, and then, was filtered by using 0.45 μm filter membrane. The filtrate obtained was the total EPS of biofilm. After dried at 105℃ for 24 h, the total mass of EPS (mg/cm^2) was obtained. Another 100 mL of biofilm suspension was used to extract soluble EPS (SEPS), bound EPS (BEPS), tightly bound EPS

(TB-EPS), and loosely bound EPS (LB-EPS) following the improved heating method (Figure 1). The content of polysaccharide (PS) in EPS was determined by phenol-sulfuric acid method, while protein (PN) content was determined by improved Lowry method [10].

Microbial community of biofilm

The total DNAs of the biofilm samples were extracted by using Soil DNA Kit (Omega Bio-tech, Norcross, GA, USA). 1% agarose gel electrophoresis was used to detect the concentration, purity, and integrity of extracted DNA [11]. The target region of bacterial colony structure analysis was V3-V4 region of 16S rRNA. The primers of 341F (5'-CCT ACG GGN GGC WGC AG -3') and 806R (5'-GGA CTA CHV GGG TAT CTA AT -3') were used for polymerase chain reaction (PCR) amplification, where N was for any base of A, G, C, T, W was for either A or T, H was for any base of A, C, T, and V was for any base of A, C, G. 20 ng of purified DNA was used as template. The PCR was carried out by using ABI Geneamp 9700 thermal cycler (Thermo Fisher Scientific, Waltham, MA, USA) with the program of denaturing at 95°C for 5 mins followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 40 s, and finally extension at 72°C for 7 mins. The PCR samples were sent to Azenta Life Science Co., Ltd. (Suzhou, Jiangsu, China) for sequencing by using Illumina MiSeq sequencing platform (Illumina, San Diego, CA, USA). Usearch software (version 7.0) (https://drive5.com/uparse/) was employed to cluster Operational taxonomic units (OTUs) at 97% threshold. The Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/) classifier software was used to classify species with the threshold of 0.8. Classification results lower than the threshold was classified as unclassified.

Statistical Analysis

All the results of experiment were shown as mean ± standard deviation (SD). Statistical analysis was conducted by using one-way ANOVA followed by LSD multiple-range test through IBM SPSS Statistics software (version 19.0 for Windows) (IBM, Armonk, NY, USA).

Results and discussion

Observation of biofilm formation process on the surface of attached plates

By macroscopic observation of the growth morphology of biofilm on the surface of attachment plates, the whole process of film formation could be divided into three periods as early-stage (0-7 days), middle-stage (7-15 days), and late-stage (15-30 days). In the early-stage of film formation, a faint yellow water film could be observed on the plate's surface, which was sticky and slippery. It was speculated that organic matter and microorganisms had attached to the surface to form a fragile covering. The adherent substance was easy to fall off when washed with water, but there was still trace amounts of viscous substance that remained on the material's surface. The biofilm was reversible attachment at this stage. In the middle-stage of film formation, the surface of the attachment plate became slippery and darker significantly, and the thickness also increased. Inhomogeneous yellow spots scattered on the surface of the plate. Even if washed with water, there were still plenty of sticky substances remained on the surface, indicating that a large number of organic substances were generated on the biofilm. These organics could adhere microorganisms to the plate's surface and stabilize the adhesion of biofilm, converting the adhesion process to irreversible. At this time, significant bacteria colloid could be observed under microscope in the covering layer. At the late-stage of film formation, the surface of the attachment plate had been entirely covered by grayish-brown substances. After washed with water, the sticky substances were not easy to fall off, indicating that the biofilm had completed the irreversible attachment process on the material at this time, which meant the film was matured.

Effect of phosphate on the mass of biofilm

The mass changes of biofilms over time formed in water with different phosphate concentrations were shown in Figure 2A and 2B, including dry weight (DW) and ash free dry weight (AFDW). DW and AFDW of biofilm showed a similar trend.

