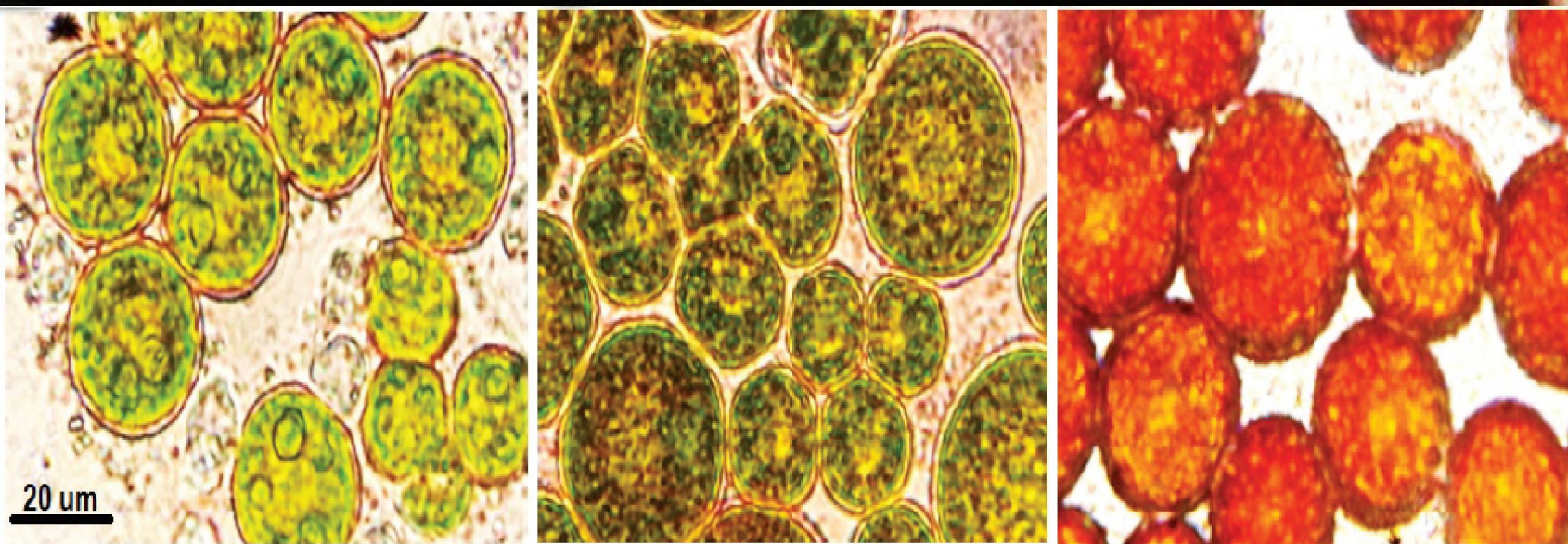


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**Influence of inoculum size, CO₂ concentration and LEDs
on the growth of green microalgae *Haematococcus pluvialis* Flotow**

Vietnam Journal of Science, Technology and Engineering (VJSTE) is a quarterly double-blind peer-reviewed academic journal. VJSTE publishes research articles and review articles in all areas of science, technology and engineering with the focus on the life sciences and environmental sciences including the following topics:

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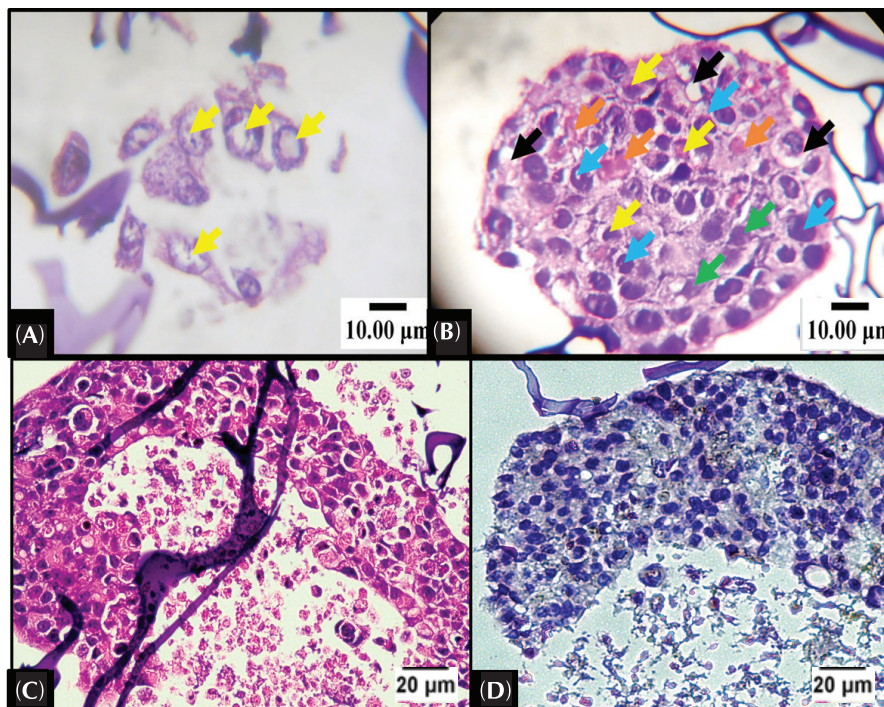
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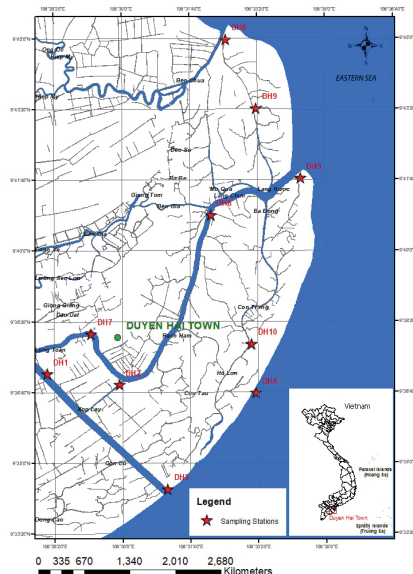
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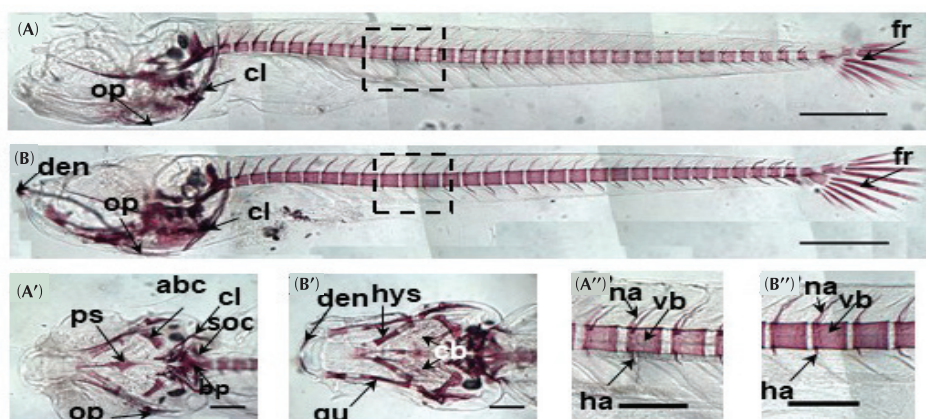
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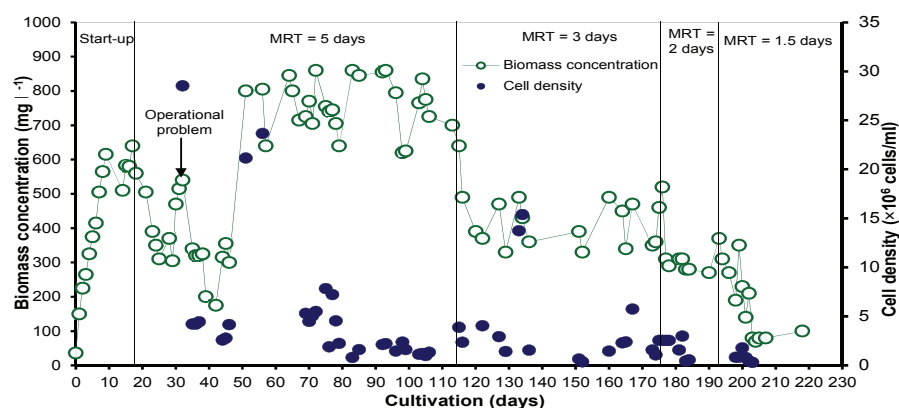
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Adsorption of Cr(VI) by material synthesized from red mud and rice husk ash

