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Evaluation of Nutrient Removal Efficiency with Chitosan: Nutrient Composition and Bacterial Removal in Effluents of Nile Tilapia (*Oreochromis niloticus*) in the Hatchery

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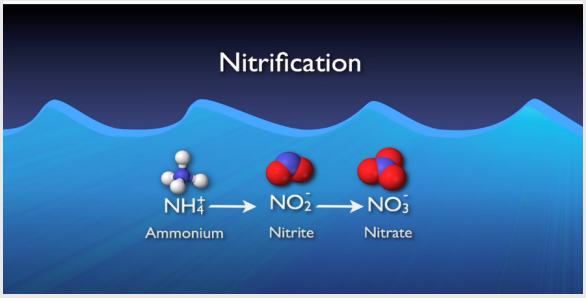
Abstract

Effluents from aquaculture systems contain large volumes of chemical substances and microbial load such as polychlorinated biphenyls and antibiotics that are often used to control infection and pathogenic bacteria originating from feed or water. These substances, if discharged, create pollution in the aquatic environment. Mitigating this problem requires implementing appropriate treatment methods. This study investigated the efficiency of uptake of nutrients in the wastewater and reduction of microbial pollution by chitosan. This product is a linear polysaccharide composed of β-linked D-glucosamine and N-acetyl-D-glucosamine and can be extracted from the shells of shrimps, lobsters, crabs and other crustaceans that are discarded in bulk quantities by seafood restaurants. The performance of laboratory-produced chitosan (S1) which was prepared from shells of Pacific white leg shrimp (*Litopenaeus vannamei*) was compared with that of the commercial grade chitosan (S2). While the latter was more effective in nitrogen and phosphorus removal and reduction of total faecal coliform, the two products were comparable in the uptake of minerals from the effluents from a tilapia culture system. The results showed that S1 and S2 adsorbed the nutrients from aquaculture effluents, especially ammonia (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-) and phosphate (PO_4^{3-}). However, differences were evident in terms of the efficiency of their removal and duration of treatment required for the purpose. In this respect, S2 performed better. Moreover, the anti-bacterial activity of S2 was higher than that of S1, and this appeared to be linked to differences in surface features of the two products. The chitosan extracted from shrimp waste and processed locally provides a low-cost solution to the environmental problems caused by aquaculture effluents.



NH_4^+, NO_2^-, NO_3^-, PO_4^{3-}

Keywords: Wastewater; Eutrophication control; Cost-effective solution; Adsorption efficiency; Environmental footprint

Introduction

Aquaculture effluents cause pollution of the receiving environment [1]. Their effects depend on several factors, including the volume and chemical composition of the discharge, and the assimilation capacity of the recipient water body [2-4]. Chemical composition of the effluent depends on several factors, including feed quality [5], ration size [6], protein content of feed [7], stocking density [8] and water exchange rate [9]. Among these factors quality of feed given to the fish is considered to a major one since it significantly influences the nature of metabolic substances generated and the products of decomposition of the uneaten feed [10]. Optimum water quality parameters for tilapia growth are dissolved oxygen (more than 3-5 mg/L), pH (7-8), temperature (26-30°C), total ammonia nitrogen,

TAN (0.17-3.87 mg/L), nitrite (0.02-0.12 mg/L), nitrate (2-219 mg/L). Regarding Biochemical Oxygen Demand, BOD, and Chemical Oxygen Demand, COD, the suitable values are less than 10 mg/L and less than 50 mg/L, respectively (ICAR, 2007). Many techniques have been adopted to treat aquaculture wastewater such as the use of chitosan and microalgae that have coagulating and flocculating ability [11], application of Recirculating Aquaculture System (RAS) which is able to ensure uptake and transformation of nutrient waste into biomass production [12,13], aquaponic system where plants act as biofilter and helps to remediate water quality [14,15], more complex system such as Integrated Multi-Trophic Aquaculture (IMTA) where multiple species of aquatic organisms from different trophic levels are integrated to utilize the waste to create a balanced system to ensure bioremediation of water quality [14,16], Biofloc Technology (BFT) that is distinguished by its ability to promote the growth of a microbial community primarily responsible for water quality maintenance [17,18], combination of aquatic organism and plants in a closed system membrane separation, electrochemical transformation and biodegradation [19]. Recently, interest is rapidly catching up in introducing the concept of circular economy inspired by nature and termed as aquamimicry that simulates natural conditions of aquatic system for integrated production. However, adsorption methods have gained popularity due to relatively low-cost, efficiency, practical convenience and environmental compatibility [7].