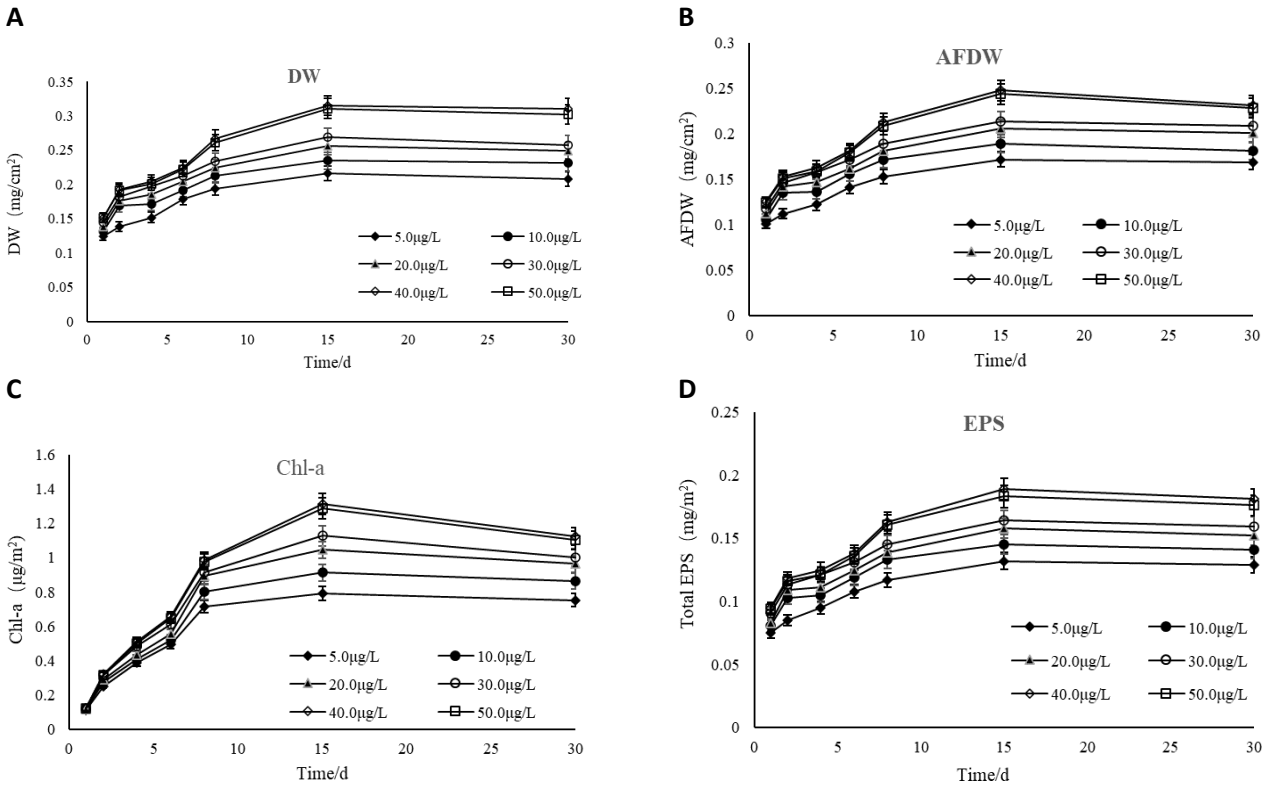

Figure 2. Effects of phosphate on the biofilm's DW (**A**), AFDW (**B**), Chl-a (**C**), and EPS (**D**).

Organics accounted for about 70-85% of the dry weight of biofilm. The DW and AFDW of the biofilm gradually increased with the increase of immersion time of the attached plate, indicating the adhere of microorganisms and microalgae on the surface of the plate. The DW and AFDW of biofilm increased slowly in the early-stage of film formation, then grew rapidly in the middle-stage, and subsequently maintained stable and slightly decreased in the late-phase. With the increase of phosphate concentrations, the mass of biofilm increased gradually and reached the maximum value of 40 μg/L, then DW and AFDW reduced slightly at a higher level of 50 μg/L.

Effect of phosphate on the content of Chl-a in biofilm

The accumulation of Chl-a in biofilm could indicate the adhesion process of algae. As shown in Figure 2C, when the attached plate immersed in seawater, the microalgae quickly attached to the surface, and the content of Chl-a reached a certain value in 1-2 days. With the extension of immersion time, the content of Chl-a in the biofilm increased slowly and reached the peak in 8-14 days before decreased slightly. The slow increase rate of Chl-a in the middle-stage of biofilm formation might be related to the three-dimensional structure of the biofilm. With the increase of film thickness, the light intensity in the biofilm was weakened, resulting in weak photosynthesis of microalgae and lower content of Chl-a in biofilm [12]. The decrease of Chl-a in the late film-forming period might be caused by the shedding or death of some microalgae. The suitable phosphate levels for microalgae growth was generally 30-50 μg/L [13], and the content of Chl-a on the surface of attached materials was relatively higher at the corresponding levels.

