Removal of total nitrogen from wastewater by a combination of Chlorella sp. and audible sound

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**ABSTRACT**

In developing countries, nitrogen in the traditional market wastewater is a critical environmental problem. In this study, the microalga Chlorella sp., which was isolated from wastewater, was used to remove the total nitrogen (TN) from conventional market wastewater in combination with audible sound (Vietnamese classical music). In addition, effects of sound exposure on removal efficiency at different initial cell densities were analyzed. Results revealed that music sound control demonstrates potential to improve the removal efficiency. TN removal efficiencies of 96\%, 69.5\%, and 4.3\% were observed for treatments with Chlorella sp./audible sound, Chlorella sp., and without Chlorella sp., respectively. The significance of probability value ($p$-value) ($<0.05$) on the paired sample $t$-test confirmed the critical role of audible sound and Chlorella sp. density on the TN removal in screening experiments. The predicted optimal conditions for TN removal were as follows: a Chlorella sp. density of 4\%, an audible sound of 52.5 dB, and a cultivation time of 4.6 days. Results based on statistical analysis revealed that the quadratic models for TN removal are significant at a low $p$-value ($<0.05$) and a high predicted coefficient of determination ($R^2 = 0.9452$) value. The obtained statistical results also indicated that most of the variables are significant for the abatement of TN from market wastewater using Chlorella sp.

**Key words:** central composite design, Chlorella sp, classical music, nutrient removal, traditional market wastewater

**HIGHLIGHTS**

- Chlorella sp. with audible sound employed to remove total nitrogen (TN) from traditional market wastewater.
- The central composite design was developed on three factors on TN removal.
- ANOVA showed a well fitted polynomial regression model with $R^2$ of 0.9908.
- The statistical results indicate audible sound has a significant effect on TN removal.
- The TN removal was reached 98.12\% at optimum condition.

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1. **INTRODUCTION**

Wastewater from traditional markets has become a serious environmental problem in developing countries (Yhdego 1992; Danial et al. 2016). Wastewater generated either from wet processes of meat, fish, poultry, fruit, vegetable, and food preparation or from public toilets, etc. has significantly contributed to effluents with specific characteristics, such as a high content of dissolved solids, complex organic compounds, and especially extremely high nutrient levels (Ha et al. 2016).

Excess nutrients lead to water eutrophication, resulting in algal blooms. These blooms can release toxins that are harmful to animals, plants, and humans (Heisler et al. 2008; Jais et al. 2017). In addition, high concentrations of chemical oxygen demand (COD) and biochemical oxygen demand (BOD) may lead to low-oxygen (hypoxic) waters that can kill fish and reduce essential fish habitats (Ha et al. 2017). Wastewater can be treated by numerous physicochemical, chemical, and biological techniques (Qin et al. 2012; Zulaikha et al. 2014). However, physicochemical-based techniques are primarily not cost-effective and not environmentally friendly and result in the production of a high amount of toxic sludge.

Owing to their ecologically friendly characteristics, cost-effectiveness, and sustainable reclamation strategy, biological methods based on microalgae have been widely used for pollutant removal (Pham & Ha 2020). In these methods, simple equipment is used, making them easier for operation and maintenance (Ha et al. 2016; Gao et al. 2019; Nguyen et al. 2019). Microalgae have been successful for the treatment of slaughterhouse wastewater (Jayangoudar et al. 1983), dairy wastewater (Woertz et al. 2009), municipal wastewater (Wang et al. 2010), textile wastewater (Devi et al. 2016), and even wastewater containing heavy metals (Mehta & Gaur 2005).