The role of chitosan in the adsorption of water pollutants has been receiving more interest in recent years [20-22]. This is considered a low-cost and eco-friendly solution. This study was carried out to investigate the effectiveness of chitosan, a linear polysaccharide composed of β-linked D-glucosamine and N-acetyl-D-glucosamine, which can be extracted from the shells of shrimps, lobsters, crabs and other crustaceans. The discarded shells of these animals are abundantly available as a waste product and can provide a rich source of the raw material free of cost for chitosan extraction.

Therefore, this investigation has the objective to evaluate the nutrient removal efficiency by chitosan based on effluents from the culture of Nile tilapia (*Oreochromis niloticus*). This fish is one of the most widely cultured species in the tropical and sub-tropical regions and ranks among the top three in the world in the aquaculture sector [23-25]. The ever-increasing tilapia culture has raised concerns about the management of effluents [23] resulting from the uneaten feed, therapeutic chemicals, solid wastes, dissolved organic matter, such as nitrogen, phosphorus and Total Suspended Solid (TSS) [26-29]. A poor water quality environment for tilapia culture is due to the high amount of toxic waste such as total ammonia nitrogen, nitrite, nitrate and organic matter that decompose, a in water and provide a plague spot environment for bacterial growth, and this condition has been linked to high levels of fish meal or fish oil in aquaculture feed [27,30]. Because tilapia is an affordable fish, only cost-effective methods for managing wastewater from its culture systems will provide a practical solution such as the one developed in this study using chitosan from shrimp shell waste.

Materials and Methods

Study site

The study was done at the Fish Hatchery and Chemical Oceanography Laboratory of Borneo Marine Research Institute, Universiti Malaysia Sabah, Kota Kinabalu, Sabah in October 2019.

Water samples collection

Tilapia wastewater samples were collected from the outlet of juvenile tilapia culture tanks in the Fish Hatchery. Thirty liters of water samples were filtered using 50 μm of white nylon net to remove solid waste for carrying out the adsorption test.

Extraction of chitosan

Sample collection: Shells of the Pacific white leg shrimp (*Litopenaeus vannamei*) were procured from the SAFMA Kota Kinabalu seafood market on September 2019, and subjected to the process for extraction of chitosan (S1). Its performance was compared with the commercial grade chitosan (trade name Kitosan) (S2) treated with sodium hydroxide obtained from Dasatim Sendirian Berhad, Malaysia. According to the company specifications, this product marketed under the name Kitosan plus TM and is extracted from crustacean shells, and is safe to use for producing organic food. These S1 and S2 products are shown in figure 1.

Demineralization: The shrimp shells were mineralized with 2.5% (w/v) of hydrochloric acid (1:20 w/v) at room temperature (27°C) for 6 hours to remove the mineral content from the ground shells. Samples were then filtered to remove the residues and then washed with tap water for at least 30 minutes until the neutral pH was achieved. Subsequently, the shell residue was dried in the oven for 24 hours at 60°C [16].

Deproteinization: The dried mass was treated with 2.0% of potassium hydroxide solution in a ratio 1:20 (w/v) with constant stirring carried out for 2 hours at 90°C to remove protein in the shells. The deproteinized shells were transferred to the oven for 24 hours at 60°C until the product was dried [16].