Effect of phosphate on the total EPS in biofilms

As the main component of biofilm, EPS not only enhances the resistance of cells to the external environment, but also plays an important role in

the microstructure and functional integrity of biofilm [14]. Therefore, it is the key factor for the stability of biofilms and plays a decisive role in the structure and adhesion strength of biofilm [15]. Figure 2D showed the trend of EPS in the biofilm on the surface of the attached plate over time. The quantity of EPS amount on the surface of the attached plate demonstrated the similar trends with DW and AFDW. The quantity of total EPS generated was very small at the initial stage, indicating that only a few microorganisms were enriched on the carrier. EPS began to increase rapidly on the fourth day and exceeded 70% of the organic matter in the biofilm. On the 15th day, EPS on the surface of the adhesive plate increased rapidly for the second time, and then, the content of EPS was relatively stable and decreased to a certain extent. According to the formation process of biofilm, the first significant increase of EPS in the biofilm at 4-8 days was caused by the attachment of microbial, and the second considerable increase of EPS at about 15th day was mainly due to the colonization of microalgae in the biofilm [16]. With the increase of the levels of active phosphate in water, the content of EPS in the biofilms increased continually and reached the peak at 40.0 μg/L, then the content of EPS in the biofilms decreased slightly at higher phosphate level (50 μg/L) (P > 0.05). According to the above results, biofilms at the days 6th, 15th, and 30th were selected in subsequent experiments to analyze changes of polysaccharides and proteins in SEPS, TB-EPS, and LB-EPS.

Effects of phosphate on polysaccharide and protein in EPS of biofilms

EPS determines the structure and adhesion strength of biofilm, and its quantity could reflect the amount of biofilm to a certain extent [17]. EPSs comprise different types of biopolymers, including polysaccharides, proteins, and nucleic acids [18], which can provide microbes with strong tolerance and favorable living environments [19]. Exopolysaccharides, the main component of the biofilm matrix, are complex substances consisting primarily of various polyanionic macromolecules as well as neutral and polycationic macromolecules [20]. Recent studies demonstrated that exopolysaccharides had a significant impact on initial biofilm formation and could strengthen extracellular electron transfer (EET) with anchored c-type cytochromes (c-Cyts) [21]. Proteins in biofilms play an important role in their degradation and construction. Extracellular enzymes are devoted to degrading biopolymers into low-molecular-weight products that can be used as energy sources. Nonenzymatic proteins usually act in surface-associated binding processes to stabilize the matrix network. Some of them are indispensable parts of biofilm formation, providing microbes with biofilm forming ability, presenting a large molecular mass, and being involved in bacterial infections [22]. EPS could be divided into soluble EPS (SEPS) and bound EPS (BEPS) according to its polymerization morphology. The BEPS was consisted of tightly adhered inner layer (TB-EPS) and loosely bound outer layer (LB-EPS) [23].

The effects of phosphate level on the contents of polysaccharide and protein in various EPS were shown in Figures 3 (SEPS), 4 (LB-EPS), and 5 (TB-EPS). The quantities of various EPS, including SEPS, LB-EPS, and TB-EPS exhibited the similar trends to that of total EPS. With the increase of phosphate concentration, the qualities of various EPS in biofilm increased gradually, as well as the content of protein and polysaccharide in EPS. Once the suitable phosphate level of microorganisms and microalgae was exceeded, the qualities of various substances decreased lightly at the highest level. The protein-polysaccharide ratio (PN/PS) increased firstly and then fell slowly with the change of phosphate level. In each type of EPS, the content of protein was higher than that of polysaccharide, which was consistent with domestic and foreign studies [24, 25]. With the extension of immersion time of adhesive materials, the quantities of protein and polysaccharide in the three types of EPS all showed a tendency to increase first and then slowly decline. However, the relative contents of protein and polysaccharide in three types of EPS showed different trends. The components of EPS