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Abstract:

In this work, the efficiency of a material prepared from red mud and rice husk ash (ZRM), in Cr(VI) absorption, without the use of acid to neutralize raw red mud (RM), was examined. The physico-chemical characteristics of the obtained material were determined by several methods, including BET nitrogen adsorption, XRD, SEM, and TEM. ZRM was employed in the adsorption of Cr(VI) in solution at 25°C with a Cr(VI) concentration of 20 ppm. The results showed that the nano particles of material were formed within the size range of 30-50 nm, and that the specific surface area of the material was 70.76 m²/g. The conditions of the adsorption process (i.e., the initial pH of the solution, the stirring rate, and the material content) were seen to significantly affect the efficiency of Cr(VI) adsorption at the material's surface. The optimum conditions for Cr(VI) adsorption via ZRM were determined as pH=2, a stirring rate of 300 rpm, and a material content of 10 g/l. With these conditions, the maximum adsorption capacity for Cr(VI) in a solution of ZRM was found to be 23.32 mg/g.

Keywords: Cr(VI) adsorption, material, red mud, rice husk ash.

Classification number: 2.2

Introduction

Nowadays, a multitude of hazardous waste is being produced as a result of rapid industrial development, with some environmental effects being particularly serious - including those involving water resources. Toxic organic compounds such as metallic ions of Cu, Zn, Pb, Ni, are some of the waste products released from petroleum oil processing, and the leather, electronics, electroplating, textile and dyeing industries. These waste compounds have been directly related to serious genetic changes, and to cancer, as well as to environmental degradation, even in small quantities. Cr(VI) can be considered one of the most hazardous of these substances. It is commonly found in waste water from a variety of industries, such as tanning, electroplating, textile dyeing, etc. Cr(VI), even in low concentrations in waste water, can cause damage to the kidneys, lungs, liver, as well as stomach [1, 2]. As a result of research, various techniques have been applied to remove Cr(VI) from waste water, including membrane filtration, ion exchange, electrolysis, adsorption, and biological techniques [3-5]. Among these, adsorption is the most attractive because of its economic efficiency [6, 7]. Discovering an appropriate adsorbent material, with high adsorption capacity and low cost, is the purpose of many current researches.

RM is one type of industrial waste which can be reused to produce low-cost adsorbent material. RM is known in the aluminium industry as a toxic waste resulting from the Bayer's process for the manufacturing of alumina from bauxite ore, following bauxite leaching by an alkali. The main components of RM are Fe₂O₃, Al₂O₃, SiO₂, CaO, and Na₂O. In Vietnam, according to the government's projection up to 2025, 15 million tons of alumina will be produced and more than 20 million tons of RM will be wasted yearly. More than 200 million tons of RM will be wasted over 10 years, therefore, this amount rising to more than 1.15 billion tons

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over 50 years [8]. There has been much research focused on recycling and increasing the value of RM. Recently, some studies have shown that it can be used as an adsorbent to remove pollutants such as arsenic [9], mercury [10], dyeing [11], as well chromium [12] from waste water. However, this involves a large amount of acid being used to neutralize the RM.

Rice husk ash (RHA) is mainly composed of SiO_2 (at about 95%), and other trace elements such as potassium, calcium, magnesium, iron, copper, manganese, and zinc. An attempt was made to investigate the synthesis of a material, with a partial zeolite structure, from RHA and RM, and with a high pH, so that the alkalinity did not require to be neutralized or acidified, thus reducing the cost of this process. The special feature of this study is that it examines synthesis of the adsorbent without processing residual alkali - an approach not previously published.

In this study, a new kind of adsorbent material was synthesized from RM and RHA, and the effect of various factors on the adsorption process of Cr(VI) investigated. The advantage of this process is not only using an agricultural by-product, but also reusing the remaining caustic soda in RM without neutralizing it with acid.

Methods and materials

The main chemicals used in the synthesis process and the testing of the adsorption properties of the material on Cr(VI) were oxalic acid (99%, Merck), $\text{K}_2\text{Cr}_2\text{O}_7$ (99.9%, Merck), and diphenylcarbazite as an indicator.

RM was obtained from Tan Rai factory, Lam Dong province, Vietnam with the composition as follows [13]: 64.2% Fe_2O_3 , 12.6% Al_2O_3 , 4.5% Na_2O , 3.7% SiO_2 , 4.13% CaO , 9.3% P_2O_5 , 0.235% TiO_2 .

RHA was collected from Sa Dec industrial park in Dong Thap province, Vietnam. After undergoing the calcination process for 2 hrs at 700°C , the composition of the RHA was as follows [13]: 95.2% SiO_2 , 0.375% P_2O_5 , 1.02% K_2O , 0.584% CaO .

The physico-chemical properties of the synthesized material were characterized using a variety of methods. An X-ray diffractometer (XRD, Bruker D8 Advance, Germany) with CuK_α radiation ($\lambda=0.15406$) was used to determine the structure and crystallite phase. The morphology of the material was investigated through use of a Scan Electronic Microscope SEM (FESEM, S4800-Hitachi, Japan) and a Transmission Electronic Microscope (TEM, JEM 1400, JEOL, Japan). The specific surface area of the synthesized powder was tested by BET (NOVA 3200e, Quantachrome Instruments, USA).

