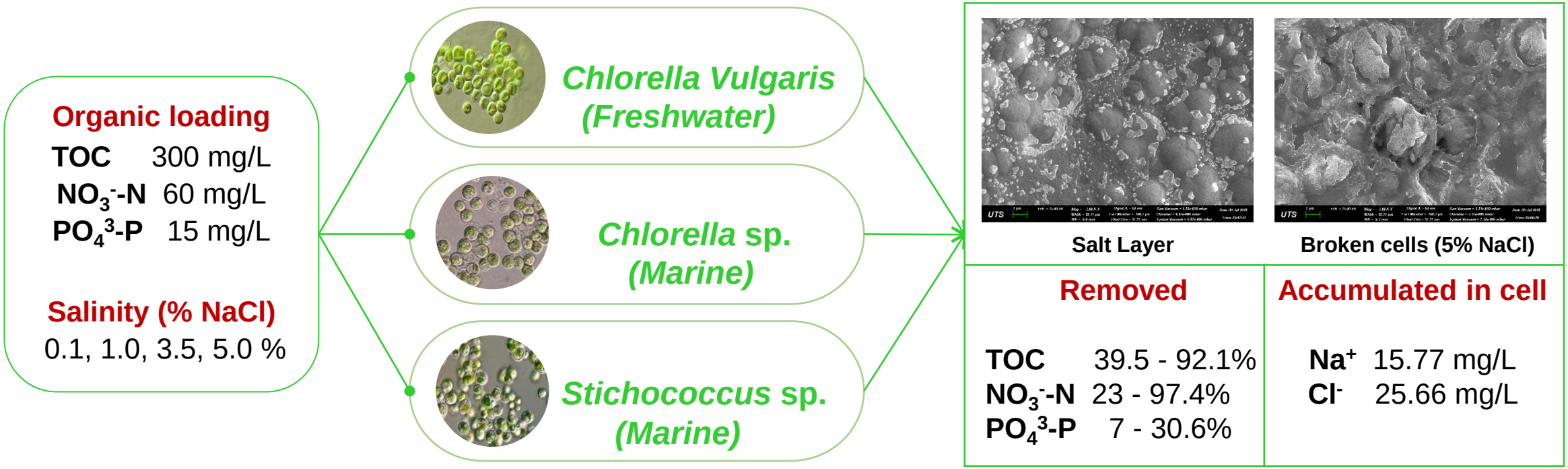
Graphical Abstract (for review)



Highlights

- Freshwater *C. vulgaris* is comparable to marine microalgae in pollutants removal.
- Unsaturated salt forms layer on microalgae cells' surface.
- Microalgae accumulate salt ions in cells proportionally to salinities in culture.
- Statistic well-confirms the negative effect of salinities on pollutant assimilation.
- Organic loading levels might alleviate salinities effect but not yet proved.

Identification of the pollutants' removal and mechanism by microalgae in saline wastewater

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Abstract

This study investigated the growth dynamics of microalgae in saline wastewater with supported biochemical performance and the pollutants removal efficiencies assimilated by microalgae strains in various salinities and the underlying effect of saline levels in which investigated by a developed method. The following percentages - 39.5-92.1%, 23-97.4%, and 7-30.6% - show that TOC, NO_3^--N, PO_4^{3-}-P were eliminated, respectively. The efficiencies in removing pollutants reduced significantly when salinities rose from 0.1 to 5%. The freshwater *Chlorella vulgaris* performed its best at 0.1 % of salinity with a focus on TOC removal. When the saline wastewater contained high N levels and salinity was 0.1 to 1%, the *Chlorella* sp. was prominent. The *C. vulgaris* could compete with marine microalgae with reference to removing pollutants in different saline levels. This study extensively explains the impacts of salinity with evidence of salt layer formation and salinity accumulation in microalgae cells.

Keywords: saline wastewater, microalgae, pollutants assimilation

1. Introduction

Saline wastewater is a recalcitrant source of pollutants, which consists of various contaminants and inorganic salts (Al-Jaloud et al., 1993). Saline wastewater is currently a major environmental problem occurring in both terrain and water reservoir contexts. Inorganic salts are known to severely compromise crop production, reduce water infiltration capacity of saline land and increase salinization of freshwater (NSW Government, 2003; QLD Government, 2013). Furthermore, such pollutants in saline wastewater are responsible for eutrophication, are toxic to ecological systems and threaten human health (Olivier Lefebvre and Moletta, 2006; Liang et al., 2017).

For these reasons the treatment of saline wastewater is a very critical issue. The presence of high concentrations of inorganic salts makes saline wastewater a refractory one. Given that saline wastewater treatment is a costly process (Wen et al., 2018), interest has grown in developing advanced technologies to manage these concerns. As such, constructed wetlands (Liang et al., 2017), zeolite (Wen et al., 2018), anaerobic processes (Xiao and Roberts, 2010) and halophilic microorganisms (Zhuang et al., 2010) are being increasingly reported as able to treat saline wastewater efficiently.

Nevertheless, less attention has been paid to saline wastewater treatment by microalgae although it was previously recognized as a low cost and 'green' or eco-friendly process. In environmental applications, microalgae have been implemented in the remediation of pollutants that are known to contain numerous contaminants. Nutrients, PPCPs, heavy metals and organic pollutants can be removed by microalgae extensively (Escapa et al., 2015; Fan et al., 2018; Qiu et al., 2017). Currently, researchers are investigating using microalgae to treat pollutants under saline conditions because salinity can alter algae's biochemical identity, change biomass yield, pigment formation, and the efficiency of contaminant removal (Babatsouli et al., 2015; Church et al., 2017; Zhou et al., 2017). Unlike other processes, microalgae offer encouraging outcomes thanks to the characteristics of biomass and pigments.

There are still shortcomings in the research and some of these are highlighted below:

- Only a limited number of microalgae species have been employed in saline wastewater treatment. Church et al. (2017) and Shen et al. (2015) utilized *Chlorella vulgaris* while Kim et al. (2016) based their study on *Acutodesmus obliquus*. Given this situation, it was not possible to generate an accurate and comprehensive comparison of microalgae's ability to treat saline wastewater.

- ❖ Efficiency in removing pollutants by microalgae is computed only by the discrepancies that appear in their influent and effluent concentrations. It ignores other processes such as precipitation, biosorption, and hydrolysis which can occur in a reactor.
- ❖ The impact of saline levels on pollutants' assimilation by microalgae is unclear and lacks solid evidence (Chen et al., 2017; Church et al., 2017).