Decolouration and dewatering: The dried shrimp shells were immersed in acetone for 10 minutes and maintained at room temperature to evaporate the solvent followed by washing with the running tap water. The product was then filtered and dried at 60°C in the oven for 24 hours [16].

Deacetylation of chitin: Chitin so obtained by the above processing was treated with 40% sodium hydroxide in the ratio of 1:15 and maintained at 105°C for 2 hours. Thereafter, the contents were filtered and washed with deionized water until the neutral pH 7 was attained. The resulting chitosan was dried at 60°C for 24 hours in the oven at 60°C [31]. The stepwise process followed for production of chitosan is shown in flow chart (Figure 2).

Adsorption test

Samples of wastewater from tilapia culture tanks in the fish hatchery were collected and filtered using a normal filter paper. Each filtered sample was transferred to a 3 L capacity beaker and 1.0 g/L of chitosan was added to the beaker while magnetic stirring continued. Aliquots measuring 10 mL were collected in triplicate at intervals from 1 to 320 minutes (1, 5, 10, 20, 40, 80, 160 and 320 minutes). The aliquots were then centrifuged for 3000 rpm and the supernatant was pipette out for transfer into clean test tubes for further water quality analysis [20].

Removal of ammonia (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-) and phosphate (PO_4^{3-}): Concentrations NH_4^+, NO_2^-, NO_3^-, PO_4^{3-} were determined using the Hach Ammonia Low-Range Standard Method 10023, Hach Nitrite Low-Range Standard Method 8192, Hach Nitrate High-Range Standard Method 8093 and Hach Phosphate Standard Method 8048, respectively. The optical density was measured using UNICO 2100 spectrophotometer.

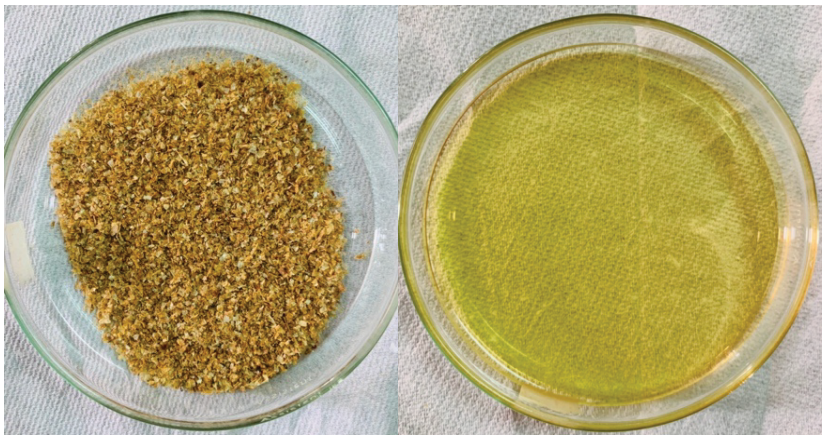


Figure 1: Chitosan products S1 (left) and S2 (right).

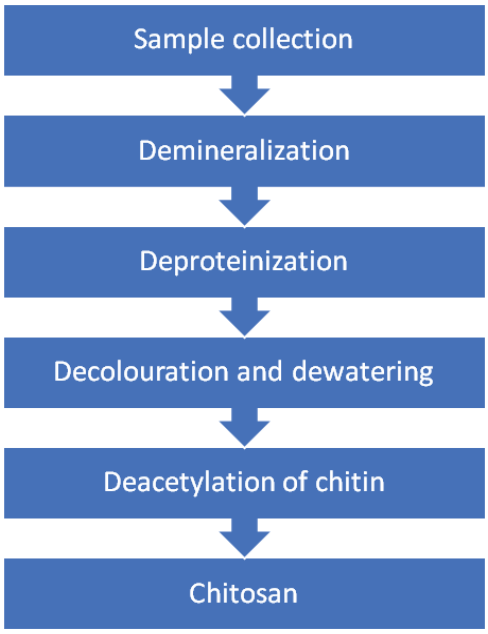


Figure 2: A flow chart showing the methodology followed for of laboratory-produced chitosan..