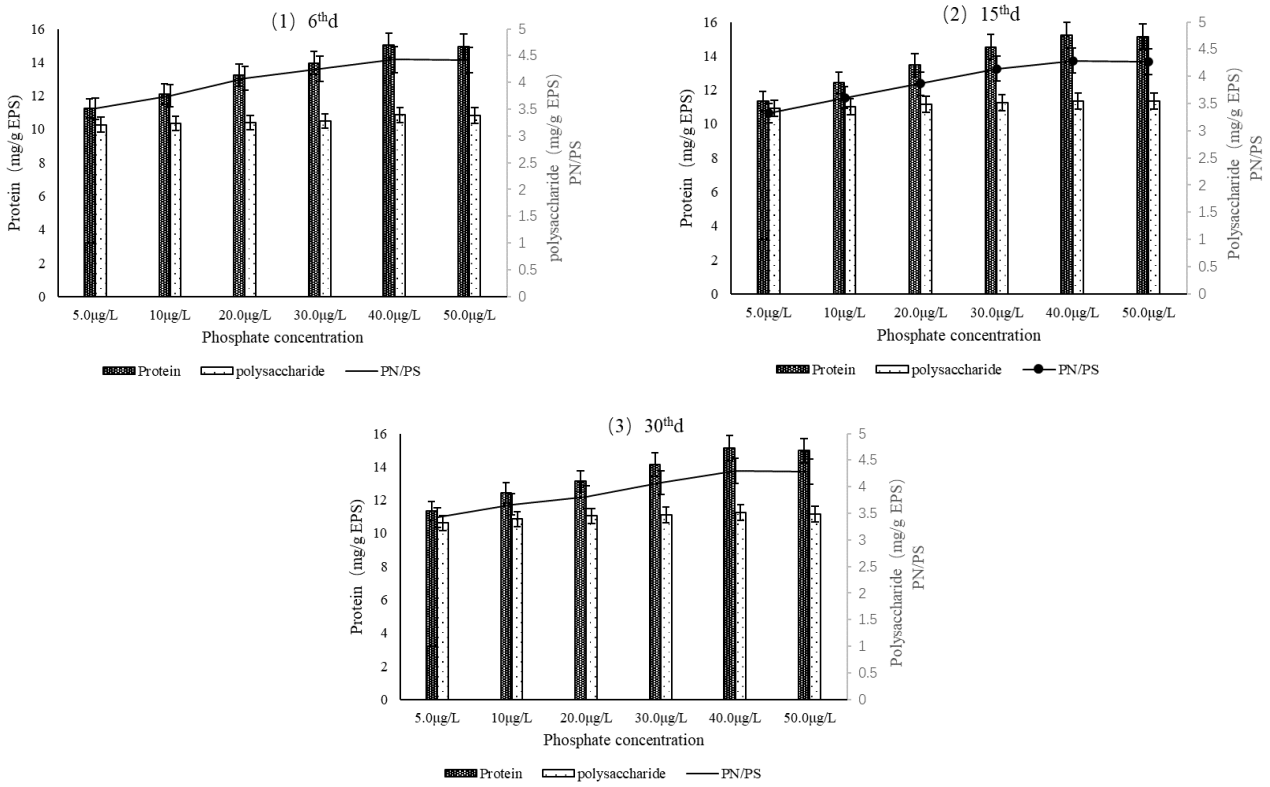

Figure 3. Effects of phosphate on SEPS of biofilms.