The absorbent material (ZRM) was synthesized from RM and RHA using the process as described in [13] with the ratio of $\text{SiO}_2/\text{Al}_2\text{O}_3$ at 1.8. The remaining caustic in the RM did not require neutralizing by acid, which is an advantage of this process. ZRM was applied to test its adsorption activity on Cr(VI). In this process, a 250 ml solution of Cr(VI) was poured into a beaker in which 10 grs of ZRM had been placed. The affecting factors were then investigated, including the pH of the initial Cr(VI) solution (2-7), the initial concentration of the Cr(VI) solutions (10-40 mg/l), and stirring rates (200-400 rpm). All experiments were conducted at room temperature (25°C). The resulting mixtures were centrifuged to separate solids from liquids, diluted with the ratio of 1:5 times, and then analyzed via UV-Vis equipment (Shimadzu, Japan) with diphenylcarbazite as an indicator at a wavelength of $\lambda=540$ nm.

For analysis of the Cr(VI) concentration in the sample according to adsorption time, the calibration curve with the dependence of Cr(VI) concentration ($C = 0; 0.5; 1.0; 2.0; 3.0$; and 4.0 mg/l) on absorbance (Abs) was constructed as follows:

$$C_i = 0.579 \cdot (\text{Abs}) \quad (1)$$

The adsorption yield was calculated by the equation (2):

$$H = (C_o - C_e) \cdot 100 / C_o \quad (2)$$

where C_o and C_e correspond to the initial and equilibrium concentrations of Cr(VI) (mg/l).

Equilibrium Cr(VI) concentration (C_e) was determined at the point at which Cr(VI) adsorption was saturated [Cr(VI) concentration did not change over time].

Results and discussion

Physico-chemical characteristics of catalysts

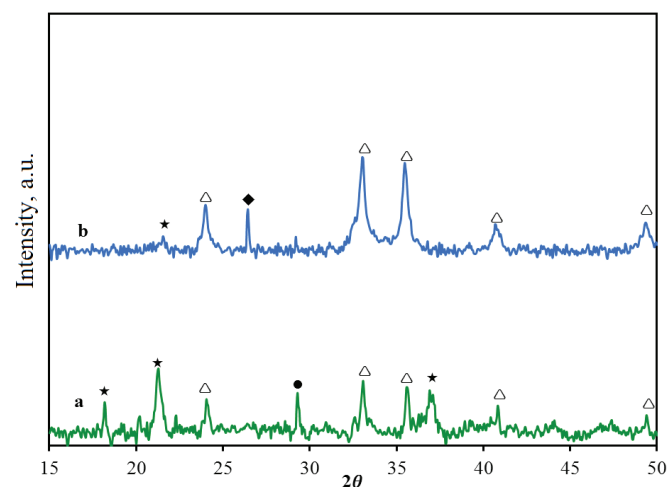


Fig. 1. XRD patterns of raw RM (a) and ZRM (b).

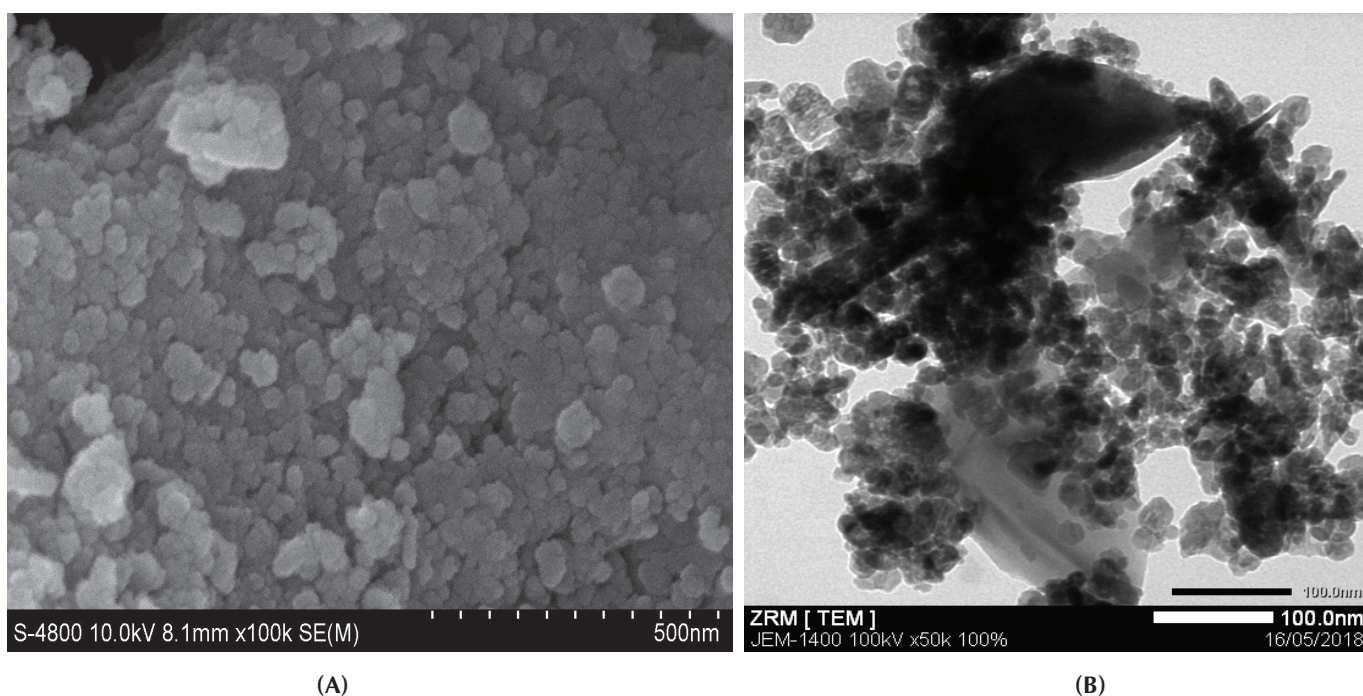


Fig. 2. SEM (A) and TEM (B) images of ZRM.

The XRD patterns in Fig. 1 show that RM is mainly composed of hematite ($2\theta = 24, 33, 35.5, 41, \text{ and } 49.4^\circ$) and gibbsite ($2\theta = 18, 21.2, \text{ and } 37^\circ$), besides the peak of calcite ($2\theta = 29^\circ$) [14]. The patterns for ZRM show that the peaks of gibbsite and calcite have disappeared, with the peaks for hematite in the same position but higher and sharper compared to that of raw RM.

The results of the specific surface areas obtained by BET analysis for RM, RHA, and ZRM were: 23.59, 28.35, and 70.76 m^2/g , respectively. According to these results, the specific surface area of ZRM is triple that of raw RM. This finding could be explained by the small amount of zeolite in phase A, formed during the synthesis process, as shown in the XRD patterns ($2\theta = 27^\circ$), and the organic compounds on the surface of raw RM being destroyed during the calcination process. The material synthesized by ZRM has an average pore diameter of 18Å and a pore volume of 0.051 cm^3/g .