Percentage removal of effluents was determined by the following equation:

$$Efficiency\ of\ removal\ (\%) = \frac{(Ic - Fc)}{Ic} \times 100 \qquad (1)$$

Where,

Ic=Initial Concentration (mg/L)

Fc=Final Concentration (mg/L)

Pollutant adsorption capacity was measured by the formula:

$$Q = \frac{(Ic - Fc)}{M} \times V \quad (2)$$

Where,

Q=Adsorption Capacity (mg/L)

Ic=Initial Concentration (mg/L)

Fc=Final Concentration (mg/L)

V= Volume of Solution (L)

M=Adsorbent Mass (g)

Determination of nutrient composition in the adsorbent

Minerals that also serve as nutrients were analysed in the adsorbents. The sample preparation was done according to the wet digestion method. Dry samples were weighed 0.5 gr each and placed in a flask followed by the addition of 10 ml of concentrated HNO_3. The samples were maintained at room temperature overnight and then heated at 125°C for 4 hours. This was followed by dilution with 12.5 ml of concentrated HNO_3 and mixing for a few minutes until amorphous substance appeared to settle at the bottom of the flask. The contents were diluted with 50 mL of distilled water and filtered on a 0.45 µm white filter paper to get a clear solution and were prepared triplicate. This sample was used for quantitative analysis of elemental composition with the help of the Inductively Coupled Plasma Optical Emission (ICP-OES) method.

Bacteria colony count (CFU/ml)

Another 1 L wastewater sample collected from Fish Hatchery was immediately put in a freezer without being filtered. The preparation of nutrient media-agar involved mixing of 0.5 gr Rosolic acid with 5 ml of 0.2 NaOH (mixture 1). Subsequently, 26 gm of m-FC agar base was added with 500 ml of distilled water (mixture 2). Next, mixtures 1 and 2 were pooled in a beaker, heated on a hotplate, and brought to simmer. Then, the agar was poured into a plate and allowed to set.

The plate count method was applied for determining the bacterial density. A series of dilutions 10-4, 10-5 and 10-6 of the water sample was done in triplicate. A measured 1.0 ml of diluted water sample was put into 100 ml of distilled water in a conical flask and shaken thoroughly. Thereafter, 100 ml of the mixed sample was filtered with a grid 0.45 µm membrane filter. The filtered contents were put on top of the nutrient agar, closed with the lid and sealed with paraffin. Finally, the sealed media was placed into an oven at 45°C for 24 hours after which the counting of bacterial colonies were observed and counted in serial dilutions of 10-4, 10-5 and 10-6.

Statistical analysis

The statistical analysis of the adsorption efficiency (%) of NH_4^+, NO_2^-, NO_3^-, PO_4^{3-} and nutrient composition of S1 and S2 were performed by Independent Sample T-test, while faecal coliform bacteria count was subjected to Paired Samples t-test in Statistical Package of the Social Sciences (SPSS) software (version of 22.0). The data variability was done in triplicate and expressed as mean standard error with significant difference of $P<0.05$, was between treatments characterised by small letter (if any).