Therefore, to explain these issues more clearly, the objectives of this research paper are to: (1) investigate the growth dynamics with supported biochemical performance; (2) examine pollutants' assimilation with developed methods; and (3) study the effect of saline levels on pollutants' assimilation by various microalgae strains in different salinities.

2. Materials and methods

2.1 Materials

2.1.1 Microalgae strains

In this research, three types of microalgae, specifically *Chlorella vulgaris* (freshwater microalgae), *Chlorella* sp. and *Stichococcus* sp. (marine microalgae), were purchased from National Algae Supply Service (Tasmania, Australia). Those microalgae strains were cultivated in 50 ml mediums and transferred to new cultures every 4 weeks for the purpose of creating stock solutions. The freshwater *C. vulgaris* was cultured in the MLA medium while the marine microalgae *Chlorella* sp. and *Stichococcus* sp. were fed in the f/2 medium. AusAqua Company (Australia) supplied the mentioned mediums. These stock cultures were operated in the following conditions: temperature was 20±1 ^{o}C; and continuous illumination intensity of 4.35 ± 0.03 klux. The illumination was provided by a LED light bulb (11W, 220-240v) (Philip, Australia). The illumination level was measured by a light meter, model QM1584 (Digitech, Australia).

2.1.2 Artificial wastewater

Artificial wastewater was experimented in this study and it was prepared by distilled water with spiked chemicals. The values of total organic carbon (TOC), NO_3^--N and PO_4^{3-}-P were adjusted to 300, 60 and 15 mg/L in the artificial wastewater, respectively. The chemicals $C_6H_{12}O_6$, KH_2PO_4, NH_4Cl were used to create TOC, NO_3^--N and PO_4^{3-}-P in artificial wastewater. The trace elements were purchased from AusAqua Company (Australia) and it was applied in the artificial wastewater with an advised dose of 1ml per 1000L medium.

2.1.3 Chemicals

The chemicals used here, $C_6H_{12}O_6$, KH_2PO_4, NH_4Cl, NaCl and Aceton, were purchased from Merck (Australia) and they were of analytical grade quality.

2.2 Experimental design

The stock microalgae were cultured in media until they were stabilized given that their concentration varied from 50 to 100 mg/L. Subsequently, 25 ml stock microalgae were transferred to experimental bottles of 1 L, which were capped to prevent air penetration. These bottles had been sterilized and filled with artificial wastewater. The magnetic stirrers were used to gently mix these cultures at a steady rate of 50 rpm. The applied temperature and light intensity were similar to those of the stock cultures. The hydraulic retention time (HRT) was 10 d with sampling being done every 2 d. The artificial wastewater was spiked with 4 levels of salinity, these being 0.1, 1, 3.5 and 5% NaCl.

2.3 Analytical methods

2.3.1 Biomass yield, optical density and growth rate

- Biomass determination

For biomass yield determination, a 150 mL sample was filtered through a pre-weighed 1.2 µm glass fiber filter paper GF/C (Whatman, Australia) (m_1). The sample was then dried at 105 °C in 24 h until a constant weight was achieved and completely dehydrated. The sample was re-weighted (m_2). The biomass yield was calculated according to the formula below:

Biomass yield = $\frac{m_2 - m_1}{v}$ (mg/L) (Eq. 1)

where

m_2: sample weight after drying (mg)

m_1: weight of filter paper (mg)

v: volume of sample (L)

❖ Optical density

Optical density (OD) at 680 nm was used to quantify cell density by a spectrophotometer (DR1900, Hach). A correlation of OD_{680} and dry biomass weight in synthetic wastewater was pre-determined as written in Eq. 2-4.

C. vulgaris: y = 0.0016x + 0.0075 (R^2 = 0.9526) (Eq. 2)

Chlorella sp.: y = 0.0021x + 0.0222 (R^2 = 0.9529) (Eq. 3)

Stichococcus sp.: y = 0.002x - 0.0049 (R^2 = 0.9809) (Eq. 4)

where

x: biomass yield (mg/L)

y: optical density

During the experiments, microalgae samples were collected as a scheduled time, analysed for OD values and converted to biomass yields via Eq. 2-4.

❖ The specific growth rate(s) (μ) (SGR(s)) is calculated using the following equation:

$$\mu = \frac{(\ln X - \ln X_0)}{(t - t_0)} \text{ (/d) (Eq. 5)}$$

where

X: the dry biomass weight at time t (mg/L)

X_0: the initial biomass weight at time t_0 (mg/L)

2.3.2 Pollutants' assimilation rates

The pollutants' assimilation rates were computed following Eq. 6 as written below:

$$R_{C, N, P} = X \text{ (mg/L)} \times \%m_{C, N, P}/d \text{ (Eq. 6)}$$

Where

$R_{C, N, P}$: assimilation rate of C, N, P at time t (mg/d)

X: biomass weigh at time t (mg/L)

$\%m_{C, N, P}$: portion weight of element C, N, P measured at time t, described in sub-section 2.3.5.

d: desired HRT to estimate assimilation rate (10 d in this case)

2.3.3 TOC, NO_3^--N, PO_4^{3-}-P concentrations

The TOC concentration was analysed by Multi N/C 3100 (Analytikjena, Germany). The NO_3^--N, PO_4^{3-}-P concentrations were analysed by test kits produced by Merck (Australia), coded 114942 and 100798, respectively. The Photometer Nova 60 (Merck, Australia) was used for NO_3^--N, PO_4^{3-}-P analysis accordingly. All the samples were filtered by RC caps filter 0.2 microns (Merck, Australia) beforehand.

2.3.4 Chlorophyll *a* content

The chlorophyll *a* analysis followed the procedure as recently used by Zhou et al. (2017). A 10 ml sample was centrifuged at 10,000 rpm in 10 min. The pellets were re-suspended in 10 mL of 90% acetone solution at 4 ^{o}C in 24 h in darkness, and then centrifuged at 4 ^{o}C, 4000 rpm in 15 min. The received supernatant was measured at four wavelengths: 750 nm, 664 nm, 647 nm and 630 nm with a spectrophotometer. The 90% acetone solution was used as the blank. The level of chlorophyll *a* was calculated as shown below:

Chlorophyll *a* (mg/L) = $11.64*(OD_{663}-OD_{750}) - 2.16*(OD_{647}-OD_{750}) + 0.1*(OD_{630}-OD_{750})$ (Eq. 7)

where

OD_{λ}: optical density at wavelength λ (nm).