The SEM image in Fig. 2 shows that particle size on the surface of ZRM is rather uniform, in the range of 30-50 nm. Furthermore, it can be seen that ZRM has high porosity and low aggregation at its surface. The TEM results for ZRM as shown in Fig. 2 show some pores on the surface were covered by other compounds found in RM, such as Fe_2O_3 , with the result that the specific surface area for ZRM is not so high.

Adsorption of Cr(VI) by ZRM

Comparison of Cr(VI) adsorption between raw RM and ZRM:

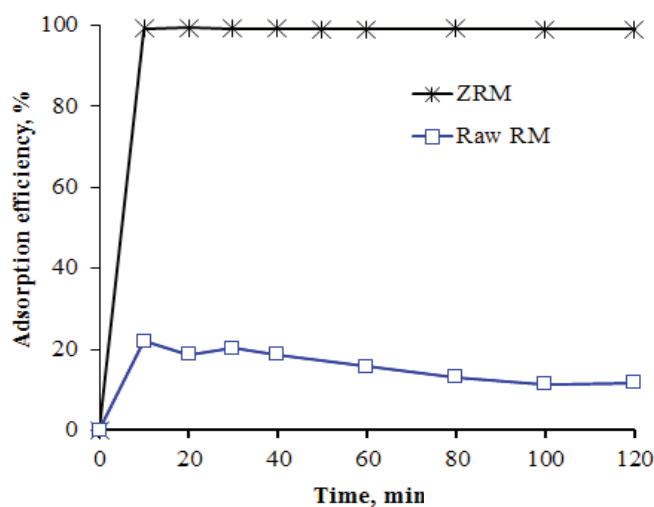


Fig. 3. Cr(VI) adsorption by raw RM and ZRM.

The adsorption capacity of raw RM and ZRM for Cr(VI) was studied at stirring velocity conditions of 300 rpm, at a temperature of 25°C, and at pH=2 (which was adjusted by use of oxalic acid); the initial concentration of Cr(VI) was 20 ppm, where the mass ratio of adsorbency was 10 g/l. The results are shown in Fig. 3. It can be seen that ZRM's capacity for absorption of Cr(VI) is much higher than that of raw RM; just 10 minutes into the adsorption process, the adsorption yield of ZRM reached 100%. This meant the absorbance (Abs) of the solution is approximately zero, while it is about 12% with raw RM.

Effect of the conditions on efficiency of ZRM's absorption of Cr(VI):

The capacity of ZRM for absorbing Cr(VI) was studied with various values of stirring capacity - from 200 rpm to 400 rpm, at pH=2, and with all other conditions remaining the same as the previous experiment. The results are shown in Fig. 4. When the stirring velocity was 200 rpm, the adsorption capacity was low, at around 40% after 30-40 min; at 300 rpm and higher, however, the adsorption yield reached 100% after 15 min with no further change.

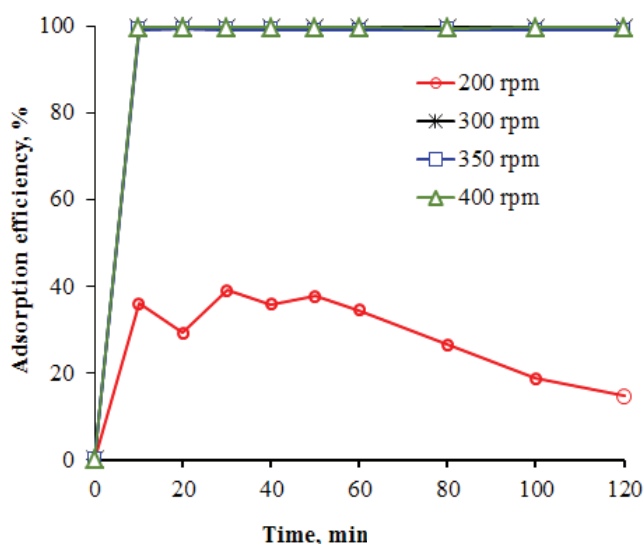


Fig. 4. Effect of stirring rate on ZRM's efficiency in absorbing Cr(VI).

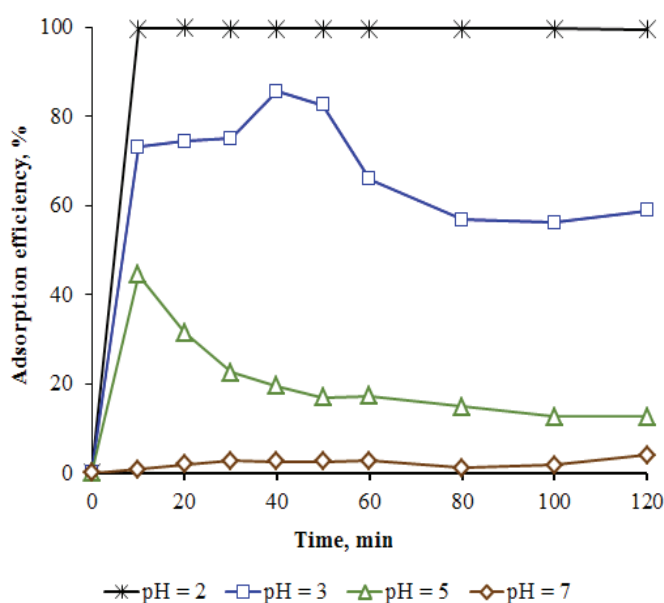


Fig. 5. Effect of pH on ZRM's efficiency in absorbing Cr(VI).

pH is one of the factors strongly affecting the adsorption of heavy metal ions. The effect of pH, adjusted by oxalic acid, on ZRM's adsorption of Cr(VI), is shown in Fig. 5, at a stirring velocity of 300 rpm and all other conditions remaining the same as in the previous experiments. The results show that when the pH is increased from 2 to 7, the adsorption capacity of ZRM is decreased. The highest adsorption yield was determined as being at pH=2, and this value is 99.73% after 10 min. This can be explained by the material surface being assembled H^+ and subsequently Cr(VI) being more easily adsorbed by the process of ion exchange. The reducing of absorption capacity when the pH is increased, might be because of the process of hydrolysis, which prevents the dispersion step in the adsorption process [15].

To determine the effect of the content of ZRM in the solution, the adsorption process was carried out with the same conditions as the previous experiments, where this varied from 5 to 15 g/l. Fig. 6 shows that when the ZRM concentration is increased, the adsorption yield also increases. However, if the ZRM concentration reaches 20 g/l, then the adsorption yield is decreased. This might be explained by the fact that at concentrations greater than 15 g/l, aggregation of the material will occur, leading to a reduction of the adsorption surface. With a ZRM concentration of 10 g/l, the maximum Cr(VI) adsorption is 23.32 mg/g which is higher than that of RM modified cetyltrimethylammonium bromide (22.20 mg/g), as reported in the work of Li, et al. [15].