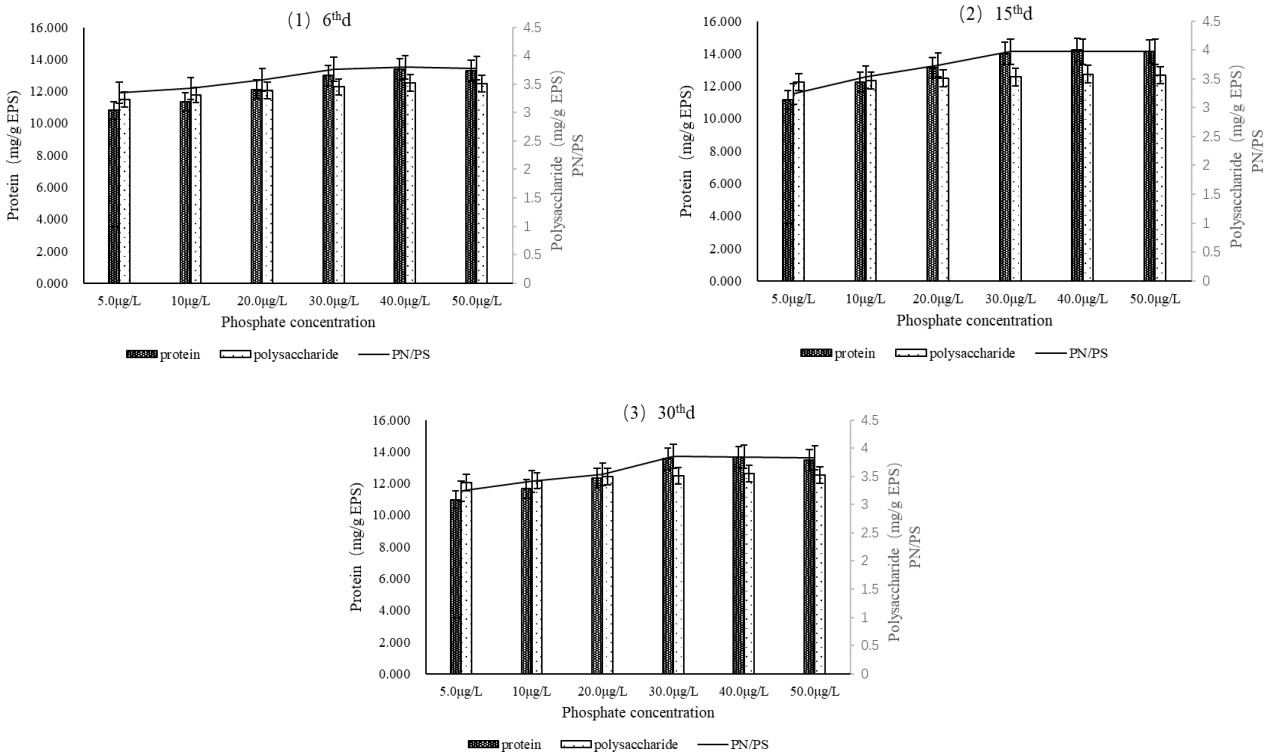

Figure 4. Effects of phosphate on LB-EPS of biofilms.

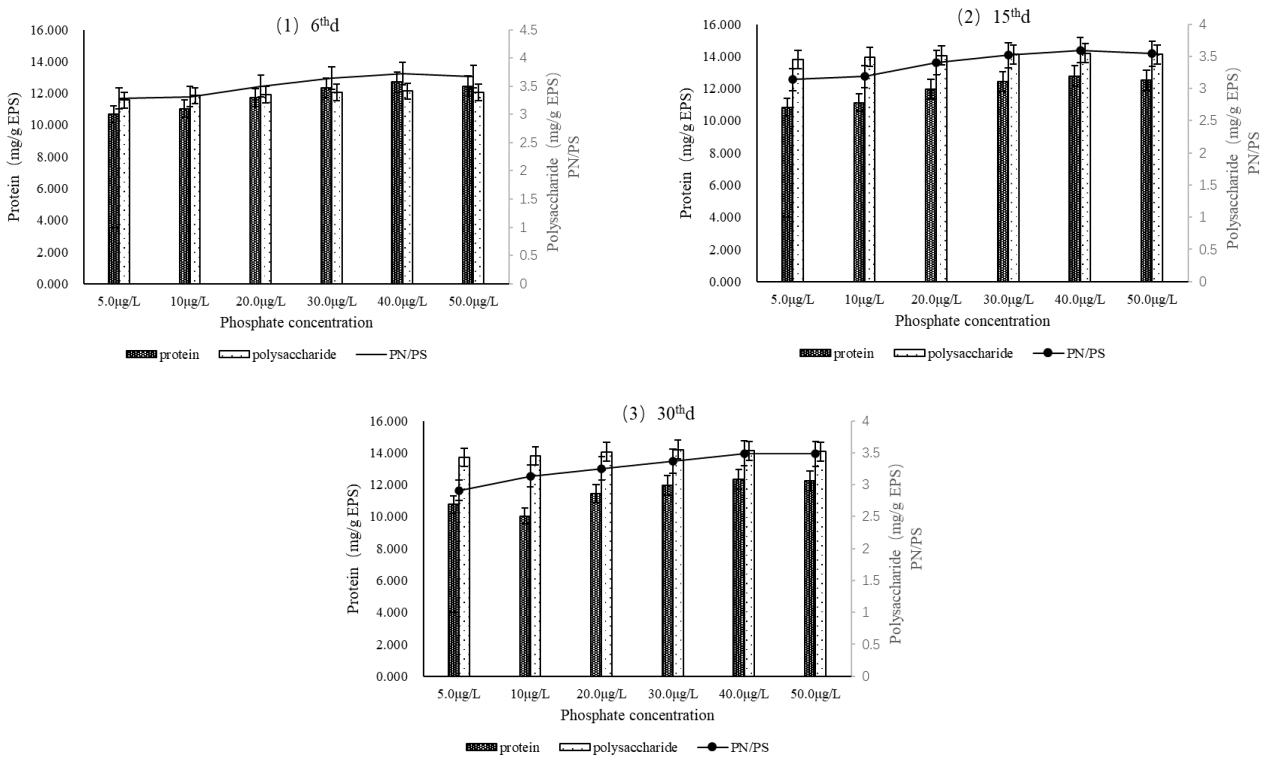

Figure 5. Effects of phosphate on TB-EPS of biofilms.