2.3.5 SEM and EDS

The surface and elemental analyses of microalgae cells were done using Scanning Electron Microscopy (SEM) and Energy Dispersive X-Ray Spectroscopy (EDS), respectively (Zeiss Supra 55VP, Carl Zeiss AG). Samples were filtered through glass fiber filter paper GF/C (Whatman, Australia), heated for 24 h at 105 ^{o}C for dehydration, and then coated by Au/Pd prior to SEM. The SEM images were operated at an accelerating voltage of 10 kV, and multiple image magnifications at various areas were achieved for each sample. The SEM analyses were employed to investigate the effect of salinities on pollutant's assimilation of microalgae. The EDS was for quantifying the pollutants' assimilation rates and salts accumulation.

2.3.6 Statistical analyses

The analyses of variance (ANOVA) was applied for the statistical purposes in this study. In details, the repeated measures ANOVA was employed to examine the effect of salinities on

biomass yield, TOC, NO_3^--N and PO_4^--P and chlorophyll *a* according to the cultured time. For pollutants' assimilation, the factorial ANOVA served to investigate the impact of salinities on C, N, P, Na and Cl assimilation efficiencies. All the data were presented as mean value ± standard deviation (Mean ± SD) with duplicated samples.

3. Results and discussion

3.1 Biomass yield and growth rate

As can be seen from Fig. 1, the biomass yield reduced significantly when salinity increased. A salinity level of 5% was observed with the lowest biomass yields in all microalgae species that were below 200 mg/L after 10 d. Similar results were achieved with salinity of 3.5%; however, the biomass yield rose steadily after day 8 indicating that the microalgae could adapt. Referring to salinity of 0.1 and 1%, the maximum biomass yields were recorded at a range from 300 to 400 mg/L, which were twice as high for the results concerning 3.5 and 5% salinity. Notably, a sudden rise was noted on day 4 when the biomass yield reached a maximum of 462 mg/L as in the case of *C. vulgaris*.

Looking at the performance of each strain, the salinity of 0.1% was well-suited for *C. vulgaris* because this strain preferred the freshwater environment ($F_{(3,15)}$= 3.48, $p<0.05$). On the other hand, the marine microalgae *Chlorella* sp. ($F_{(3,15)}$= 5.73, $p<0.05$) and *Stichococcus* sp. ($F_{(3,15)}$= 3.65, $p<0.05$) grew substantially in saline conditions from 0.1 to 1%. The biomass yield of these strains was a remarkable outcome with reference to salinity at 3.5% after day 10; however, it would be impractical for onsite application because the long HRT needed more cultured volume and this made it a costly process.

Compared to other studies, the achieved biomass yield in this experiment was competitive. As such, Zhou et al. (2017) explored the biomass yield of *Spirulina platensis* cultured for an array of salinity that ranged from 0.93 to 3.2%. Consequently, the maximum biomass yield

was approximately 800 mg/L after 12 d, which corresponded to a salinity level of 2.24%. Although the applied nutrient concentrations as documented by Zhou et al. (2017) were higher than this study (COD=900 mg/L, TN=130 mg/L, TP=15 mg/L), the resulting biomass yield was comparable. This indicated that *S. platensis* might have similar biomass productivity, though Zhou et al. (2017) experimented with different light:dark cycle of 14:10 h. In another study, Kim et al. (2016) witnessed a biomass yield of *A. obliquus* as being 6 g/L at 4 d HRT with salinity of 5.2%. This value was significantly higher than reported in our study; nevertheless, it could be reasonably explained by the extremely high nutrient concentration of piggery wastewater implemented by Kim et al. (2016). Thus, the effect of salinity could be alleviated by utilizing high-loading nutrient concentration (TOC = 3935 mg/L, TN = 981 mg/L, TP = 81 mg/L). To ensure its accuracy and validation, this observation requires more in-depth research. Church et al. (2017) also remarked that higher salinity decreased biomass yield accordingly and it confirmed the findings of this present study. Notably, none of the above mentioned studies explained what caused biomass yield reduction using the evidence provided and it was fixed in the work given in the latter sections.

[Insert Fig. 1]

With reference to SGR, a similar trend was reported wherein an increase in salinity would reduce SGR of microalgae (Pandit et al., 2017; Shen et al., 2015).

Herein, the correlation of SGRs and saline levels fitted well with high reliability (R^2>0.81). Furthermore, the achieved SGRs were in the 0.1 to 0.6/d range. For *C. vulgaris*, Church et al. (2017) discovered its SGR was from 0.06 to 0.27/d which was lower than that reported in this study. Pandit et al. (2017) also explored the SGRs of *C. vulgaris* and *A. obliquus* were at their highest of 0.127/d when salinity ranged from 0 to 2.3%. Based on this data, this suggests that

C. vulgaris is a better option for removing saline wastewater, and especially when the salinity ranged from 0.1 to 1%. Alternatively, this study illustrated another higher salinity application using *Chlorella* sp. and *Stichococcus* sp.

3.2 Performance of the biochemical system

The photosynthesis process of microalgae includes photosystem (PSI) and photosystem II (PSII) (Kebede, 1997). For PS I, the photosynthetic activity, taking into account the light-harvesting efficiency, is made possible by chlorophyll *a* pigment. The performance of chlorophyll *a* is important for evaluating the adaptation of microalgae in environmental stress conditions, including salinity.

The production of chlorophyll *a* was initiated actively on day 2 of the experiment to three microalgae; however, levels of chlorophyll *a* produced by each microalga were different (Fig. 2). For *C. vulgaris*, its maximum chlorophyll *a* concentration of 13.6 mg/L was reported at a salinity of 0.1% indicating it preferred this saline level ($F_{(3,15)}$= 7.66, $p<0.05$). Regarding *Chlorella* sp., the gradual increase of chlorophyll *a* was identified at salinity levels of 0.1 and 1% ($F_{(3,15)}$= 6.57, $p<0.05$). Likewise, *Stichococcus* sp. produced a significant amount of chlorophyll *a* at salinity 0.1 and 1%, provided that a higher chlorophyll *a* concentration was observed compared to the other microalgae ($F_{(3,15)}$= 8.44, $p<0.05$). After day 8, chlorophyll *a* concentration started to decline which coincided with the reduction in biomass yield in the reactor. The chlorophyll *a* concentration of microalgae at salinity levels of 3.5 and 5% was very low and this explained the low nutrient consumption at those corresponding salinities.

[Insert Fig. 2]

With reference to chlorophyll *a* in microalgae, it was previously highlighted that the chlorophyll *a* and *c* concentrations in brackish algae were higher than the marine one of 25%

and 60%, respectively. Based on this evidence, marine algae were more adaptable to salinity stress because their biochemical systems fluctuated less (Gylle et al., 2009).