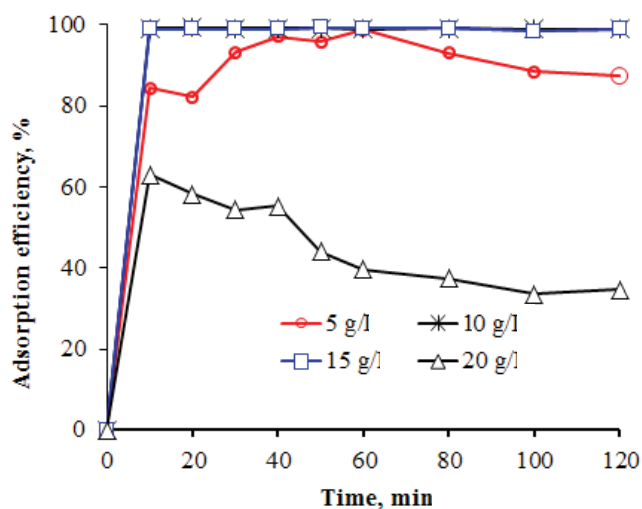


Fig. 6. Effect of ZRM concentration in the solution, on adsorption of Cr(VI).

Conclusions

The material ZRM for adsorption of Cr(VI) in solution, synthesized from RM and RHA without the use of acid to neutralize it, has a surface area of 70.76 m²/g and a particle size of 30-50 nm. The optimum conditions for Cr(VI) adsorption by ZRM at 25°C were determined as pH=2, a stirring rate of 300 rpm, and material content of 10 g/l. With these conditions, based on the equilibrium adsorption result, the maximum adsorption capacity for Cr(VI) in a solution of ZRM is 23.32 mg/g - three times higher than that of raw RM. This study has suggested a way of synthesizing cheap material from two waste resources, RM and RHA, for Cr(VI) adsorption in solution, and with high efficiency.

ACKNOWLEDGEMENTS

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The authors declare that there is no conflict of interest regarding the publication of this article.

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Optimisation of heat-moisture treatment conditions for producing high amounts of resistant starches from purple sweet potato and yam starches using response surface methodology

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Abstract:

Heat-moisture treatment, a physical modification method of starch, causes changes in the internal structure of starch and thereby produces resistant starch (RS). In this study, the heat-moisture treatment conditions (moisture content, heating temperature, and incubation time) was optimized to maximise the RS contents of the treated sweet potato and yam starches, using the Box-Behnken design and response surface analysis. The predicted maximised RS content of the treated sweet potato starch (43.9%) was obtained under optimal conditions of 34.76% moisture content, heating temperature of 100.11°C, and incubation time of 6.01h; the predicted maximised RS content of the treated yam starch (36.8%) was obtained under optimal conditions of 30.06% moisture content, heating temperature of 109.68°C, and incubation time of 6.59h using a quadratic model within the range of various process variables. The experimental RS contents of the treated sweet potato and yam starches obtained under optimal treatment conditions were 42.4% and 35.4%, respectively; this confirms that the models were valid and adequate because the predicted data and experiment data did not differ significantly. The results also indicate that the RS contents of the treated sweet potato starch were significantly higher than that of the treated yam starch. As a result, both starch structure and treatment conditions were determined to significantly affect the formation of RS in the heat-moisture treated starches.

Keywords: heat-moisture treatment, resistant starch, response surface methodology, sweet potato starch, yam starch.

Classification number: 2.2

Introduction

In modern times, the number of patients who are overweight or suffer from diabetes is rapidly increasing both among children and adults because of their high intake of saturated fatty acids, high total fat intake, and inadequate consumption of dietary fibre [1-4]. Many studies have reported that diabetes-related diseases such as obesity, cardiovascular disease, and type 2 diabetes have been prevented or controlled by increasing amounts and varieties of fibre-containing foods [5]. However, the increase in dietary fibre content in food products significantly affects sensory and textural properties of these foods, such as negative effects on the final bread quality, which results in reduced volume and altered texture and consistency of the bakery product [6]. Recently, the RS from various starch sources has been widely used as a ‘low-carbohydrate’ ingredient in food formulations [7] because its health benefits resemble those of dietary fiber [8-9]. Englyst, et al. [10] used the term ‘RS’ to describe a small fraction of starch that resisted to hydrolysis by exhaustive α -amylase and pullulanase treatment *in vitro*. Currently, RS is defined as the fraction of dietary starch which escapes digestion in the small intestine and does not contribute to the blood glucose levels of healthy individuals [11]. Therefore, WHO recommends consuming 27-40 g of RS per day to prevent colon diseases [9].

Recently, physical, chemical, and enzymatic modifications have been developed to produce RS from various starch sources. Among these methods, the heat-moisture treatment (MHT) is a well-known hydrothermal method of increasing levels of SDS and RS in starches without destroying their granular structures [12]. The starch is treated at low moisture levels (<35% moisture, w/w) and at high temperatures (84-120°C) for 15 min to 16h [13]. Hung, et al. [14] reported that the RS contents

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of sweet potato and yam starches increased from 14.7% and 21.6% in native form to 27.2% and 31.0% in treated starches, respectively, after heat-moisture treatment at a moisture content of 30% and at a heating temperature of 110°C for 8h. Conversely, Huang, et al. [15] report that the RS content of the heat-moisture-treated sweet potato starch at a moisture content of 30% and at a heating temperature of 100°C for 2h (one cycle) decreased from 25.94% to 13.73% during the first cycle and then increased to the maximum amount of 20.99% after five treating cycles. The increase in RS content was also observed for corn, pea, and lentil starches after heat-moisture treatment under conditions of 30% moisture content, a heating temperature of 100°C, and treatment time of 2h [16]. In addition, Chung, et al. [16] also demonstrated that the amounts of RS of corn, pea, and lentil starches treated at 120°C were higher than those treated at 100°C. Therefore, the differing treatment conditions of the hydrothermal method resulted in different degrees of RS formation. Although many heat-moisture treatment conditions have been applied to investigate changes in physicochemical properties and digestibility of starches [17], the results of RS production after heat-moisture treatment of sweet potato starch remained variable because of the differing treatment conditions used in these studies [14, 15, 18]. In addition, the amylose content and starch characteristics, such as crystallinity and chain-length distribution, also affect the RS formation of starch after heat-moisture treatment [17]. Therefore, the objective of this study is to optimise heat-moisture treatment conditions (moisture content, heating temperature, and incubation time) to obtain the highest RS contents of sweet potato and yam starches using Box-Behnken designs and response surface analysis. Sweet potato and yam starches were selected in this study because these tuber starches possess different starch characteristics such as amylose content, chain-length distribution, and crystallinity [14]. In addition, limited information concerning RS formation of sweet potato and yam starches has been previously discovered.