Recently, attention has been focused on audible sound, which can support the growth of organisms, and audible sound with appropriate frequencies can stimulate microbial growth. Christwardana & Hadiyanto (2017) have reported that audible sounds affect the growth and metabolism of microorganisms. Audible sound has been reported to decrease thermodynamic phase transition, which can enhance the fluidity of the cell wall and membrane in plant cells. This process helps cells to grow and divide more rapidly and easily (Lovelli et al. 2012; Christwardana & Hadiyanto 2017). In addition, sound waves help plant cells manage cell stress via an increase in the capacity of indole-3-acetic acid metabolism and inhibition of the abscisic acid metabolism during division (Lovelli et al. 2012). During the fermentation of rice vinegar in Ajinomoto factories, vinegar is listened subjected to classical music to increase the fermentation efficiency and afford a better quality of monosodium...
glutamate product (Ajinomoto-Vietnam 2021). These results have indicated that audible sound positively affects the cultivation of bacteria and can be applicable on a practical or an industrial scale. Although effects of sound on bacterial and microalgal growth have been investigated, only a few studies have reported the effect of audible sound on the removal efficiency of microalgae.

Response surface methodology (RSM) is a vital tool for designing experiments, simulating models, evaluating the response of variables, and determining optimal conditions for operation control via the combination of mathematics and statistical techniques (Lee & Chen 2015). Central composite design (CCD) is an RSM method that is widely applied for the optimization of conditions required for the removal treatment of pollutants (Ha 2017, 2018). This technique is applied to indicate the optimized conditions total nitrogen (TN) removal among various treatment factors.

In this study, for the first time, effects of audible sound using Vietnamese classical music on the TN removal efficiency of the microalgae Chlorella sp. from traditional market wastewater were investigated. In addition, different initial cell densities, audible sound levels, and cultivation durations were investigated. The optimal condition was determined by CCD. Furthermore, the kinetic order of the treatment process was examined.

2. MATERIALS AND METHODS

2.1. Chemicals and wastewater

All chemicals, i.e., sodium nitrate (NaNO₃), potassium hydrogen phosphate (K₂HPO₄), magnesium sulfate heptahydrate (MgSO₄·7H₂O), calcium chloride (CaCl₂·2H₂O), citric acid (CH₃COOH), ferric ammonium citrate ([Fe(C₆H₄O₇)]·2H₂O), ethylenediaminetetraacetic acid disodium salt dihydroxide (Na₂EDTA), sodium carbonate (Na₂CO₃), boric acid (H₃BO₃), manganese(II) chloride tetrahydrate (MnCl₂·4H₂O), zinc sulfate heptahydrate (ZnSO₄·7H₂O), copper sulfate pentahydrate (CuSO₄·5H₂O), sodium molybdate dihydrate (Na₂MoO₄·2H₂O), and cobalt(II) nitrate hexahydrate (Co(NO₃)₂·6H₂O) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and solutions were prepared using deionized water with a resistivity of no less than 18.2 MΩ·cm. Wastewater was collected from Hocmon market, Ho Chi Minh City, Vietnam, and filtered through a 0.45-μm membrane by vacuum filtration and sterilized before experiments.

2.2. Collection and isolation of microalgae

Wastewater samples were enriched in different flasks with BG11 medium containing 17.6 mM NaNO₃, 0.23 mM K₂HPO₄, 0.5 mM MgSO₄·7H₂O, 0.24 mM CaCl₂·2H₂O, 0.031 mM citric acid·H₂O, 0.021 ferric ammonium citrate, 0.0027 mM Na₂EDTA·2H₂O, 0.19 mM Na₂CO₃, and 1 mM BG-11 trace metal solution prior to isolation (Hong et al. 2016). The enriched samples were dominant with Chlorella sp. A single cell of Chlorella sp. was isolated using a micropipette and washed into glass tubes containing BG-11 medium. The culture was grown under laboratory conditions at 25 °C, a light intensity of 50 μmol photons/m²/s, and 12:12 h light:dark cycle. Then, the culture was enriched in 1-L glass beakers containing 800 mL BG-11 medium prior to the experiment. Algal cell density was quantified with a Neubauer-improved counting chamber under an Olympus light microscope.

