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Visualization of the Microbial Community and Elemental Mapping of *Anadara granosa* Media Used in a Slow Sand Filter Using a SEM-EDS

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Abstract: The removal of contaminants in slow sand filters occurs mainly in the biofilm above the filter media called schmutzdecke - a thin biological layer consisting of various microbial communities of algae, bacteria, diatoms and zooplankton. The layer formed ripens along with continuous straining and adsorption mechanism of impurities in raw water. *Anadara granosa* shell has been broadly used as an adsorbent to trap organic matter, turbid particles and heavy metal ion in raw wastewater. This research is aimed to visualise the microbial community grown on schmutzdecke in 2-weeks ripening period and maps the elemental characterisation of a grinded *Anadara granosa* shell media after the ripening period using a Scanning Electron Microscope with Energy Dispersive X-ray Spectroscopy (SEM-EDS). The result shows that mostly algae and diatoms have been recognised without species identification. Calcium (67%) and oxygen (21%) dominate the major chemical element contained in grinded *Anadara granosa* shell media, indicating that calcium carbonate and calcite can replace conventional sand as a more-efficient slow sand filter media, with longer maturing period. Such result can lead to further research about the increase of clamshell usage as a slow sand filter media to treat any types of wastewater, especially in rural areas in developing countries

Key words: Visualization; Schmutzdecke; slow sand filter; *Anadara granosa* shell.

Introduction

It is well understood that access of safe and healthy clean water is one of the basic needs of human beings. Access to clean water shows that human rights are being highly appreciated. Clean water also sustains life. An inadequate production of safe clean water contributes to the increase in mortality and morbidity in the third world countries. Therefore, an effort to improve the quality of sanitation and clean water must be carried out immediately. Indonesia, as one of the developing countries, has tried to fulfill this global program of Sustainable Development Goals goal 6, to achieve 100% universal access of clean water and sanitation. The

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concrete evidence to boost the supply of clean water and sanitation in Indonesia was regulated in President Decree No. 185, 2014.

Slow sand filtration is one of the conventional technology and most successful potable water treatment techniques available for rural regions. High efficiency of water treatment is mostly achieved by the slow filtration rate in range of 0.1 to 0.3 m/hour and fine effective size grain in range of 0.15 to 0.35 mm. In comparison with rapid sand filter, biological process which occurs in the upper layer of sand filter bed plays the most important role. The contaminant removal mainly occurs in the schmutzdecke – a biological active layer or biofilm formed at the surface of sand filter bed (Campos et al., 2002).

Long schmutzdecke ripening period required at the beginning of filter run is the main limitation while operating slow sand filters. Schmutzdecke ripening involves continuous complex physical and biological mechanisms. As the filter runs, the biologically active layer keeps on developing and contributes to removal of water impurities (Dizer et al., 2004). Apart from the advantages of relatively high removal efficiency, slow sand filter is also profound in the other aspects, namely its simple design, ease to operate and maintain and low cost in construction. The operation does not require any electric supply, electric equipment and additional particular chemical substances. Furthermore, it does not need special cognitive skills from the workers and operators. All materials needed for constructing filter tank, filter bed, plumbing system and clean water reservoir are widely available in low price. Especially for the filter bed media, there is no particular type of sand used and so it is a possible option to utilise existing nearby local natural resources instead (Khudair and Jasim, 2018).

One of the potential material that acts as an alternative filter media is seashell solid waste. There are some types of seashells quite well-known by the Indonesian people, such as blood cockle (*Anadara granosa*), green mussel (*Perna viridis*), cockle (*Anadara antiquata*) and baby clam (*Paphia undulata*). The *Anadara granosa*, in particular, has been in existence along the seashore where the sandy mud substrate is found between 10 and 30 meters depth. In terms of its price, *Anadara granosa* is very economical because it is as cheap as IDR 7000 per kg (Suwignyo, 2005). In 2010, its production quantity level might reach as high as 34.482 metric tons with 5% to 10% annual growth based on data at the Ministry of Marine and Fisheries Republic of Indonesia (Pemerintah RI, 2011).