According to Gu et al. (2012), the rising level of salinity diminished *Nannochloropsis oculata*'s growth rate and pigment contents (e.g., chlorophyll *a*, and carotenoid), especially from 45 to 55 g/L of salinity concentration. More specifically, the chlorophyll *a* concentration decreased to 2.03 mg/g. In this study, the results confirmed that salinity exerted a great impact on biomass yield and pigment concentration. In addition, the combined effect of salinity and other environmental stresses (e.g., temperature, acid rain) could worsen the effect. For example, low salinity and acid rain inhibited the photosynthesis process, as illustrated through the concentration of chlorophyll *a* in *Ulva prolifera* (Li et al., 2017).

3.3 Pollutants' removal

3.3.1 TOC removal

Organic carbon is an important nutrient source of microalgae for cell build up and it presents in numerous saline wastewater types, such as food processing (Qin et al., 2017), agricultural run-off (Karimov et al., 2009) and mariculture (Feng et al., 2004). The removal of TOC by microalgae differs in terms of effectiveness as determined by various saline concentrations (Fig. 3).

The observed trend of TOC removal efficiency was consistent with biomass yield received beforehand. The highest removal efficiency happened for saline levels of 0.1 and 1%. Typically for *C. vulgaris*, 92% of TOC was removed in 10 d and this corresponded to 0.1% salinity ($F_{(3,15)}$= 14.15, $p<0.05$). At 1% salinity, it eliminated approximately 50% of TOC at similar HRT. For *Chlorella* sp., a similar amount of TOC was removed (60-80%) when salinity was 0.1 and 1% ($F_{(3,15)}$= 14.84, $p<0.05$), while *Stichococcus* sp. illustrated better TOC removal at 1% salinity ($F_{(3,15)}$= 9.35, $p<0.05$). With regard to 3.5 and 5% salinity, a steady

improvement in TOC removal efficiency was observed; however, it fell to less than 50% after 10 d. The TOC removal efficiency could increase after 10 d but this increased the HRT, reactor volume and associated costs.

From those results, it can be seen that salinity wielded a critical influence on TOC assimilation efficiency for all microalgae species. Each microalgae strain evidently adopted its own particular saline level. The freshwater microalgae removed TOC at high salinity (e.g., 3.5 and 5%) compared to marine microalgae. In another study, Kim et al. (2016) discovered that *Acutodesmus obliquus* could eliminate 70% of dissolved organic carbon in piggery wastewater at a salinity level of 5.2%. Elsewhere, *S. platensis* could remove 62 to 96% COD in mixed saline wastewater (Zhou et al., 2017). As mentioned earlier, the nutrient concentrations in influent wastewaters documented by Kim et al. (2016) and Zhou et al. (2017) were much higher than in this study. Thus, the comparison was relatively easy to make. Pollutants' removal efficiencies undertaken in our study provided better outcomes because the effects of different saline levels were considered.

[Insert Fig. 3]

3.3.2 NO_3^--N removal

Apart from C, N is a much needed element for microalgae growth in which NO_3^--N was used in this work as a nutrient source. Both *C. vulgaris* ($F_{(3,15)}$= 17.8, $p<0.05$) and *Chlorella* sp. ($F_{(3,15)}$= 19.5, $p<0.05$) well assimilated NO_3^--N at salinity of 0.1 and 1% in which 95% of NO_3^--N was removed accordingly (Fig. 4). Especially, *Chlorella* sp. consumed NO_3^--N rapidly during the first two days while *C. vulgaris* only did so gradually. *Stichococcus* sp. utilized more NO_3^--N at salinity of 0.1% compared to 1% ($F_{(3,15)}$= 12.43, $p<0.05$). Furthermore, it can be suggested that *Chlorella* sp. has the potential to treat NO_3^--N because its rapid consumption rate reduces the reactor/pond volume substantially. Referring to von

Alvensleben et al. (2013), *Picochlorum atomus* was stated as assimilating NO_3^--N more than 85% (C_o=60-70 mg/L) in a saline environment ranging from 0.2 to 3.6%. Notably, the used microalgae strain was halo-tolerant and the optimal saline level for microalgae was indicated as being 1.1%. Consequently, it was clear that marine microalgae species, when associated with salinity 3%, performed at their best at a salinity level of around 1%.

[Insert Fig. 4]

3.3.3 PO_4^{3-}-P removal

In this study, the removal of PO_4^{3-}-P by these microalgae was moderately successful in that the best removal efficiency was 30% (Fig. 5). With reference to *C. vulgaris* ($F_{(3,15)}$= 18.86, $p<0.05$) and *Chlorella* sp. ($F_{(3,15)}$= 13.12, $p<0.05$), the effect of salinity on PO_4^{3-}-P removal efficiency was the same as TOC and NO_3^--N. Specifically, 30% of PO_4^{3-}-P was consumed by salinity of 0.1% which was higher than other salinity levels. However, the consumption of PO_4^{3-}-P by *Stichococcus* sp. was different. No clear discrepancy was observed by this microalga with reference to levels of salinity implemented ($F_{(3,15)}$= 3.25<F_{cri}=3.28, $p>0.05$). It can be concluded that that PO_4^{3-}-P consumption by *Stichococcus* sp. was not critically influenced by a wide range of salinity levels.

[Insert Fig. 5]

To date the results regarding PO_4^{3-}-P assimilation by microalgae have been controversial and non-conclusive. For example, dissolved inorganic phosphorus was found to be removed only in very small amounts at the initial concentration of 15 mg/L after 5 d (Shriwastav et al., 2017). Later on, the bacteria and microalgae consortium could increase P removal efficiency to 100% with an initial concentration of 6.53 mg/L in 3 d (Shriwastav et al., 2018). Thus, the bacterial consortium played an important part in P consumption. Compared to Shriwastav et al. (2017), Al Ketife et al. (2016) remarked that P removal performance was notably better

given that microalgae could remove 90% of initial concentrations of 4 to 6 mg P/L within 10 d. This was due to the higher biomass concentration of 200-500 mg/L whereas Shriwastav et al. (2017) could only generate a biomass yield less than 120 mg/L. Furthermore, the P yield efficiency as reported by Al Ketife et al. (2016) was 24 mg/g biomass which indicated the microalgae's more dynamic assimilation. Interestingly enough, both studies used a model with the same microalgae, that is *C. vulgaris* (Al Ketife et al., 2016; Shriwastav et al., 2017). Other authors agreed with Al Ketife et al. (2016) in that P was removed substantially regardless of the microalgae species involved (Shen et al., 2015; von Alvensleben et al., 2013; Zhou et al., 2017).