2.3. Experimental design

Screening experiments with and without audible sound or algae for 10 days were conducted to investigate the effect of the two factors on the TN removal efficiency. For treatment, a glass reactor with a volume of 4 L of effluent per treatment was used (Figure 1). Concentrations of Chlorella sp. were mixed in wastewater to achieve three desired densities (viz. 2.5%, 5%, and 7.5% corresponding to 1.25 × 10⁵ cells/mL, 2.5 × 10⁵ cells/mL, and 7.75 × 10⁵ cells/mL, respectively). The airflow rate and light intensity were maintained constant at 3 L/min and 50 μmol photons/m²/s, respectively, and the speaker sound was adjusted to 60 dB (recorded using a 407730 digital sound level meter, Extech, China), playing the Song of the Black Horse (Ly Ngua O) presented by the Vietnam Traditional Orchestra. All screening trials were performed at a room temperature range of 20 °C to 25 °C (± 1.0 °C), and unadjusted pH from 6.5 to 7.5 (± 1.2) was utilized. Then, CCD was conducted to determine the conditions optimal for the TN removal via the algae process. Three independent variables were applied: algae density (X₁), audible sound (X₂), and cultivation time (X₃). The level of each code ranged from low (−1) to high (1), as shown in Table 1 based on our previous screening experiments.

Twenty experimental sets were designed, including eight sets from 2 k of a factorial point, six sets from 2 k of the axial point, and six sets from the central point (Table 2), where k is the number of independent variable factors (Ha 2017). Each set contained a speaker, four glass tube reactors, an air compressor, and light (Figure 1). The TN removal efficiency,
as calculated by Equation (1), was defined as the response of the dependent variable:

\[
\text{TN removal efficiency (\%) = } \frac{(\text{TN}_0 - \text{TN}_i)}{\text{TN}_0} \times 100
\]  

(1)

where \(\text{TN}_0\) is the concentration of TN at the initial time, and \(\text{TN}_i\) is the concentration of TN at any time \(t\) of cultivation.

The mathematics model was evaluated using Minitab version 18.1 software and expressed by a quadratic model equation as shown in Equation (2). Least-square regression was employed to calculate the coefficient. \(\beta_0, \beta_i, \beta_{ii}, \beta_{ij}\), and \(e\) represent the constant coefficient, linear coefficient, quadratic coefficient, interaction coefficient, and statistical error, respectively. The adequacy and significance of the model were justified by the analysis of variance (ANOVA):

\[
Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k} \sum_{j=2}^{k} \beta_{ij} X_i X_j + e
\]  

(2)

The three confirmed experiments were also conducted to verify the prediction under optimal conditions (algae density of 4\%, an audible sound of 52.5 dB, and a cultivation time of 4.6 days).

2.4. Analytical methodology

First, 20 mL of the treated samples was collected at the designated time period (7 a.m.), filtered, and analyzed for TN and TOC contents using a TOC Vcph/cpn instrument (SHIMADZU, Japan). Other parameters such as pH, conductivity, \(\text{NO}_3^-\text{N}\), \(\text{NO}_2^-\text{N}\), and \(\text{NH}_4^+\text{N}\) were evaluated according to the Standard methods for the examination of water and
Statistical analysis was performed using the Minitab 18.1 version (Minitab Inc, USA) and analyzed using the paired $t$-test. A $p$-value of less than 0.05 was thought to be significant.

### 3. RESULTS AND DISCUSSION

#### 3.1. Algae community in the wastewater

Characteristics of wastewater effluents were described as follows: initial pH of 6.4–7.5, total organic carbon (TOC) of 150–190 mg/L, TN of 66.9–75.7 mg/L, ammonium (NH$_4^+$–N) of 47.5–60.2 mg/L, and nitrate (NO$_3^-$–N) of 14.1–21.2 mg/L, with no detected nitrite (NO$_2^-$–N).

Enriched wastewater samples contained several types of microalgae, including cyanobacteria (*Planktothrix* sp., *Pseudanaabaena* sp.), Chlorophyta (*Chlorella*, *Ankistrodesmus* sp., *Scenedesmus* sp.), and Bacillariophyta (*Aulacoseira* sp., *Cyclotella* sp., *Nitzschia* sp.), where *Chlorella* sp. was the most abundant in the samples. The isolation and culture stock of *Chlorella* sp. revealed that the *Chlorella* cells are spherical, with a diameter of 5–8 μm and without flagella (Figure 2).