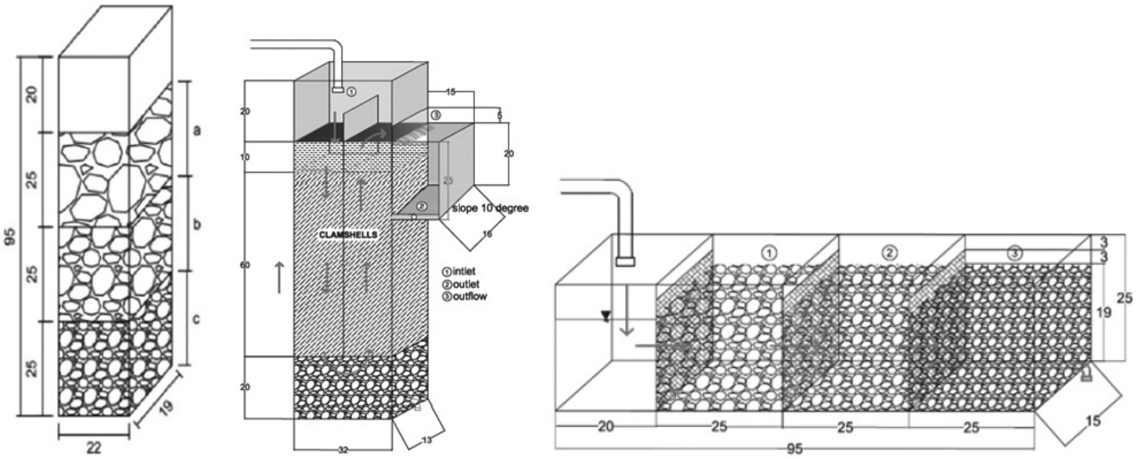
It turns out that the increasing quantity level of *Anadara granosa* production pushes the increase of number of its shell solid waste consequently. Nowadays, this waste is mostly being used as raw materials of seashell craft, room decoration, as well as food for cattle, whereas the number of shell solid waste absorbed are still definitely low (Agustini et al., 2011). Yet, Awang-Hazmi et al. (2005) stated that clamshell as a commodity contains approximately 98% content of calcium carbonate ($CaCO_3$), which is potentially substitutable for usage in water filtration. Moreover, research done by Surest et al. (2012) observed that high calcite (CaO) content obtained from grinded *Anadara granosa* shell solid waste could remove BOD, COD, TSS and turbidity water quality parameters in treating swamp water. This still huge quantity of *Anadara granosa* shell solid waste but high pollutant removal efficiency of *Anadara granosa* shell in treating polluted water had then inevitably brought about the new concept to take place this seashell solid waste as an alternative of the slow sand filter media.

Previously, SEM has been used to visualise the sand samples removed from a slow sand filter with varying success and describing further about its morphological information (Joubert and Pillay, 2008; Law et al., 2001). In contrast, energy dispersive X-ray spectroscopy (EDS) chemical element analysis feature has not previously been studied together with the SEM visualisation of microbial community within a slow sand filter. Therefore, it is envisaged that this research could aid in the study of the development of microbial community on schmutzdecke grown on grinded *Anadara granosa* shell particles of a slow sand filter. In addition, the chemical element was observed with EDS analysis software programme linked with the SEM tools. The microorganisms found on the micrograph were generally identified based on any morphological evidences compared to the control sample.

Materials and Methods

Design and Operation of Slow Sand Filter

An experimental laboratory-scale slow sand filter reactors had to be designed and constructed, including vertical-flow and horizontal-flow roughing filter as pretreatment units. These reactors were designed and construction met the standard design criterias regulated in SNI 3981:2008 about 'design of slow sand filter installation'. The dimensions of the reactors are described in Figure 1 and Table 1.



(a) (b) (c)

Figure 1: Schematic representation of the (a) vertical-flow roughing filter, (b) horizontal-flow roughing filter, and (c) slow sand filter.

Table 1: The dimensional details of vertical-flow roughing filter (VRF), horizontal-flow roughing filter (HRF), and slow sand filter (SSF)

Design	*VRF*	*HRF*	*SSF*
Grain/gravel size (mm)	10–30	5–30	0.25–0.42
Filter length (cm)	22	95	32
Filter width (cm)	19	15	13
Freeboard height (cm)	10	3	20
Supernatant height (cm)	10	3	10
Amount of compartment	3	4	-
Compartment height (cm)	25	25	-
Sand bed depth (cm)	-	-	60
Gravel bed depth (cm)	-	19	20
Filter height (cm)	95	25	110

The slow sand filters were constructed using 10 mm-thick flat glass sheet, as this material is more anti-fuites, longlasting, does not erode, easy to clean and has a cheaper cost to maintain. While the vertical-flow roughing filter and horizontal-flow roughing filter were assembled using 10 mm-thick flat acrilic sheet, as this material is easily obtainable, low cost and light in weight yet strong. The filter was made by local aquarium-made craftmen. The original dry clean shell was mechanically crushed and grinded, and then using sieve/mesh no. 40 and 60 in order to get an effective size of range 0.25–0.42 mm.