This study agreed with Shriwastav et al. (2017) in that P removal by microalgae was limited. As mentioned previously, calculating the efficiency in removing P through the difference between influent and effluent concentration could entail errors being hidden (Vo et al., 2018). Hence, the EDS technique was employed in this study to explain the issue previously raised in sub-section 3.1.5. Additionally, the Redfield constant also noted the C:N:P ratio for microalgae consumption was 106:16:1. The, moderate efficiency in removing P in our study complied with the Redfield ratio accordingly. A comparison of pollutants' removal by various microalgae and salinities is illustrated in Table 1.

[Insert Table 1]

3.3.4 Pollutants' assimilation rates

The pollutants' assimilation rates as supported by EDS are illustrated in Table 2. Generally, these assimilation rates decreased when the saline levels increased ($F_{(3,24)} = 6.15$, $p<0.05$).

The C and N assimilations were slightly affected by salinity, especially at 5%, in which the assimilation rate reduced by 35 to 50% compared to assimilation rates when salinity was 0.1%. For *C. vulgaris* and *Chlorella* sp., the C and N assimilation rates were the highest at the

salinity range from 0.1 to 1%. With reference to *Stichococcus* sp., it achieved the most significant assimilation rate when salinity ranged from 1 to 3.5%.

Regarding P uptake, *C. vulgaris* and *Chlorella* sp. suffered severely from salinity given that the assimilation rates diminished from 50 to 77%. Nevertheless, it was observed that *Stichococcus* sp. wielded only a slight influence, specifically, the assimilation rate reduced by 35%. This confirmed the P removal efficiency described in sub-section 3.3.3. Salinity had a much less significant impact on P uptake when *Stichococcus* sp. was involved.

According to Kim et al. (2016), the N and P assimilation rates of *A. obliquus* were 175 and 1.5 mg/g biomass.d respectively. As noted earlier the applied N and P concentrations in the influent were higher, these being 981 and 81 mg/L, respectively. Also, the N and P consumption rates as summarized by Shriwastav et al. (2017) were 600 and 80 mg/g biomass.d. Elsewhere, Al Ketife et al. (2016) modelled pollutants' assimilation with total C, N, P yield coefficiencies of 500, 200 and 24 mg/g biomass. It should be noted that that this study investigated the assimilation rates of pollutants based on the EDS technique. The experimental platform and calculated unit were, consequently, different. Doing this eliminated the potential errors due to the involvement of other processes such as biosorption and precipitation. The influence of inlet organic loading levels on pollutants' assimilation rates needed to be examined extensively.

[Insert Table 2]

3.4 Identifying the effect of salinities on pollutants' assimilation

The microalgae's efficiencies in assimilating pollutants were clearly evident as reported beforehand. Most authors have agreed that salinity hindered the pollutants' assimilation by microalgae (Borecka et al., 2016; Zhang et al., 2016). Previously, the numerous hypotheses

and findings on this issue are not conclusive (Pandit et al., 2017; Shen et al., 2015; von Alvensleben et al., 2013; Zhou et al., 2017). Through the literature, the effects of salinity on pollutants' removal efficiency were highlighted with an emphasis on two major topics: (i) the competition of salt ions and pollutants in the bulk liquid; and (ii) the internal accumulation of salt ions in microalgae cells.

With reference to the first assumption, via the SEM technique, an unsaturated salt layer was found as attached on microalgae cells' surface at salinity levels of 3.5 and 5% (Fig. S2). These layers might be responsible for the reduction of pollutants' accumulation efficiency. When salinity was 5%, the broken cells of *C. vulgaris* were found (Fig. S2d). On the other hand, the cells of *Chlorella* sp. and *Stichococcus* sp. stayed normal and this underlines their good adaptation in salinity of 5% (Fig. S2h & S2n).

For the second mechanism, Na^+ has been known to be involved in the co-transport of P into microalgae cells; however, this was limited to a saline level below 100 mg/L (Mohleji and Verhoff, 1980). Church et al. (2017) agreed that Na^+ would impair the assimilation rate, especially at 4.5% salinity because Na^+ would stream into microalgae cells and inhibited the photosynthesis reaction accordingly. In this study, the solid evidence is provided in Table 3. The highest Na^+ and Cl^- accumulations were 15.77 and 25.66 mg/L, respectively, with reference to *Stichococcus* sp.. As such, it was found that the concentration of Na^+ and Cl^- ions increased steadily when the salinity also increased ($F_{(3,15)}$= 28.29, $p<0.05$). *C. vulgaris* is a and its good accumulation of those ions makes it able to compete with *Chlorella* sp. and *Stichococcus* sp ($F_{(5,15)}$= 0.59, $p>0.05$). This perhaps led to the damaged cells of *C. vulgaris* as observed.

[Insert Table 3]

To the best of our knowledge, this work is the first to unlock the salt layer and salt accumulation in microalgae cells. It updates previous assumptions and observations and also establishes a background for extensive research on saline wastewater treatment by microalgae. Saline wastewater removal includes two major objectives which are: the removal of pollutants and desalination. Salinity is the main obstacle to pollutants' removal when biological processes are employed. To remove pollutants efficiently, the salts in wastewater need to be eliminated simultaneously. Although bioprocesses such as activated sludge and anaerobic processes have been commented on as having great capability in removing pollutants' in saline wastewater (Ahmadi et al., 2017; Gebauer and Eikebrokk, 2006; O. Lefebvre et al., 2005), desalination is still a major technical challenge.

4. Future perspectives and practical applications

The results obtained in this study have to some extent resolving the shortcomings of previous research. Our analysis is able to offer practical applications. Firstly, the appropriate microalgae species applied in saline wastewater treatment have been identified. *C. vulgaris* was implemented in salinity of 0.1% with the focus on TOC removal. If the saline wastewater contained high N concentration and salinity from 0.1 to 1%, *Chlorella* sp. was the ideal candidate. The fast N consumption rate by *Chlorella* sp. will help in reducing reactor volume and operational costs. When microalgae are employed for pigment, instead of removing the pollutants, *Stichococcus* sp. produces superior chlorophyll *a* content. This study confirmed that saline levels only had insignificant influence on P removal in the case of *Stichococcus* sp..

Furthermore, the pollutants' assimilation rates and SGR estimated in this study can serve as a manual for future saline wastewater treatment designs. The HRT, biomass retention time (BRT) and reactor/pond volume can be calculated accordingly. In continuous operation, the

BRT, which was calculated from the maximum specific growth rate ($1/\mu_{max}$ ~ BRT), was computed. The μ_{max} could be retrieved with a known influent salinity of wastewater. The BRT should be maintained in the reactor/pond by harvesting the excess microalgae biomass in order to sustain the maximum microalgae growth rate.