#### 3.2. Screening experiments

Results revealed that TN removal efficiencies of 96%, 69.5%, and 4.3% are achieved by *Chlorella* sp./audible sound, *Chlorella* sp., and control treatment, respectively (Figure 3). TN was rapidly removed by the *Chlorella* sp./audible sound and *Chlorella* sp. treatments, but barely eliminated under control conditions (neither *Chlorella* sp. nor audible sound). The TN removal followed the second-order model, with a removal rate of 0.035 L/mg·day for treatment by *Chlorella* sp./audible sound, and the third-order model, with a removal rate of 1.939 $\times$ 10$^{-2}$ L$^2$/mg$^2$·day by only *Chlorella* sp. treatment (Table 3). The results suggested that the TN removal efficiency is significantly improved by the combination of audible sound on *Chlorella* sp.; this significance was also confirmed by a paired $t$-test between three groups of *Chlorella* sp., *Chlorella* sp./audible sound, and control, respectively, with a $p$-value of $\sim$0.000 (Table 4). The results suggested that the increase in the TN removal efficiency by

### Table 2 | Matrix of the CCD experiment and the corresponding experimental data

| Exp. run no. | Algae density (%) | Audible sound (dB) | Cultivation time (days) | TN removal (%) |
|--------------|-------------------|-------------------|-------------------------|--------------|
|              |                   |                   |                         | Actual       | Predicted  |
| 1            | 7.5               | 30.0              | 7.0                     | 66.24        | 64.75      |
| 2            | 5.0               | 9.5               | 5.0                     | 68.12        | 69.33      |
| 3            | 5.0               | 60.0              | 5.0                     | 96.16        | 95.28      |
| 4            | 9.2               | 60.0              | 5.0                     | 74.67        | 76.23      |
| 5            | 5.0               | 60.0              | 5.0                     | 95.71        | 95.28      |
| 6            | 5.0               | 60.0              | 5.0                     | 97.75        | 95.28      |
| 7            | 5.0               | 60.0              | 5.0                     | 93.75        | 95.28      |
| 8            | 7.5               | 30.0              | 3.0                     | 77.43        | 76.96      |
| 9            | 2.5               | 30.0              | 7.0                     | 76.25        | 74.69      |
| 10           | 2.5               | 90.0              | 7.0                     | 67.63        | 66.15      |
| 11           | 2.5               | 30.0              | 3.0                     | 84.42        | 84.32      |
| 12           | 2.5               | 90.0              | 3.0                     | 68.85        | 68.39      |
| 13           | 5.0               | 110.5             | 5.0                     | 49.52        | 51.07      |
| 14           | 5.0               | 60.0              | 8.4                     | 62.18        | 65.03      |
| 15           | 7.5               | 90.0              | 3.0                     | 64.18        | 63.79      |
| 16           | 5.0               | 60.0              | 1.6                     | 77.28        | 77.18      |
| 17           | 7.5               | 90.0              | 7.0                     | 60.82        | 58.97      |
| 18           | 5.0               | 60.0              | 5.0                     | 95.64        | 95.28      |
| 19           | 5.0               | 60.0              | 5.0                     | 95.18        | 95.28      |
| 20           | 0.8               | 60.0              | 5.0                     | 87.26        | 88.46      |
using audible sound is mainly related to the enhanced cell growth of *Chlorella* sp. by sound waves via the modulation of the endomembranous transport of algae (Ying et al. 2009). Previous studies (Ganeshkumar et al. 2018; Gao et al. 2019) also have reported that TN in wastewater was successfully eliminated via assimilation uptake into cells of *Chlorella* sp. Based on the results obtained from screening experiments, the two factors, viz. *Chlorella* sp. and audible sound, respectively, combined with cultivation time, are included in CCD experiment to determine the conditions optimum for TN removal.