Raw municipal wastewater from one of low-middle apartment in Surabaya City was previously pre-treated by making it flow through the vertical-flow roughing filter and horizontal-flow roughing filter by means of one-way sequence. This should be done in order to meet the quality of the slow sand filter intake water, which requires turbidity as low as 5 NTU. The rate of filtration was controlled by the inflow valve exactly 0.1 m/hr. The water debit was monitored twice a day. The water level above the horizontal-flow roughing filter media was continuously kept fully submerged. The entire reactors were housed outdoor in a partially shaded place that received minimum direct sunlight. Consequently, this was carried out to prevent the excessive growth of the algae.

Sampling of Sand

Samples of filter media and schmutzdecke were taken once at the end of two-week ripening period. In addition, a control sample (clean media) was taken at the beginning of the filter run (time 0). A sterile modified long-handed spatula was inserted into the grinded shell to a depth of approximately 1 cm below the surface of media to make the particles remain intact. Only a small amount of 2 g sample is needed that is a sufficient quantity for SEM-EDS analysis. The execution was taken carefully to ensure minimal handling and possible disturbance of samples.

Scanning Electron Microscopy and Energy Dispersive X-Ray Spectroscopy (SEM-EDS)

No specific preservation method was performed to prepare samples. Samples were stored in glass bottles and dried naturally under room temperature. Dry samples were mounted onto aluminium stubs and stuck using carbon double-sided tape. Initially, the specimens were coated with gold (Au) ion under vacuum condition using the COXEM SPT-20 Ion Sputter Coater. These samples were viewed under high vacuum (approximately 10^{-6} Torr) to obtain high quality images, with HITACHI FlexSEM 1000 VP-SEM at 15.0 kV, 6.1 mm Work Distance (WD) in four different magnification (5.0k, 10.0k, 15.0k and 30.0k). Images have a detector labelled as SE on the databar. Then, one micrograph with 5.0k magnification was analysed using an Energy Dispersive X-Ray Spectroscopy (EDS) software programme to map eight chosen chemical elements (i.e., O, Na, Mg, Al, Si, K, Ca and Fe). The obtained data was visualised in sum spectrum graphic and smart quantitive result.

Results and Discussion

Scanning Electron Microscopy (SEM) Visualization Analysis

Under the SEM visualisation, it is observed that grinded *Anadara granosa* shell particles takes irregular forms and its edges are sharp in nature. Unfortunately, there is no empirical evidence on the attachment of microbial communities or other particles on them. This sample acted as a control variable and a comparison for post ripening period sample. Each grinded shell particle dimension could not be described precisely, but the majority of the particles fell into the size range between 250 and 400 µm in accordance to the sieving standard. In this context, the image demonstrates that initially grinded shell particles are still independent and are not attached to other particles. Joubert and Pillay (2008) reported the same bareness result of sand particles control sample in their SEM micrograph.

After two-weeks ripening period of schmutzdecke, there was a small evidence of difference in colonisation of grinded shell particles by bacteria and diatoms. It is beyond the normal confidence that bacteria appear to be the initial inhabitant occupying in the schmutzdecke layer. The obvious evidence was found that first, a sticky glue-like mucilage footage had been spotted, as shown in Figure 2. Surely, extracellular polymeric matrix had been firmly in contact, attached and covered surrounding the shell particle. Therefore, the initial formation of an attached-growth with schmutzdecke is aided by the production of mucilage excreted by bacteria. This could not be done unless there is an enough supply of nutrient or organic impurities contained in the slow sand filter intake raw water. This mucilage product acts as push on towards the attachment process of bacteria to surface of filter media particles (Law et al., 2001).

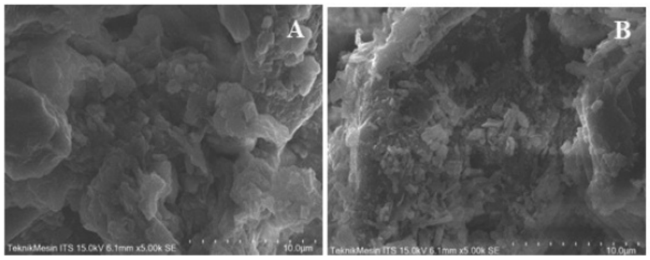


Figure 2: (a) Micrograph of the control sample and (b) sample after two-weeks ripening period showing the sign of mucilage production by bacteria and diatoms (magnification 5.00 k) Energy Dispersive X-Ray Spectroscopy (EDS) Elemental Mapping

Due to some inevitable handicaps and limitations, there is a limited biofilm drying procedure, which takes place at room temperature. It is not advised to store this delicate sample under room temperature in an open air, but should be prepared very carefully. During the biofilm preparation step, some special approaches ought to be done, for example the fixation in glutaraldehyde cross-linking, ethanol dehydration, critical-point drying (CFD) or less-invasive low-temperature drying by vacuum sublimation (lyophilisation) method.