Results

Nutrient removal by adsorption

The initial concentrations of NH_4^+, NO_2^-, NO_3^-, PO_4^{3-} in the tilapia wastewater were 3.3 mg/L, 3 mg/L, 3 mg/L and 4.9 mg/L, respectively. The data shows (Figure 3) the efficiency of NH_4^+ removal with the S2 treatment increased after 20 minutes whence it was 55.4%. It was more efficient than S1 treatment that achieved 47.9% removal. After 80 minutes, the adsorption was constant, reaching the equilibrium or saturated state. The removal of NO_2^- by S2 treatment amounted to 53% after 40 minutes and 52% with S1 treatment. After 40 minutes, the adsorption of NO_2 became constant with both S1 and S2 treatments until the end of the experiment. It was different in the case of NO_3^- where the highest removal was observed after 1 minute, amounting to 56.7% in S2, but 48.3% in S1 and that too after 20 minutes of treatment. Interestingly, after 80 minutes the graph for S2 showed a declining trend by the release of adsorbate back to the solvent, marking a desorption condition after a saturated stage of adsorption. The pattern of removal of PO_4^{3-} was different, with 32% in S2 and only 10.2% in S1 treatment after 1 minute of agitation. The maximum removal was observed after 80 minutes when it reached 72.0% for S2 and about 50% removal can be seen in S2 at 80 min. Statistically, there is no significant difference ($p>0.05$) detected between S1 and S2 treatments in the removal efficiency (%) of NH_4^+, NO_2^-, NO_3^-, but was significant for PO_4^{3-} ($P<0.05$).

Adsorption capacity test

The dosage of 1.0 g/L of chitosan used in this study did not result in linear equilibrium in adsorption of some nutrients, most likely due to competition with other substance and chemicals such as dyes, metals, ions, drugs or hormones, antibiotics that may present in the solution for the active sites known as interference in adsorption and desorption behavior. It may also because the dosage of chitosan added into the treatment was not be sufficient to bind with the wastewater molecules. The maximum adsorption of NH_4^+, NO_2^-, NO_3^-, PO_4^{3-} by S1 and S2 is shown in table 1. However, there was no significant difference as far as the adsorption capacity of S1 and S2 is concerned.

Determination of nutrient composition of chitosan

The nutrient composition of S1 and S2 are shown in figure 3, figure 4A and 4B. The results revealed that there were no heavy metal elements in the treatments. This could be because the source of S1 and S2 was the same (shrimp shells). They have almost the same nutrient composition where Na and Ca occurred in significant amounts. Minor differences ($P>0.05$) were noticed in the concentrations of K, Ba, Al, Zn, Fe, Mg as well as Rb, Sr, Se, Sr and Mn.

Bacterial colony count (CFU/ml)

The faecal coliform count was used to monitor the water quality conditions and the risk of waterborne disease in aquatic ecosystems [28]. The initial bacteria colony count of faecal coliform was 1.62 × 106 (Figure 5A). After treatment with S1, it decreased to 1.08 × 106 (Figure 5B), and a sharp decline to 0.23 × 106 was observed