### 3.3. Response surface design analysis for TN removal

Table 5 lists the coefficients of the models and the evaluated statistical significance. Statistical results revealed that all of the interactions between the three variables analyzed (i.e., algae density with audible sound or cultivation time) do not exhibit significant effects on the removal efficiency of TN from wastewater. This statement may be confirmed through the Pareto chart with 95% confidence (Figure 4).

Based on the program analysis, the TN removal efficiency model was expressed by a quadratic equation with significant terms (Equation (3)). The significant terms were considered as a p-value less than 0.05 (Ha 2017):

\[
\text{TN removal (\%)} = 9.88 + 5.96 X_1 + 1.2729 X_2 + 18.36 X_3 \
- 0.7319 X_1^2 - 0.0138 X_2^2 - 2.137 X_3^2
\]

**Figure 2** | Microscopic images of *Chlorella* sp. in BG-11 medium. (a) View at ×100 magnification. (b) View at ×400 magnification. Scale bars, 20 µm.

**Figure 3** | TN removal efficiencies by *Chlorella* sp. (5% density) with and without audible sound (60 dB) and control (neither algae nor audible sound).
The adequacy of the model is considered from the *p*-value of lack-of-fit and the regression coefficient ($R^2$). The *p*-value of the model should be less than 0.05, whereas the *p*-value of the lack-of-fit in comparison to the pure error should be greater than 0.05 (Ha 2018). The *p*-value for the TN removal model equation was less than 0.05, while the *p*-value for the lack-of-fit was 0.141 (Table 5). Moreover, the quadratic model exhibits a maximum-adjusted and predicted $R^2$. The predicted $R^2$ of 0.9452 was in acceptable agreement with that of adjusted 0.9825, and the good correlation between measured data and predicted data calculated using Equation (3) ($R^2$) was 0.9908. These data indicated that these model equations are adequate for predicting TN removal efficiency.

### 3.4. Conditions optimum for the removal of TN under *Chlorella sp./sound* treatment

Figure 5(a)–5(c) shows the response contour plot between TN removal efficiency and interaction of two variables by maintaining one variable constant. The contour line represents the TN removal percentage under the effect of algae density ($X_1$) and audible sound ($X_2$) at a constant cultivation time (5.5 days), as shown in Figure 5(a).

### Table 3 | Reaction kinetic model parameters for the TN removal of traditional market wastewater

| Factors          | Reaction order | Regression equation                              | $R^2$  | Rate constant | $t_{1/2}$ (day) |
|------------------|----------------|--------------------------------------------------|--------|---------------|-----------------|
| *Chlorella sp./sound* | 1             | $y = 0.3182x + 0.3390$                           | 0.9497 | 0.3182        | 2.178           |
|                  | 2             | $y = 0.0535x – 0.0217$                           | 0.9685 | 0.0535        | 0.419           |
|                  | 3             | $y = 0.0108x – 0.0210$                           | 0.8248 | 0.0108        | 0.027           |
| *Chlorella sp.*   | 1             | $y = 0.1094x + 0.2249$                           | 0.9080 | 0.1094        | 6.356           |
|                  | 2             | $y = 0.0331x + 0.0165$                           | 0.9770 | 0.0331        | 4.524           |
|                  | 3             | $y = 1.959 \times 10^{-4}x + 1.674 \times 10^{-4}$ | 0.9996 | $1.959 \times 10^{-4}$ | 0.152           |
| Control          | 1             | $y = 0.0018x + 0.0299$                           | 0.1799 | 0.0018        | 385.1           |
|                  | 2             | $y = 2.655 \times 10^{-5}x + 0.0144$             | 0.1771 | $2.655 \times 10^{-5}$ | 19.212.7        |
|                  | 3             | $y = 7.329 \times 10^{-7}x + 0.0002$             | 0.1743 | $7.329 \times 10^{-7}$ | 11.5            |