Hence, it is advisable to apply the latest method mentioned above, lyophilisation, as it is suitable for the preservation method of bacterial cells sample and many forms of extracellular matrix structure, this method is a fast and inexpensive nondestructive preparatory method for SEM analysis of biofilm. Lyophilised material could be imaged with high resolution using Conventional High-Vacuum SEM (CSEM) (Karcz et al., 2012). Under difficult circumstances, the final method that can be applied is by visualising under Environmental SEM (ESEM) tool, which can accept wet biofilm sample condition in similar high resolution image quality (Joubert and Pillay, 2008).

According to the sum spectrum graphic result as shown in Figure 3 and quantitative results in Table 2, calcium and oxygen were seen as two dominant

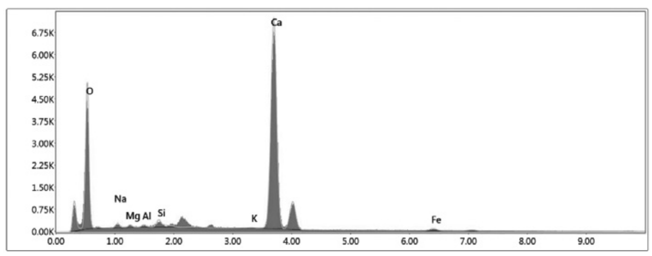


Figure 3: The sum spectrum graphic of EDS chemical element analysis.

Table 2 : The smart quantitive results of EDS chemical element analysis

Element	*Weight (%)*	*Atomic (%)*	*Net Int.*	*Error (%)*	*K ratio*	*Z*	*A*	*F*
O	47.65	69.21	75.44	10.24	0.0803	1.0824	0.1557	1.0000
Na	0.81	0.82	2.51	15.31	0.0029	0.9797	0.3603	1.0012
Mg	0.28	0.27	1.52	22.24	0.0015	0.9955	0.5147	1.0022
Al	0.24	0.20	1.51	21.35	0.0015	0.9578	0.6577	1.0041
Si	0.64	0.53	4.84	8.91	0.0049	0.9779	0.7721	1.0068
K	0.08	0.04	0.36	63.32	0.0007	0.9028	0.9924	1.0664
Ca	48.86	28.33	183.78	2.00	0.4509	0.9184	1.0022	1.0032
Fe	1.44	0.60	2.16	16.21	0.0117	0.8115	0.9770	1.0274

chemical contents in the grinded *Anadara granosa* shell sample. It had been done because the grinded clamshells mostly consist of calcium carbonate compound which took effect to the level of shell hardness. In accordance with Afranita et al. (2013) results, *Anadara granosa* shell consists of several chemical compounds such as calcium carbonate, calcium hydroxyapatite, calcium phosphate and chitin. Combined with calcium carbonate, chitin produces a much stronger composite. Addition of only 1% calcium carbonate can reduce the turbidity value on water treatment as grinded shell has more amounts of microporouses. Microporouses take an important role to trap organic impurities and heavy metal which had existed in the inflow raw water of a slow sand filter. A rich-nutrient environment provides an ideal condition for the schmutzdecke to grow and develop.

Conclusion

This research result visualises the variety of microbes that grew in the biologically active schmutzdecke layer of a modified slow sand filter with grinded *Anadara granosa* shell media. After two weeks ripening time to develop the schmutzdecke layer, it can be concluded that bacteria and diatoms are two microorganisms which promote the formation of this complex matrix layer. It was also concluded that due to improper sample preparation handling, visualisation of bacteria and diatoms morphology using SEM was not well-executed and difficult to find out the best enlargement spot for further analysis. The EDS element analysis proved that calcium and oxygen element dominates the main composition of shell media, in accordance with clamshell which consists approximately 97% to 99% calcium carbonate ($CaCO_3$) or calcite (CaO) compound. Higher content of calcium and oxygen has pushed on the performance of pollutant straining and adsorption mechanism. Hence, it created an ideal environment for enhancing the attachment process of exopolysaccharides or extracellular polymeric mucilage by bacteria, followed by diatoms. Unfortunately, it is recommended that further research should be done to investigate the correlation between the chemical elements composition of media and microbial biodiversity, microorganism identification, water quality analysis of the slow sand filter outflow during the ripening period, as well as doing the proper biofilm sample preservation method, to explain in depth about the relationship between the usage of grinded *Anadara granosa* shell and the pollutant removal efficiency. With such a conventional technology such as slow sand filter, the sixth goal of the 17 Sustainable Development Goals (SDGs) of creating well management and supply of clean water and sanitation can be achieved. This research can be further improved and modified to another project with similar objectives and needs.

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