Materials and methods

Materials

Starches used in this study were directly isolated from fresh purple sweet potatoes (*Ipomoea batatas*) and yams (*Dioscoreaceae atatas*) in the laboratory, as previously reported by Hung, et al. [14]. The isolated sweet potato contained 1.1% protein, 0.9% lipid, 0.1% ash, and 97.9% total carbohydrate, while the yam starch contained 0.8% protein, 1.3% lipid, 0.1% ash, and 97.8% total carbohydrate [14]. Amylose contents of sweet potato and yam starches were 18.7 and 22.3%, respectively [14].

Alpha-amylase from *A. oryzae* (~30 U/mg, product # 10065) and amyloglucosidase from *A. niger* (≥300 U/ml, product # A7095), which were purchased from Sigma-Aldrich Co. (St. Louis, MO, US), were used in this study. Other chemicals were purchased from Merck Co. (Darmstadt, Germany).

Starch characteristics

Thermal characteristics of starches were determined using a differential scanning calorimeter (DSC-60, Shimadzu Co., Kyoto, Japan) [19]. An aluminum vessel which contained 3.0±0.1 mg of starch and 10 µl of distilled water was sealed and remained at room temperature for over 30 min for equilibration. The vessel was then heated from 30 to 120°C at a rate of 10°C/min by a DSC-60 heater. An empty vessel was used as a reference. The initial, peak, and recovery temperatures and transition enthalpy were automatically calculated using a TA-60WS program (Shimadzu Co.).

Crystalline characteristics of starches were determined using an X-ray diffractometer (Rigaku Co., Ltd, Rint-2000 type, Tokyo, Japan). The XRD system was operated at 40 kV and 80 mA, and diffractograms of the starches were recorded from 2° 2θ to 35° 2θ, with a scanning speed of 8°/min and a scanning step of 0.02° [19].

Box-Behnken designs for heat-moisture treatments of starches

The heat-moisture treatment of starches was performed based on the method of Hung, et al. [14]. The starches (100 g) were directly weighed and mixed with water at a desired moisture content level. The sample was well-dispersed and equilibrated at room temperature for 24h before being heated in a forced air oven at a specific temperature for a controlled duration. After heat-moisture treatment, the starch samples were cooled and then dried at 45°C for 24h to a moisture content of approximately 10%.

Table 1. Coded levels of variables selected for the experiments.

Variable	Coded	Range and level		
		-1	0	+1
Moisture content (%)	X ₁	25	30	35
Heating temperature (°C)	X ₂	100	110	120
Incubation time (h)	X ₃	6	7	8

A three-factor Box-Behnken design and optimisation [20] was used to optimise the heat-moisture treatment conditions for all variable factors to obtain the highest RS content. Three important factors, including moisture content (X₁), heating temperature (X₂), and incubation time (X₃)

were selected as the independent variables, and RS content (Y) was selected as the dependent response variable. Three different levels of each independent variable, including moisture contents (25, 30, and 35%), heating temperatures (100, 110 and 120°C), and incubation times (6, 7 and 8h) were used and coded as -1, 0, and +1 for low, middle, and high levels, respectively, as presented in Table 1. A total of 15 experiments were conducted for three independent variables based on the Box-Behnken design.

The mathematical relationship between response (Y) and independent variables (X) was demonstrated by the following regression equation.

Where Y is the quadratic response, β_0 , β_i , β_j and β_{ij} are the regression coefficients for intercept, linear, quadratic, and interaction terms, respectively. X_i and X_j are the coded values of the i^{th} and j^{th} independent variables. The variables $X_i X_j$ represent the first order interaction between X_i and X_j for ($i < j$).

The optimal values of the selected variables were calculated by solving the regression equation and also by analysing the response surface contour plots using a design expert software (version 7.0.0, STAT-EASE Inc., Minneapolis, MN, USA). The validity and adequacy of the predictive models were determined through experimental analysis at optimal conditions suggested by the design expert [20].

Determination of starch fractions (RDS, SDS, RS)

After heat-moisture treatment, the native and treated starches were then measured for rapid digestible starch (%RDS), slowly digestible starch (%SDS), and RS (%RS) based on the methods of Englyst, et al. [21], as previously described by Hung, et al. [14]. Starch (0.3 g, db) was mixed with 20 ml of sodium acetate buffer (pH 6.0) and boiled for 30 min in a water bath. The sample was then equilibrated at 37°C for 15 min prior to adding an enzyme solution (5 ml) of α -amylase (1,400 U/ml) and amyloglucosidase (13 AGU/ml). The starch solution was incubated with shaking at 37°C for 120 min, and the total glucose concentrations of the 20 min-digested and 120 min-digested hydrolysates (G_{20} and G_{120} , respectively) were determined using the phenol-sulfuric acid method. The remaining residue was intensively hydrolysed with amyloglucosidase (50 AGU/ml) after hydrolysing by 7M KOH. The final hydrolysate was then determined for total glucose concentration (TG). The total glucose levels at different digestive times (G_{20} , G_{120} and TG) were used to calculate for RDS, SDS, and RS as follows [21]:

$$RDS = G_{20} \times 0.9$$

$$SDS = (G_{120} - G_{20}) \times 0.9$$

$$RS = (TG - G_{120}) \times 0.9$$

Statistical analysis

The statistical analysis was performed through an analysis of variance (one-way ANOVA) with Duncan's multiple-range test to compare treatment means at $p < 0.05$, using SPSS software version 16 (SPSS Inc., USA).

The regression and graphical analysis of the data was conducted using a design Expert software (version 7.0.0, STAT-EASE Inc., Minneapolis, MN, USA).

Results and discussion

Starch characteristics

Table 2. Amylose content and thermal characteristics of sweet potato and yam starches*.

Starch	Amylose content (%)	Thermal characteristic			
		Onset (°C)	Peak (°C)	Completion (°C)	Enthalpy (J/g)
Sweet potato	18.7 ^a	71.2 ^a	75.3 ^a	80.2 ^a	17.8 ^a
Yam	22.3 ^b	75.5 ^b	79.3 ^b	86.0 ^b	35.8 ^b

*Data followed by the same letter in the same column are not significantly different ($p \leq 0.05$).

Thermal characteristics of starches isolated from sweet potato and yam tubers are indicated in Table 2. The gelatinisation temperature of the sweet potato starch was between 71.2 and 80.2°C, which was significantly lower than that of the yam starch (75.5 to 86.0°C). Transition enthalpy of the sweet potato starch was also significantly low relative to the yam starch. The higher transition enthalpy of yam starch relative to that of the sweet potato starch was due to the higher amylose content of yam starch relative to sweet potato starch (Table 2). This result aligns with the previous reports which stated that the root starches containing higher amylose contents had a larger transition enthalpy relative to those containing lower amylose contents [19, 22, 23].