### Table 4 | Descriptive statistics and paired sample t-tests for the TN removal of traditional market wastewater by the three types of treatment

| Sample                | n | Mean  | StDev | SE mean | Lower CI for μ difference | Upper CI for μ difference | t   | p-value |
|-----------------------|---|-------|-------|---------|---------------------------|---------------------------|-----|---------|
| Control               | 11| 3.80  | 1.37  | 0.41    | 0.00                      | 5.60                      | –   | –       |
| *Chlorella sp.*       | 11| 50.33 | 21.27 | 6.55    | 0.00                      | 69.50                     | –   | –       |
| *Chlorella sp./sound* | 11| 74.44 | 30.65 | 9.24    | 0.00                      | 96.00                     | –   | –       |
| Control – *Chlorella sp.* | 22| −46.53| 20.74 | 6.25    | −60.47                    | −32.60                    | −7.44| <0.001  |
| Control – *Chlorella sp./sound* | 22| −70.64| 25.59 | 8.92    | −90.52                    | −50.76                    | −7.92| <0.001  |
| *Chlorella sp.*- *Chlorella sp./sound* | 22| 24.10 | 9.38  | 2.83    | 17.80                     | 30.41                     | 8.52| <0.001  |

*aComparison between groups.*

The adequacy of the model is considered from the *p*-value of lack-of-fit and the regression coefficient ($R^2$). The *p*-value of the model should be less than 0.05, whereas the *p*-value of the lack-of-fit in comparison to the pure error should be greater than 0.05 (Ha 2018). The *p*-value for the TN removal model equation was less than 0.05, while the *p*-value for the lack-of-fit was 0.141 (Table 5). Moreover, the quadratic model exhibits a maximum-adjusted and predicted $R^2$. The predicted $R^2$ of 0.9452 was in acceptable agreement with that of adjusted 0.9825, and the good correlation between measured data and predicted data calculated using Equation (3) ($R^2$) was 0.9908. These data indicated that these model equations are adequate for predicting TN removal efficiency.

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The density of *Chlorella* sp. and sound intensity affected the TN removal efficiency. Generally, musical sound of 60–80 dB is beneficial to stimulate the microalgae growth rate, and a high initial algal density could be more beneficial for nutrient removal (Lee & Chen 2015; Sarvaiya & Kothari 2015; Christwardana & Hadiyanto 2017; Gao et al. 2019). However, few studies on the combined effects of the initial algal density and sound power level on the removal efficiency have been conducted. In our experiment, the TN removal efficiency increased with the increase in the density of *Chlorella* sp. and audible sound from 1.0 to ∼6.5% and from ∼30 to 70 dB, respectively. However, at a *Chlorella* sp. density of greater than ∼6.5% and audible sound of greater than 70 dB, the TN removal efficiency decreased. Living cells can rapidly respond to sound stress under transcriptional and post-transcriptional levels, and some potential mechanisms may be involved in the responses of living cells to sound stress (Gu et al. 2016). Our results are in agreement with previous studies in that musical sound at a suitable intensity level can increase the density of the *Chlorella* sp. or enhance the removal efficiency; however, a high

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**Table 5 | ANOVA analysis for TN removal efficiency**

| Source          | DF | Coef | SS      | MS      | F-value | P-value | Note     |
|-----------------|----|------|---------|---------|---------|---------|----------|
| Model           | 9  | 95.280 | 3,897.38| 433.04  | 119.60  | < 0.001 | Significant |
| X₁ - Algae density | 1 | -3.636 | 180.53  | 180.53  | 49.86   | < 0.001 | Significant |
| X₂ - Audible sound | 1 | -5.429 | 402.50  | 402.50  | 111.16  | < 0.001 | Significant |
| X₃ - Cultivation time | 1 | -3.612 | 178.22  | 178.22  | 49.22   | < 0.001 | Significant |
| X₁² | 1 | -4.575 | 301.57  | 301.57  | 83.29   | < 0.001 | Significant |
| X₂² | 1 | -12.404 | 2,217.29| 2,217.29| 612.37  | < 0.001 | Significant |
| X₃² | 1 | -8.547 | 1,052.68| 1,052.68| 290.73  | < 0.001 | Significant |
| X₁ X₂ | 1 | 0.690 | 3.81    | 3.81    | 1.05    | 0.329   | Not significant |
| X₁ X₃ | 1 | -0.645 | 3.33    | 3.33    | 0.92    | 0.360   | Not significant |
| X₂ X₃ | 1 | 1.848 | 27.31   | 27.31   | 7.54    | 0.021   | Not significant |
| Lack-of-fit | 5  | 26.70 | 5.34    | 5.34    | 2.81    | 0.141   | Not significant |
| Pure error | 5  | 9.51 | 1.90    | 1.90    |         |         |          |
| Total | 19 |      | 3,933.59|         |         |         |          |