exhibited a gradient change through the whole thickness of the films. The protein content was the highest in surface SEPS, which gradually decreased along the direction of biofilm depth, while polysaccharide showed the opposite. This meant that SEPS and LB-EPS contained more protein and TB-EPS contained more polysaccharide. These were because the structures of the biofilm gradually tightened along the direction of the depth of the biofilm, the metabolism of microorganisms and microalgae decreased gradually due to obstructed exchange of substances [26]. The activities of microorganisms and microalgae in the surface biofilm were higher, and more extracellular enzymes were produced, which were components of proteins in EPS, resulting in the higher protein content in the surface layer. While polysaccharides were gradually consumed by microorganisms as they metabolized. Some extracellular protein was ectoenzyme, which could hydrolyze extracellular substances, such as polysaccharides, proteins, lipids, and chitin, into small molecules, which could be absorbed by microorganisms, resulting in the reduction of polysaccharides [27]. In the three types of EPS, the values of PN/PS decreased gradually from the outermost SEPS to the innermost TB-EPS.

Effect of phosphate on microbial composition of biofilm

The changes of microbial composition in the biofilms formed on the surface of attached plates under different water temperatures were shown in Figure 6. The microbial communities among these biofilms formed in different phosphate levels showed no significant difference. The microorganisms in biofilms mainly included *Bacillus*, *Pseudomonas*, *Vibrio*, *Aeromonas*, *Flavobacteria*, *Halomonas*, *Photobacterium*, *Xanthomonas*, *Acinetobacter*, and *Alcaligenes*, among which the predominant species were *Bacillus*, *Aeromonas*, *Pseudomonas*, and *Vibrio*. The main microalgae in biofilms were diatoms, and the dominant species were *Nitzschiella* and *Navicula*, *Rhizosolenia* and *Pleurosigma*.