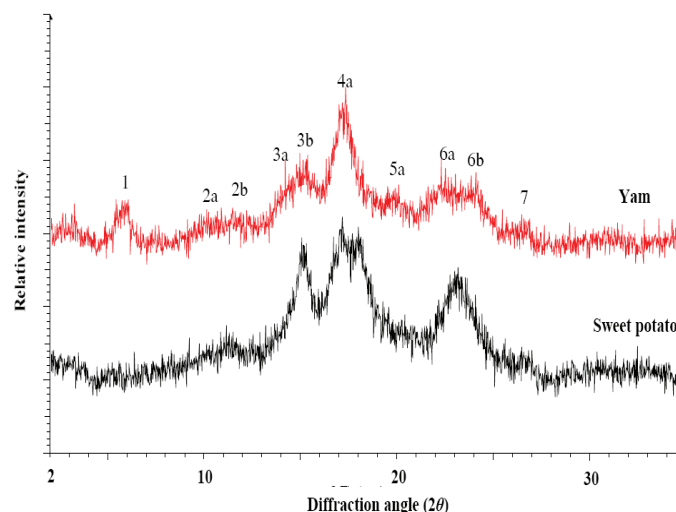


Fig. 1. X-ray diffraction patterns of sweet potato and yam starches.

The X-ray diffraction patterns demonstrated that native sweet potato and yam starches had different crystalline structures (Fig. 1). The native sweet potato starch had major peaks around d-spacings 5.8 Å (line 3b), 5.2, and 4.8 Å (line 4a, 4b) and 3.8 Å (line 6a), while yam starch peaks at 15.8 Å (line 1), about 5.9 Å (line 3a, 3b), 5.2 Å (line 4a), and 4.0 and 3.7 Å (lines 6a, 6b). As a result, the native sweet potato starch exhibited the A-type crystal, whereas the native yam starch displayed the B-type crystal, as classified by Zobel [24]. These results are consistent with those previously reported by Hoover [25].

Therefore, the differences in amylose contents, thermal characteristics, and crystalline structures of the native sweet potato and yam starch may affect RS formation under heat-moisture treatment.

Optimisation of RS content through response surface methodology

Significant factors used in heat-moisture treatment, including moisture content, heating temperature, and incubation time, were optimised using Box-Behnken design to maximise RS contents of sweet potato and yam starches. Table 3 illustrates the design matrix with three independent variables (coded values) and experimental responses (RS contents (% w/w, db) of treated sweet potato and yam starches). Based on the treatment conditions formulated by the Box-Behnken design, the highest RS content of the treated sweet potato starch was 41.64% after treating the native starch under the conditions of 30% moisture content at a heating temperature of 100°C for 6h, while the lowest RS content of this type of starch was 24.96%; this was obtained under the conditions of 25% moisture content and a heating temperature of 120°C for 7h. The highest RS content of the treated yam starch was 34.57%, which was obtained under treatment conditions of 35% moisture content and a heating temperature of 120°C for 7h, while the lowest RS content of this type of starch was 15.57% under the conditions of 35% moisture content and a heating temperature of 110°C for 8h. The data were then analysed through multiple regression analysis, and the regression coefficients for the equation concerning the relationship between three variables and a response were determined and presented in Table 4. Moreover, the results of the analysis of variance (ANOVA) with the Fisher's statistical test using Design Expert software (Design Expert 7.0.0) are indicated in Table 5. The coefficient of determination (R^2), which can be defined as the ratio of the explained variation to the total variation, was used to evaluate the fitness and adequacy of the model. The empirical model fits the actual values if the R^2 value is near unity. The R^2 values of the regressed models

which predicted the RS contents of the treated sweet potato and yam starches were 0.9763 and 0.9565, respectively; this suggests a high dependence and correlation between the measured and predicted values of the responses.

Table 3. Full factorial Box-Behnken design matrix with three independent variables in coded units and experimental responses.

Trial run	Coded variable			Experimental response (RS content, %)	
	Moisture content (%)	Heating temperature (°C)	Incubation time (h)	Sweet potato	Yam
	X_1	X_2	X_3	Y_1	Y_2
1	+1	0	+1	31.96	15.57
2	0	-1	+1	37.65	19.64
3	+1	-1	0	35.15	20.08
4	0	-1	-1	41.64	32.40
5	-1	0	-1	34.08	32.28
6	0	+1	-1	32.44	34.12
7	+1	0	-1	38.34	31.45
8	-1	-1	0	32.93	32.20
9	0	+1	+1	27.48	25.19
10	0	0	0	26.90	30.67
11	0	0	0	27.24	31.35
12	-1	+1	0	24.96	34.46
13	+1	+1	0	29.66	34.57
14	0	0	0	27.10	32.79
15	0	-1	+1	33.95	19.62

Table 4. Coefficients of the response function to predict resistant starch content of sweet potato and yam starches through regression analysis.

Factor	Coefficient estimate	
	Sweet potato	Yam
Intercept	27.08	31.63
X_1	1.15	- 2.11
X_2	- 4.10	3.00
X_3	- 1.93	- 6.28
X_1X_2	0.62	3.06
X_1X_3	- 1.56	-0.80
X_2X_3	- 0.24	0.96
X_1^2	1.69	- 2.19
X_2^2	1.91	0.92
X_3^2	5.81	- 4.68

X_1 : moisture content (%); X_2 : heating temperature (°C); X_3 : incubation time.

Table 5. Analysis of variance for the response surface quadratic model.

Source	df	Sweet potato		Yam	
		F-value	p-value	F-value	p-value
Model	9	22.89	0.0015*	12.22	0.0066*
X ₁	1	6.71	0.0489*	6.91	0.0466*
X ₂	1	85.58	0.0002*	13.98	0.0134*
X ₃	1	18.98	0.0073*	61.13	0.0005*
X ₁ X ₂	1	0.98	0.3684	7.25	0.0432*
X ₁ X ₃	1	6.20	0.0551	0.50	0.5101
X ₂ X ₃	1	0.15	0.7150	0.71	0.4376
X ₁ ²	1	6.68	0.0492*	3.44	0.1229
X ₂ ²	1	8.53	0.0330*	0.60	0.4735
X ₃ ²	1	79.31	0.0003*	15.69	0.0107*
Residual	5				
Lack of fit	3	89.19	0.0111	6.67	0.1331
R ²		0.9763		0.9565	

*Significant (p-value < 0.05); X₁: moisture content (%); X₂: heating temperature (°C); X₃: incubation time.