SS: Sum of squares; df: Degree of freedom; MS: Mean square.

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**Figure 4 | Pareto chart for the analysis of the experimental data obtained to remove TN from traditional market wastewater using cultivated *Chlorella* sp. with 95% confidence.**

The density of *Chlorella* sp. and sound intensity affected the TN removal efficiency. Generally, musical sound of 60–80 dB is beneficial to stimulate the microalgae growth rate, and a high initial algal density could be more beneficial for nutrient removal (Lee & Chen 2015; Sarvaiya & Kothari 2015; Christwardana & Hadiyanto 2017; Gao et al. 2019). However, few studies on the combined effects of the initial algal density and sound power level on the removal efficiency have been conducted. In our experiment, the TN removal efficiency increased with the increase in the density of *Chlorella* sp. and audible sound from 1.0 to ∼6.5% and from ∼30 to 70 dB, respectively. However, at a *Chlorella* sp. density of greater than ∼6.5% and audible sound of greater than 70 dB, the TN removal efficiency decreased. Living cells can rapidly respond to sound stress under transcriptional and post-transcriptional levels, and some potential mechanisms may be involved in the responses of living cells to sound stress (Gu et al. 2016). Our results are in agreement with previous studies in that musical sound at a suitable intensity level can increase the density of the *Chlorella* sp. or enhance the removal efficiency; however, a high
sound intensity might cause stress and reduce the cellular mechanisms (Ying et al. 2009; Gu et al. 2016; Christwardana & Hadiyanto 2017).

Figure 5(b) shows effects of the density of Chlorella sp. and cultivation time at an audible sound of 60 dB on the TN removal efficiency. The highest removal was observed at a cultivation time of 3–6 days. At this time, the Chlorella sp. density between 1.0 and 7.0% can remove TN from the wastewater. At this cultivation time, the TN removal efficiency gradually declined at an audible sound range of 30–70 dB (Figure 5(c). As can be observed in previous studies, an excessive increase in the cultivation time under a constant initial nutrient can reduce the TN removal efficiency because the remaining nutrient cannot provide sufficient supply for algal growth (Gao et al. 2019; Nguyen et al. 2019).

From the model, the optimization of the Chlorella sp./audible sound treatment was determined on the basis of the theoretical highest TN removal efficiency of 97.07%, with a desirability function value of 0.942 (Figure 6). The optimal Chlorella sp. density, audible sound, and cultivation time were 4%, 52.5 dB, and 4.6 days, respectively. Then, two additional experiments
were performed to verify the optimum results. The average TN removal obtained by experiment was 98.12%, which was in good agreement with the predicted response value.

4. CONCLUSION

In this study, effects of musical sound exposure on the removal efficiency under different initial cell densities of the microalga *Chlorella* sp. were analyzed. Results indicated that the combination of *Chlorella* sp. with audible sound exhibits good efficiency for the removal of TN from traditional market wastewater. The *Chlorella* sp./audible sound treatment can almost completely degrade TN. As an enhancement factor for *Chlorella* sp., the use of audible sound was limited because its frequency and noise should be utilized and not just simply noise in this treatment. Moreover, the mechanism via which musical sound increased the growth of *Chlorella* sp. is still not clear, necessitating better evaluation of the characteristic of algae in further analysis. Other types of music with the same noise or frequency also should be examined for this method to understand its behavior in terms of audible sound as support treatment.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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