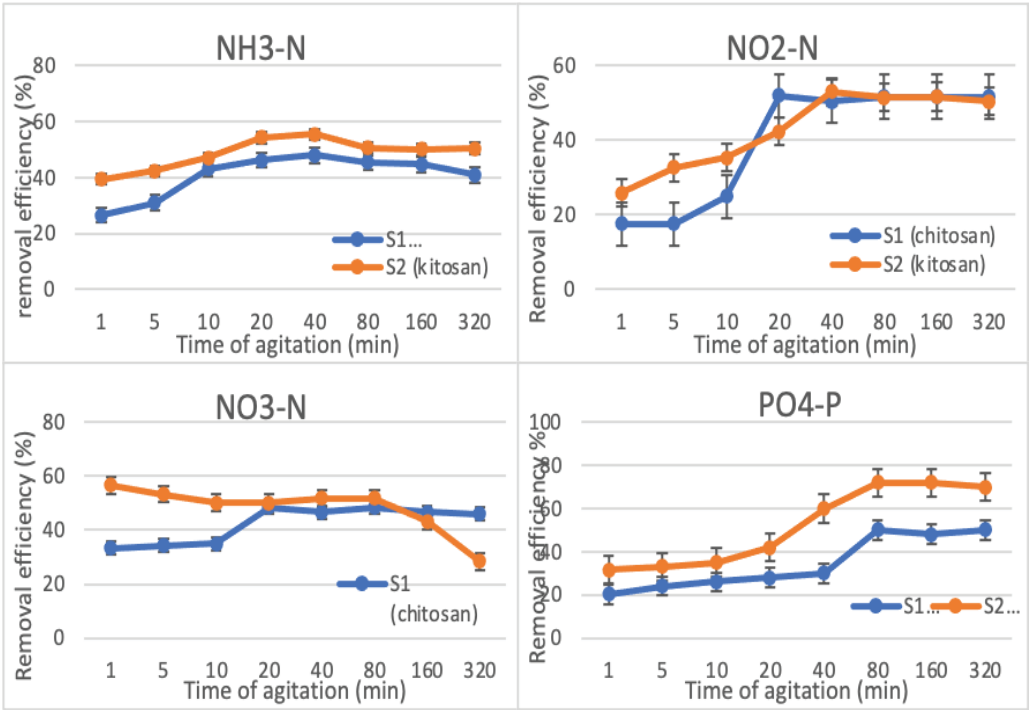


Figure 3: Effect of duration of treatment by agitation (min) on the removal efficiency (%) of NH_4^+, NO_2^-, NO_3^- and PO_4^{3-} for S1 and S2.

Table 1: Adsorption efficiency of S1 and S2 for NH_4^+, NO_2^-, NO_3^- and PO_4^{3-}.

Treatment	Ammonia, Q (mg/g)	Nitrite, Q (mg/g)	Nitrate, Q (mg/g)	Phosphate, Q (mg/g)
S1	10.8 ± 0.1	7.80 ± 0.3	1.25 ± 0.15	12.25 ± 0.2
S2	11.1 ± 0.1	7.95 ± 0.3	8.5 ± 0.1	17.15 ± 0.2

Table 2: Average efficiency of S1 and S2 in removing faecal bacteria in the tilapia wastewater.

Treatment	Average efficiency (%)
S1	32.82
S2	85.45

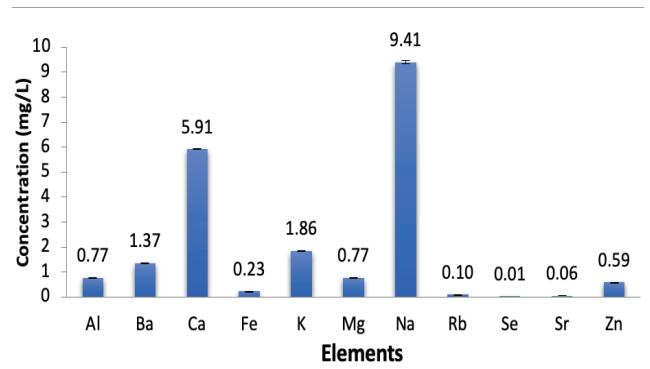


Figure 4A: Nutrient composition of S1.

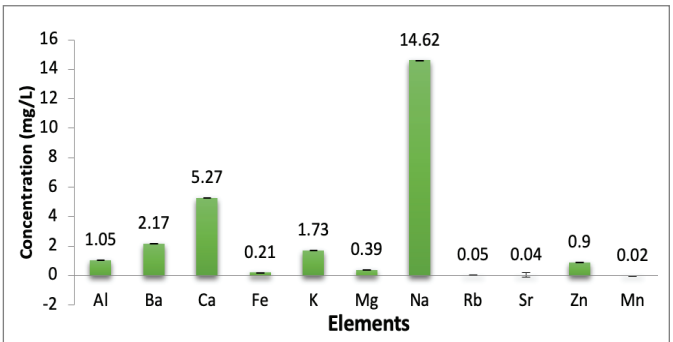


Figure 4B: Nutrient composition of S2.

following the treatment with S2 (Figure 5C). It is evident from table 2 that S2 removed a higher percentage of faecal coliform than S1. The antibacterial activity of chitosan can be affected by factors such as temperature, dosage (concentration), purity and target bacteria [32]. A paired t-test reveals a statistical difference ($P<0.05$) between S1 and S2 treatments in the faecal coliform count.