This study clearly describes the impact of salinity with evidence of salt layer formation and salinity accumulation in microalgae cells. This established a platform for in-depth research tailored at reducing salinity's influence and enhancing biomass yield and pollutants' assimilation efficiency. Another objective is to achieve higher organic loading because the literature and some prior studies' comparisons have indicated that the impact of salinity can be alleviated.

5. Conclusion

Saline wastewater treatment by microalgae is proved feasible. However, high salinity concentration resulted in low removal efficiency of TOC, NO_3^--N, PO_4^{3-}P. The freshwater *C. vulgaris* demonstrated its capability to compete in assimilating pollutants' compared to marine microalgae. The new approach to explore microalgae's assimilation of pollutants could help to document the underlying effects of salinity. Future research of investigating efficiency of assimilating pollutants in high saline conditions is necessary.

E-supplementary data for this work can be found in e-version of this paper online.

Acknowledgement

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[Figure captions]

Fig. 1. Biomass yield of (a) *C. vulgaris*, (b) *Chlorella* sp. and (c) *Stichococcus* sp. Value and error bars are the average and standard deviation of two samples

Fig. 2. Chlorophyll *a* performance of (a) *C. vulgaris*, (b) *Chlorella* sp. and (c) *Stichococcus* sp. Value and error bars are the average and standard deviation of two samples

Fig. 3. TOC removal percentage of (a) *C. vulgaris*, (b) *Chlorella* sp. and (c) *Stichococcus* sp. Value and error bars are the average and standard deviation of two samples

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Fig. 5. PO_4^{3-}-P removal percentage of (a) *C. vulgaris*, (b) *Chlorella* sp. and (c) *Stichococcus* sp. Value and error bars are the average and standard deviation of two samples

[List of tables]

Table 1. Comparison of pollutant removal efficiency.

No.	Microalgae	Salinity	COD/TOC Removal Efficiency (%), Initial Concentration (mg/L)	Nitrogen Removal (%), Initial Concentration (mg/L)	Phosphorus Removal (%), Initial Concentration (mg/L)	HRT	References
1	*C. vulgaris*	0.1 - 5%	39.5 - 92.1%, 300 mg/L	23 - 97.4%, 60 mg/L	7 - 30.6%, 15 mg/L	10 d	This study
	Chlorella sp.	0.1 - 5%	28.4 – 79.6%, 300 mg/L	20.9 - 94.2%, 60 mg/L	5.5 - 29.9%, 15 mg/L		
	Stichococcus sp.	0.1 - 5%	53.4 – 78.3%, 300 mg/L	20.9 - 86.4%, 60 mg/L	13.1 - 25.4%, 15 mg/L		
2	*Acutodesmus obliquus* KGE-17	5.2%	55% COD, 11000 mg/L*	40% TN, 981 mg/L*	70% TP, 81 mg/L	140 h	Kim et al. (2016)
3	*S. platensis*	0.93 - 3.2%	90.02% COD, 1200 mg/L	79.96% TN, 180 mg/L	93.35% TP, 20 mg/L	12 d	Zhou et al. (2017)
4	*Picochlorum atomus*	2 - 36 ppt	n.d	13 - 15 mg NO_3^--N/L.d,	1.3 - 2.4 mg P/L.d, 6	4 d	von Alvensleben et

				55 mg/L	mg/L		al. (2013)
5	*C. vulgaris*	0 - 4.5% NaCl	n.d	100% NH_4^+-N, 20-50 mg/L	100% TP, 2-6 mg/L	200 h	Church et al. (2017)
6	*C. vulgaris Cv* (strain: CCAP 211/11B, CS-42)	n.d	n.d	80-99%, 70 mg/L 60%, 207 mg/L	100% TP, 7-8 mg/L*	10-13 d	Al Ketife et al. (2016)
7	*C. vulgaris* and *Chlamydomonas reinhardtii*	n.d	n.d	50% NO_3^--N, 23.3 mg/L*	5% Inorganic P, 16.4 mg/L*	5 d	Shriwastav et al. (2017)

572 *Retrieved from graph

573 n.d: no data

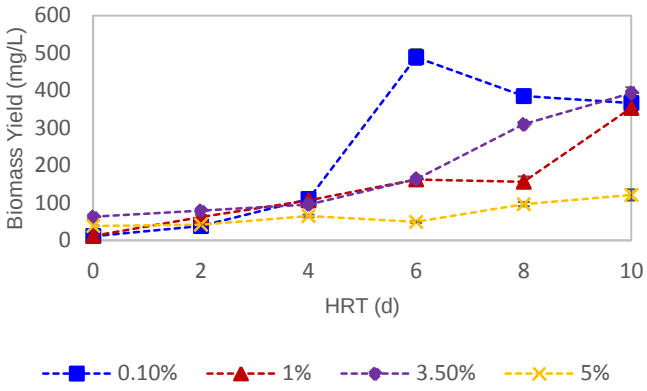
Table 2. Pollutants assimilation rates at day 10th (mg/d)

Element/ Salinity (%)	*C. vulgaris* 0.1	1	3.5	5	*Chlorella* sp. 0.1	1	3.5	5	*Stichococcus* sp. 0.1	1	3.5	5
C	15.55 ± 0.19	15.41 ± 0.14	14.72 ± 0.18	3.32 0.03	18.82 ± 0.13	19.8 ± 0.16	16.06 ± 0.21	2.00 ± 0.03	9.01 ± 0.8	9.51 ± 0.5	17.32 ± 0.23	6.00 ± 0.4
N	5.29 ± 0.06	5.35 ± 0.01	4.91 ± 0.04	0.77 ± 0.02	6.25 ± 0.08	5.41 ± 0.07	5.40 ± 0.04	0.71 ± 0.03	3.14 ± 0.07	2.98 ± 0.1	5.86 ± 0.08	1.79 ± 0.04
P	0.64 ± 0.05	0.58 ± 0.04	0.28 ± 0.02	0.05 ± 0.003	0.77 ± 0.03	0.39 ± 0.02	0.45 ± 0.04	0.05 ± 0.004	0.31 ± 0.01	0.21 ± 0.01	0.39 ± 0.02	0.13 ± 0.01

Table 3. Weight of salts accumulation in microalgae cells (mg/L)