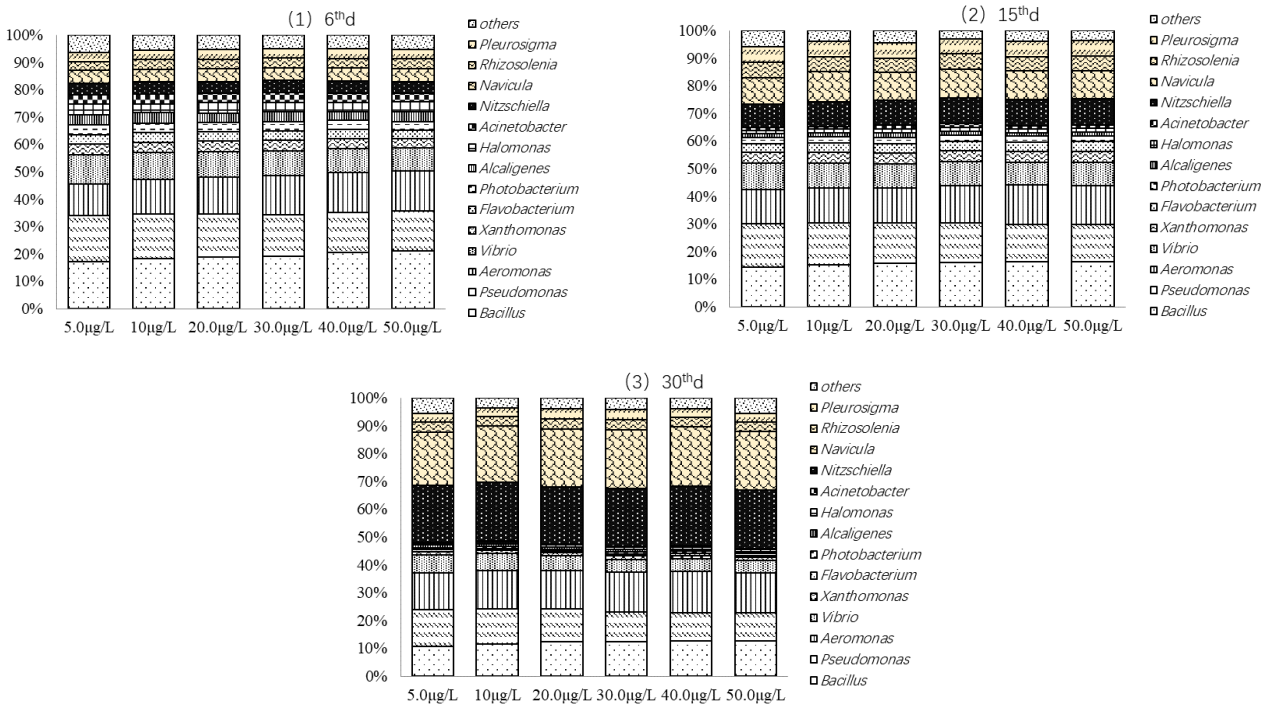

Figure 6. Effect of phosphate on microbial composition of biofilm.

On the 6th day, the biofilm was dominated by microorganisms of *Vibrio*, *Bacillus*, *Aeromonas*, and *Alcaligenes*, while the proportions of other microorganisms and diatoms were lower relatively. With the extension of time, the proportions of bacteria decreased. The ratios of *Pseudomonas*, *Vibrio*, *Bacillus*, and *Alcaligenes* decreased significantly ($P < 0.05$), while the proportion of *Aeromonas* slightly increased ($P > 0.05$). The diatoms in biofilms increased gradually over time. On the 30th day, the proportion of microorganisms in the biofilm further decreased, and the ratio of diatoms increased to more than 50%. At the same immersing time, the proportions of *Vibrio* and *Pseudomonas* in the biofilm decreased with the increase of water temperature, while the proportions of *Bacillus* and *Aeromonas* increased. The proportion of diatoms in the microbial population also increased with the increase of phosphate level, especially the *Navicula* and *Nitzschiella*, which changed significantly with the increase of phosphate concentration ($P < 0.05$).

Conclusion

The effects of phosphate level on the formation and dynamic succession of biofilm on the surface of attached plates were studied by indoor simulation experiments. The amount of biofilm, Chl-a, EPS, contents and proportions of polysaccharide and protein in EPS were analyzed. The results showed that 30-50 μg/L phosphate was more suitable for the biofilm formation. The changes of microbial community species during biofilm formation suggested that diatoms played a vital role in the maturation of biofilms. In view of the effect of biofilm on the aquaculture, this study also provided primary data and research ideas for the green development of the factory aquaculture.