Discussion

The presence of reactive hydroxyl functional group (-OH) and amine (-NH2) in the chitosan chemical structure makes it effective as an adsorbent material for the removal of contaminants from wastewater [7]. A high percentage of nitrogen on the porous surface of chitosan suggests the existence of an amino-functional group that facilitates adsorption interactions between chitosan and wastewater molecules [31]. The modified or treated chitosan that develops by grafting amino, quaternary ammonium, carboxyl and thiol groups possesses a higher density of reactive groups [33]. This enhances the ability of the product to carry out more functions with higher selectivity and affinity in a wide range of applications.

Since most of the compounds causing pollution are negatively charged, the unique features of chitosan as biopolymers are more effective than other mineral coagulants such as aluminium sulphate in removing undesirable chemicals from the aqueous solution. By virtue of being a single cationic biopolymer the chitosan neutralizes and removes the anionic suspended colloidal particles by coagulation-flocculation [34].

The adsorption of NH_4^+ into chitosan is mainly due to the ionic interaction between this positively charged molecule and negative adsorption sites of the adsorbent (-COO-). This is further proved by performing an adsorption study involving a mixed solution containing positively charged NH_4^+ and negatively charged PO_4^{3-}. At the end of the trial, the concentration of PO_4^{3-} was equivalent to the initial concentration of NH_4^+, indicating that the adsorbed electrostatic attraction is most likely responsible for the adsorption of NH_4^+ [35]. Similarly, for the other nutrients, namely, NO_2^-, NO_3^-, PO_4^{3-} the adsorption mechanism was mainly due to electrostatic attraction between quaternary sites' positively charged cations and the negatively charged anion [7]. A rapid and high level of absorption of nitrate into chitosan (Figure 2) can be attributed to the presence of nitrogen functional group $N^+(C_2H_5)_3$. The evidence pointing to the ion exchange as being responsible for this adsorption mechanism has been presented by Appunni S, et al. [36].

Nitrate is the final product of the nitrification process in the wastewater and phosphate is a part of the chemicals in the waste originating from fish culture, including the uneaten feed and excrement. Generally, these two compounds have a noticeable presence in tilapia wastewater. Figure 2 shows that the percentage removal of phosphate is 15.3% higher than that of nitrate. This is mainly because phosphate adsorption capacities are greater than nitrate due to the ionic potential [37]. These findings serve to show that the polymers like chitosan tend to have a greater affinity towards ions with higher atomic number and valence.

This accounts for the three anions: $PO_4^{3-}>OH^->NO_3^-$ having higher adsorption of phosphate compared to nitrate [7]. Even though, most of the fish can tolerate very high concentration of nitrate up to 90 mg/L without any bad side effect and concentration of more than 300 mg/L can cause nitrate toxicity and cause disturbance on osmoregulation of the fish [26].

Generally, the percentage of removal of NH_4^+, NO_2^-, NO_3^-, PO_4^{3-} was better in S2 than S1 because of the differences in physical features of their surface. The product S1 was in the form of flakes, whereas S2 was in a powder state. Differences in these physical features are known to influence the adsorption [25]. The chitosan flakes have a rough surface and are lumpy, whereas chitosan powder has numerous interparticle pores that are highly diffused. Wu FC, et al. [38] also discussed this matter and highlighted that chitosan flakes have more rigid pore structures that are a steric hindrance to amino groups compared to powder that has a loose pore structure that facilitates the adsorption. The adsorption capacity was higher with smaller particle size [39] because the chemical reaction mostly occurs on the surface layer of chitosan so obviously smaller and more numerous particles will be more effective in adsorption [40]. Results presented in table 1 are consistent with these reports. The highest adsorbed wastewater nutrient was PO_4^{3-} (12.25 ± 0.2 mg/g) followed by NH_4^+, NO_2^- and