Salt ion/ Salinity (%)	*C. vulgaris*				*Chlorella* sp.				*Stichococcus* sp.			
	0.1	1	3.5	5	0.1	1	3.5	5	0.1	1	3.5	5
Na	3.26 ± 0.27	4.77 ± 0.35	15.77 ± 0.63	7.90 ± 0.58	5.87 ± 0.34	5.19 ± 0.47	14.08 ± 1.13	5.79 ± 0.36	4.75 ± 0.51	3.39 ± 1.73	15.79 ± 1.06	8.94 ± 0.61
Cl	0.87 ± 0.05	3.71 ± 0.27	20.93 ± 0.86	15.57 ± 0.93	1.57 ± 0.09	3.42 ± 0.39	18.68 ± 0.72	8.75 ± 0.47	0.51 ± 0.06	1.73 ± 0.17	25.66 ± 1.26	14.77 ± 0.94

[List of figures]



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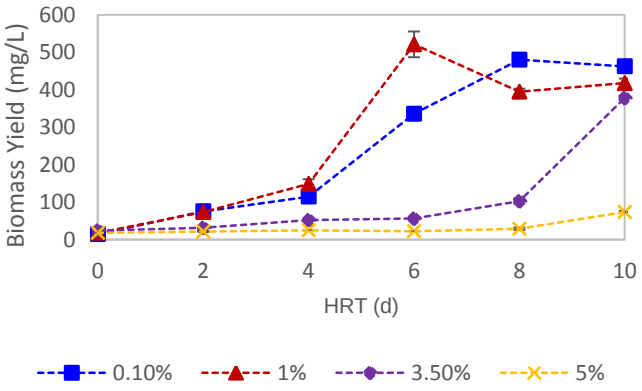

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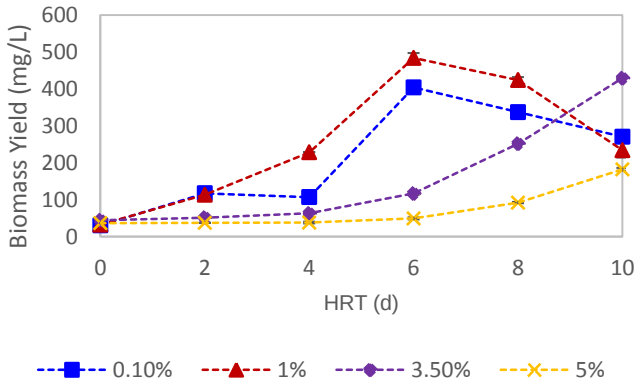

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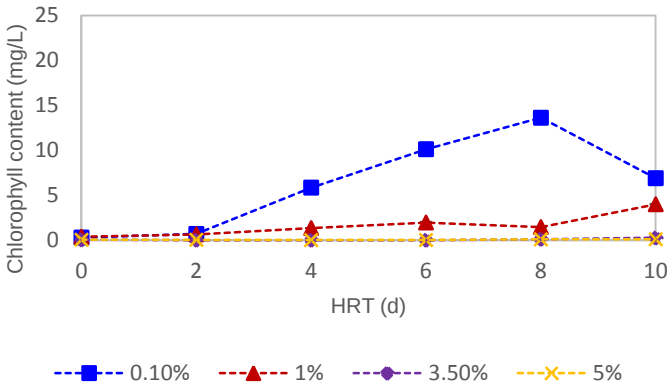

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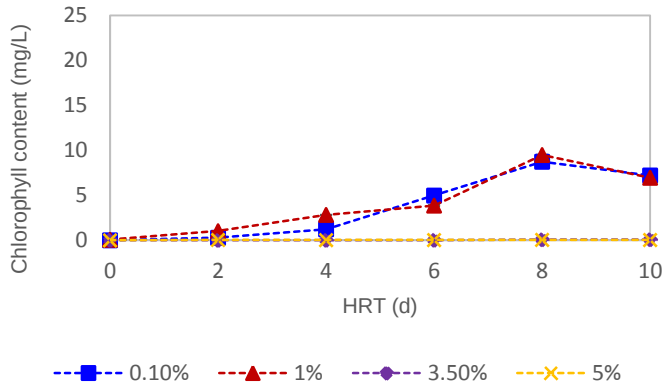

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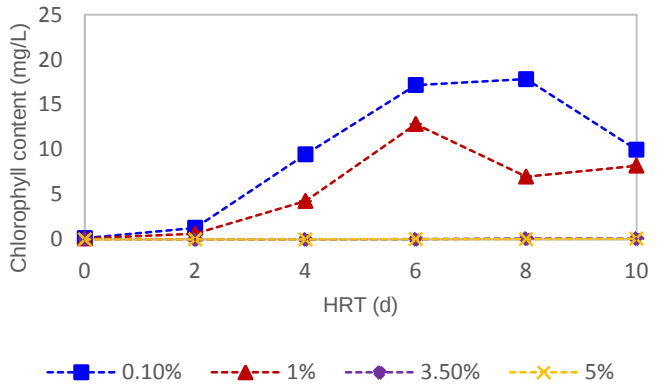

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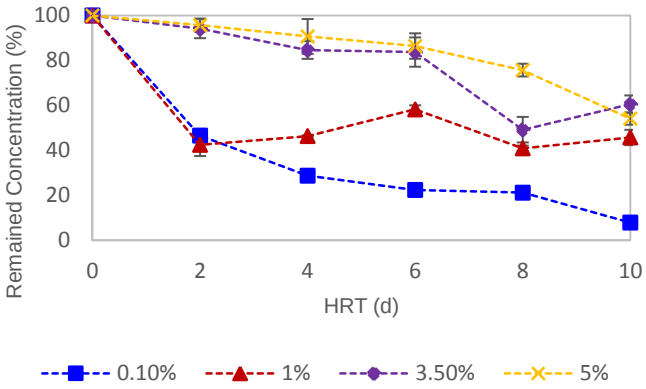
585



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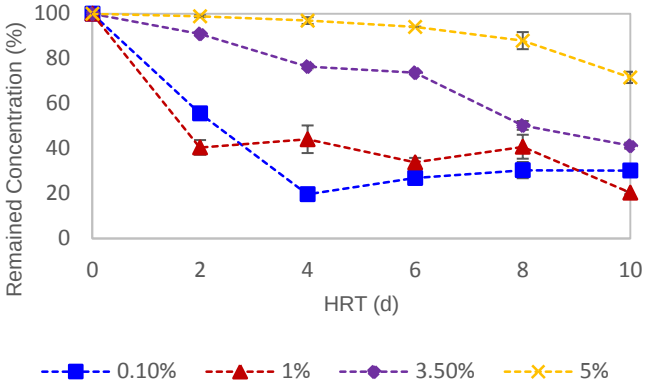