Acknowledgments

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References

1. Bayliss SC, Verner-Jeffreys DW, Bartie KL, Aanensen DM, Sheppard SK, Adams A, *et al*. 2017. The promise of whole genome pathogen sequencing for the molecular epidemiology of emerging aquaculture pathogens. Front Microbiol. 8:121.
2. Zheng LH, Li J, Liu SL, Guo ZC, Wang FC, Liu JL. 2020. The main problems and effective countermeasures in pond culture of sea cucumber. J Aquacult. 41:1-3.
3. Xu Y, Sun Y, Wang L, Chen WB, Shang HX, Chen Y. 2021. Prevention for disease in sea cucumber culture. Scientific Fish Farming. 11:54-55.
4. Gao Q, Gong JX. 2019. Construction of integrated system of ecological marine aquaculture in China from the perspective of coupling mechanism of composite ecosystem. Journal of Ocean University of China (Social Sciences Edition). 3:15-20.
5. Mangwani N, Shukla SK, Rao TS, Das S. 2014. Calcium-mediated modulation of *Pseudomonas mendocina* NR802 biofilm influences the phenanthrene degradation. Colloids Surf B: Biointerfaces. 114:301–309.
6. Wu YQ, Zhao KF, Liu LM, Zhang YX, Du RB. 2016. Effects of substrate type on succession of periphyton and intermediate seedling culture of sea cucumber, *Apostichopus japonius Selenka*, in net cages set in an earthen pond. Marine Sciences. 40:22-31.
7. Flemming HC. 2016. EPS-then and now. Microorganisms. 4:41.
8. Siddik A, Satheesh S. 2019. Characterization and assessment of barnacle larval settlement-inducing activity of extracellular polymeric substances isolated from marine biofilm bacteria. Scientific Reports. 9:1-15.
9. Chiu J, Thiyagarajan V, Tsoi M, Qian P. 2005. Qualitative and quantitative changes in marine biofilms as a function of temperature and salinity in summer and winter. Biofilms. 2:183–195.
10. Therkelsen GH. Indstrial Gums: Polysaccharides and their derivatives. San Diego: Academic Press Inc. 1993.
11. Khalid ZM, Sameena ML, Rovidha SR. Advanced Methods in Molecular Biology and Biotechnology: Chapter 2 - Agarose gel electrophoresis. Academic Press. 2021. Pages 7-12.
12. Liu LM, Du RB, Zhang XL, Dong SL, Sun SC. 2017. Succession and seasonal variation in epilithic biofilms on artificial reefs in culture waters of the sea cucumber *Apostichopus japonicus*. Chin J Oceanol Limnol. 35:132-152.
13. Gruber-Brunhumer M, Nussbaumer M, Jerney J, Ludwig I, Zohar E, Lang I, *et al*. 2016. Two-stage cultivation of N-rich and N-deprived *Acutodesmus obliquus* biomass: Influence of cultivation and dewatering methods on microalgal biomass used in anaerobic digestion. Algal Res. 17:105–112.
14. Camacho-Chab JC, Lango-Reynoso F, Castaneda-Chavez MR, Galaviz-Villa I, Hinojosa-Garro D. 2016. Implications of extracellular polymeric substance matrices of microbial habitats associated with coastal aquaculture systems. Water. 8:1-21.
15. Rasamiravaka T, Labtani Q, Duez P, El Jaziri M. 2015. The formation of biofilms by *Pseudomonas aeruginosa*: a review of the natural and synthetic compounds interfering with control mechanisms. Biomed Res Int. 2015:1-17.
16. Yadav P, Verma S, Bauer R, Kumari M, Dua M, Johri AK, *et al*. 2020. Deciphering streptococcal biofilms. Microorganisms. 8:1-31.
17. Borlee BR, Goldman AD, Murakami K, Samudrala R, Wozniak DJ, Parsek MR. 2010. *Pseudomonas aeruginosa*, uses a cyclic-di-GMP-regulated adhesin to reinforce the biofilm extracellular matrix. Mol Microbiol. 75:827-842.
18. Li Z, Wang XY, Wang J, Yuan XY, Jiang XY, Wang YY, *et al*. 2022. Bacterial biofilms as platforms engineered for diverse applications. Biotechnol Adv. 57:107932.
19. Lu JY, Hu XC, Ren LJ. 2022. Biofilm control strategies in food industry: Inhibition and utilization. Trends Food Sci Technol. 123:103–113.
20. Fulaz S, Vitale S, Quinn L, Casey E. 2019. Nanoparticle-biofilm interactions: the role of the EPS matrix. Trends Microbiol. 27:915-926.
21. Zhuang Z, Yang GQ, Zhuang L. 2022. Exopolysaccharides matrix affects the process of extracellular electron transfer in lectroactive biofilm. Sci Total Environ. 806(3):150713.
22. Lasa I, Penadés JR. 2006. Bap: a family of surface proteins involved in biofilm formation. Res Microbiol. 157(2):99-107.
23. Thi MTT, Wibowo D, Rehm BHA. 2020. *Pseudomonas aeruginosa* biofilms. Int J Mol Sci. 21:1-25.
24. Dieguez AL, Balboa S, Magnesen T, Jacobsen A, Lema A, Romalde J. 2020. Comparative study of the culturable microbiota present in two different rearing systems, flow-through system (FTS) and recirculation system (RAS), in a great scallop hatchery. Aquac Res. 51: 1-15.
25. Hou JP, Wang C, Rozenbaum RT, Gusnaniar N, de Jong ED, Woudstra W, *et al*. 2019. Bacterial density and biofilm structure determined by optical coherence tomography. Nat Scientific Reports. 9:9749.
26. Flemming HC, Wingender J, Szewzyk U, Steinberg P, Rice SA, Kjelleberg S. 2016. Biofilms: An emergent form of bacterial life. Nat Rev Microbiol. 14:563-575.
27. Wilking JN, Zaburdaev V, De Volder M, Losick R, Brenner MP, Weitz DA. 2013. Liquid transport facilitated by channels in *Bacillus subtilis* biofilms. Proc Natl Acad Sci USA. 110:848–852.