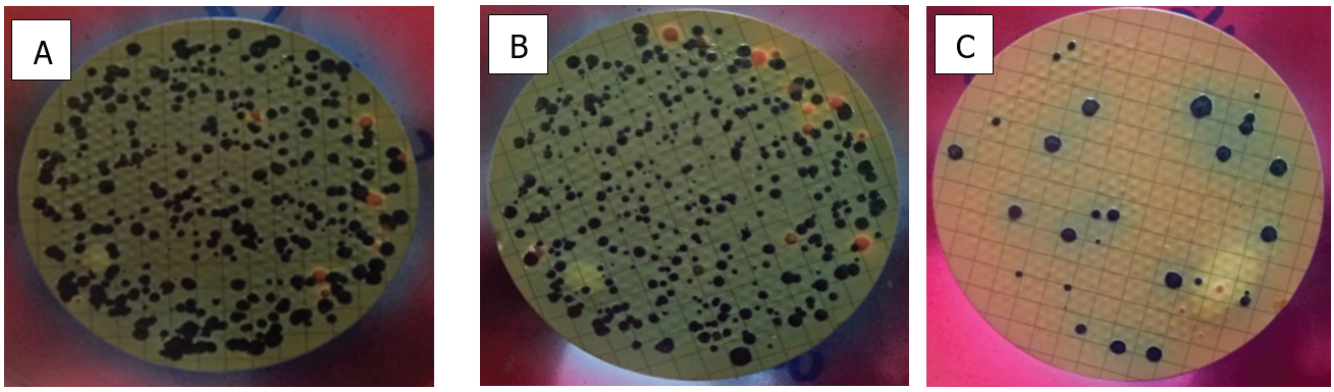


Figure 5: Faecal bacterial colonies (CFU/ml) in the tilapia wastewater effluents (A) after S1 (B) and S2 (C) treatments.

NO_3^-. Concordant results were obtained earlier by Zadinelo IV, et al. [31] and Bernardi F, et al. [20] who suggested that chitosan structure selectivity tends to PO_4^{3-} compared to the other wastewater compound (NH_4^+, NO_2^- and NO_3^-), in disagreement with the views of Wu FC, et al. [35].

Concentrations of the trace elements (Be, B, Na, Mg, P, K, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Cd, Sn, Sb, Hg and Pb) in the chitosan were low and of normal occurrence [41], posing no risk [42]. Relatively high levels of Ca in S1 and S2 (Figures 3 and 4) is due to calcium carbonate being one of its major components [22]. The source of the high level of Na is obviously because of its uptake from the seawater [43-48].

Regarding the effect of chitosan on the coliform bacteria, it is evident from the data presented in figure 5 that S1 treatment did not make any appreciable difference from the untreated effluent. However, S2 treatment markedly reduced the bacterial density. This antibacterial activity appears to be linked to the surface area and particle size of chitosan. Ardila N, et al., [49] have suggested that a decrease in chitosan particle size increases the antibacterial activity. S2 provided a larger specific surface area compared to S1. Chung YC, et al. [50] have provided details of the mechanism of antibacterial activity of chitosan. These authors have suggested a two-step process: separation between the bacteria cell wall and cell membrane and followed by the destruction of the cell wall by chitosan. The presence of inorganic substances, including Ca, Mg, Ba, Na influences this process but it depends on their relative proportions [32]. These reports support role of chitosan as a natural bactericide and emphasize its use in aquaculture effluent treatment [51-54].

Conclusion

It can be concluded that chitosan possesses the attributes required for adsorption of nutrients that cause pollution if released into the environment. These nutrients include NH_4^+, NO_2^-, NO_3^- and PO_4^{3-} in addition to faecal coliform bacteria that are encountered in the waste water from Nile tilapia culture systems. The fact that the product was extracted from discarded shrimp shells makes it economical to use. This approach is also consistent with the concept of circular economy that is currently being promoted to transform aquaculture into a more environment-friendly and low-carbon food production system. Further investigations can be carried out for enhancing the adsorbing efficiency of the local preparation by combining it with low-cost carbonaceous materials or biochar from weeds or discarded biomass with high surface area and porosity.

Acknowledgements

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