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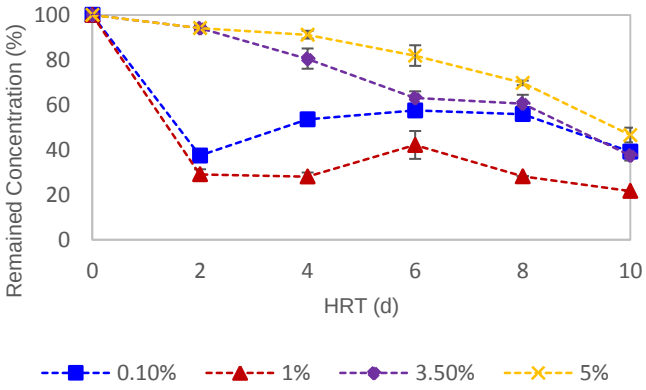

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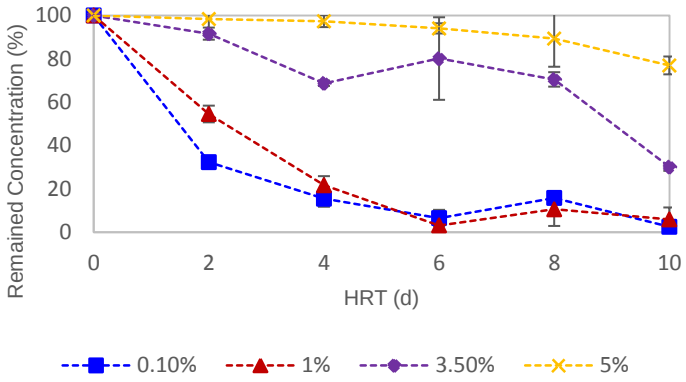
588



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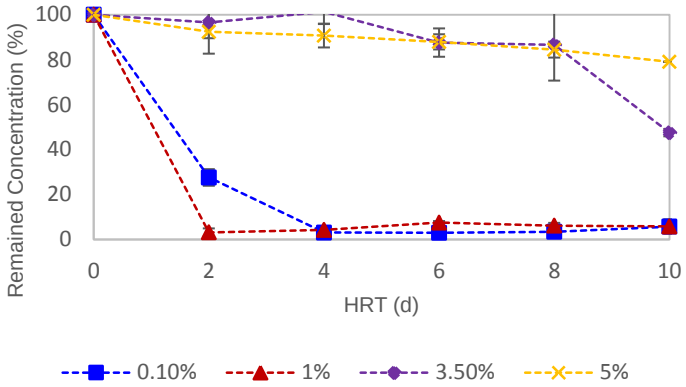

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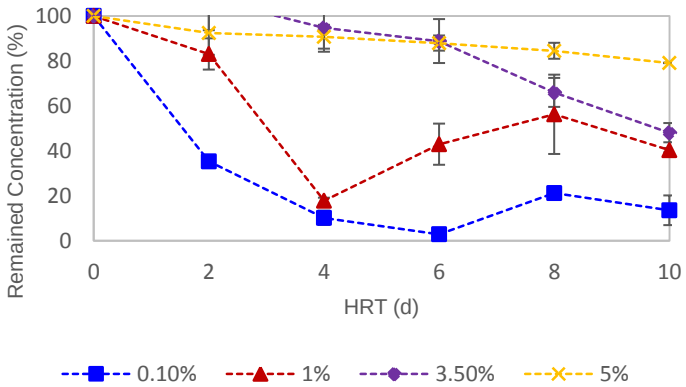

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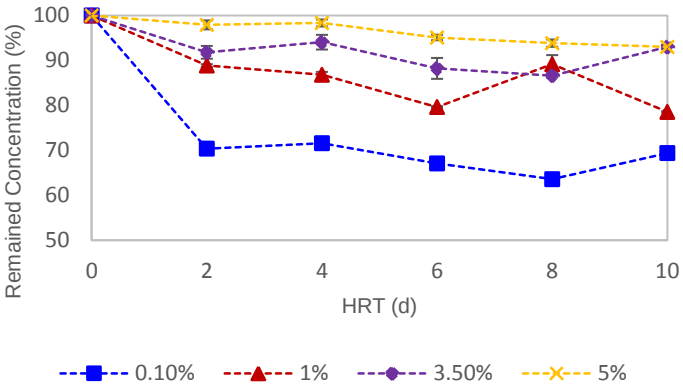
589 Fig. 4. NO_3^--N removal percentage of (a) *C. vulgaris*, (b) *Chlorella* sp. and (c) *Stichococcus*

590 sp. Value and error bars are the average and standard deviation of two samples

591



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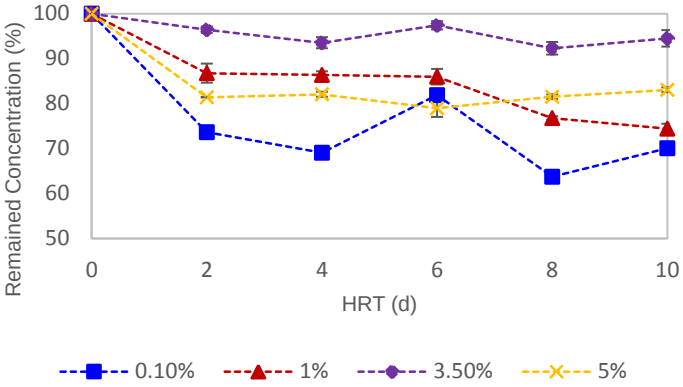

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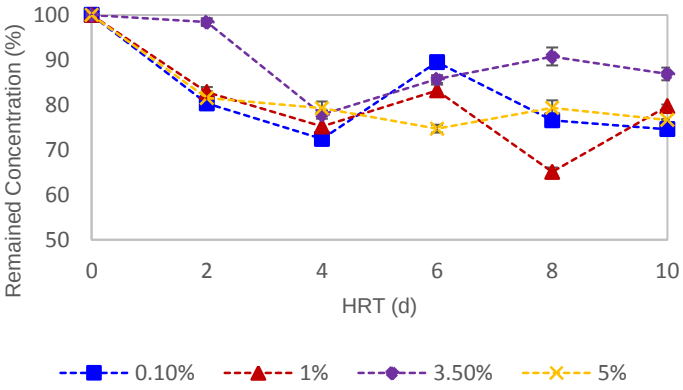

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592 Fig. 5. PO_4^{3-}-P removal percentage of (a) *C. vulgaris*, (b) *Chlorella* sp. and (c) *Stichococcus*

593 sp. Value and error bars are the average and standard deviation of two samples

Supplementary Interactive Plot Data (CSV)

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