The F-test and *p*-value obtained through the analysis of variance (ANOVA) were used to determine the significance of each coefficient. The *p*-value denotes the probability value. The *p*-values of the adjusted models were 0.0015 and 0.0066 for both sweet potato and yam starches, respectively; these were lower than 0.05, which indicates that both models were significant. For the model of sweet potato starch, the X₁, X₂, X₃, X₁², X₂², and X₃² factors were the most significant among the factors which influenced the response because the *p*-values of these variables were all lower than 0.05. For the model of yam starch, X₁, X₂, X₃, and X₃² were also the significant factors which influenced the response, while the *p*-value of X₁X₂ was 0.0432 (lower than 0.05) which indicates that the interaction between moisture content and heating temperature affected the response. As a result, the equations which illustrated the relationship between three variables including moisture content, heating temperature, and incubation time, and the responses are formed in a reduction form as follows:

$$Y_1 = 27.08 + 1.15X_1 - 4.10X_2 - 1.93X_3 + 1.69X_1^2 + 1.91X_2^2 + 5.81X_3^2$$

$$Y_2 = 31.63 - 2.11X_1 + 3.00X_2 - 6.28X_3 + 3.06X_1X_2 - 4.68X_3^2$$

where: Y₁ and Y₂ are the predicted responses of the RS contents of the sweet potato and yam starches, respectively, X₁, X₂, and X₃ are coded variables for moisture content, heating temperature, and incubation time, respectively.

Table 6. Optimal conditions for producing high amounts of resistant starches.

Variable	Sweet potato		Yam	
	Calculation	Confirmation	Calculation	Confirmation
Moisture content (%)	34.76	35	30.06	30
Heating temperature (°C)	100.11	100	119.68	120
Incubation time (h)	6.01	6	6.59	6.5
RS (%)	43.9	42.4	36.8	35.4

The optimisation of the process variables to maximise RS contents of the heat-moisture treated sweet potato and yam starches was performed by solving the quadratic models using the studied experimental range of various variables. Table 6 presents the predicted values of the responses under optimal conditions (in the range constraint) for the models. For the sweet potato starch, the optimal conditions included a moisture content of 34.76%, a heating temperature of 100.11°C, and an incubation time of 6.01h to achieve the highest RS content of 43.9%. For the yam starch, the highest RS content was 36.8%, which was achieved under the optimised conditions of a moisture content of 30.06%, a heating temperature of 119.68°C, and an incubation time of 6.59h. These models were experimentally assessed to confirm the RS contents of the treated sweet potato and yam starches. However, it is difficult to maintain the recommended conditions during processing. Therefore, optimal conditions were targeted using the rounded numbers of all factors, as displayed in Table 6. As a result, the experimental RS content of the treated sweet potato starch under the experimental conditions of heat-moisture treatment, including moisture content of 35%, heating temperature of 100°C, and incubation time of 6h, was 42.4%; this did not significantly differ from the calculated data (43.9%). Likewise, the experimental RS content of the treated yam starch (35.4%), which was obtained under the experimental conditions of heat-moisture treatment, including a moisture content of 30%, a heating temperature of 120°, and an incubation time of 6.5h, did not significantly differ from the calculated data (36.8%). Therefore, the model conditions were targeted to be optimal for the development of RS contents of the heat-moisture-treated sweet potato and yam starches, and the data obtained confirmed the validity and adequacy of the models.

The formation of RS during heat-moisture treatment was caused by the formation of some interactions during heat-moisture treatment that have survived after gelatinisation and partly resisted the accessibility of starch chains through the hydrolysing enzymes [16]. Therefore, both the treatment conditions, including moisture content, heating temperature, and time, and starch characteristics,

such as amylose-lipid interactions and amylose-amylose or amylose-amylopectin interactions, exerted a significant influence on the digestibility of starches [26]. The results of this study indicate that the heat-moisture treatment exerted a greater impact on the sweet potato starch than the yam starch under optimal treatment conditions. As a result, the higher RS content of the treated sweet potato at optimal treatment conditions was obtained relative to that of the treated yam starch. These results were caused by the fact that the formation and lateral association of double helices involving amylopectin chains in the heat-moisture treated B-type starches would be significantly slower, more difficult, and less strong relative to the heat-moisture-treated A-type starch [22]. In addition, the sweet potato starch required a low temperature (100°C) but a high moisture content (35%) to form the highest RS content, while the yam starch required a high temperature (120°C) but a low moisture content (30%) to maximise RS content. Therefore, the optimal condition to maximise the RS content of starch differed based on the nature of the starch.

RS contents of native and treated starches

Amounts of rapid digestible starch (RDS), slowly digestible starch (SDS), and the RS of native and treated sweet potato and yam starches are indicated in Table 7. Amounts of RDS and SDS in native sweet potato starch were higher than those in native yam starch. However, the RS content of the native sweet potato starch was lower relative to the native yam potato. Under optimal heat-moisture treatment conditions, the amounts of SDS and RS of the treated sweet potato and yam starches significantly increased relative to those of the native starches. The SDS and RS contents of the treated sweet potato under optimal treatment conditions were significantly higher than those of the treated yam starch, although the amount of RS of the treated yam starch was higher relative to the treated sweet potato starch when these starches were heat-moisture-treated under the same conditions of 30% moisture content and a heating temperature of 110°C for 8h, as reported by Hung, et al. [14]. In addition, the RS contents of the treated sweet potato and yam starch obtained in this study under optimal treatment conditions were significantly higher than those obtained by Hung, et al. [14]. Therefore, the formation of RS in the starch through heat-moisture treatment was not only affected by the internal structures and amylose contents of the starches but was also affected by the heat-moisture treatment conditions. The highest amount of RS is obtained if the starch is treated under optimal conditions specific to each starch based on the type and structure of the starch.

Table 7. RDS, SDS, and RS of native and heat-moisture-treated sweet potato and yam starches*.

Sample	RDS (%)	SDS (%)	RS (%)
Sweet potato starch			
Native	78.7±2.0 ^d	6.6±0.5 ^a	14.7±1.5 ^a
Heat-moisture	43.2±2.3 ^a	14.4±2.9 ^c	42.4±0.6 ^d
Yam starch			
Native	73.8±2.2 ^c	4.7±1.0 ^a	21.6±1.8 ^b
Heat-moisture	55.7±0.3 ^b	8.9±1.8 ^b	35.4±1.5 ^c

*Data followed by the same letter in the same column are not significantly different ($p \leq 0.05$).

Conclusions

The RS contents of the heat-moisture-treated sweet potato and yam starches were maximised using the Box-Behnken design and the response surface analysis. The results indicate that moisture content, heating temperature, and incubation time were the most pivotal factors which affected the RS formation of the heat-moisture-treated starch. The quadratic models within the studied experimental range of various process variables were used to maximise the RS contents of the treated starches. As a result, the experimental RS contents of the treated starches obtained using the optimal conditions of heat-moisture treatment did not significantly differ from the data calculated using the quadratic models, meaning that the models were valid and adequate. Under optimal treatment conditions, the RS content of the treated sweet potato starch was higher relative to the treated yam starch because of the differences in the internal structures and amylose contents of these starches. Therefore, the heat-moisture treatment condition must be optimised for each starch to obtain the highest RS content of starch.

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Germination and shoot development of *Pisum sativum* L. under exposure to arsenic, lead, and copper in laboratory conditions

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Abstract:

Metal contamination and pollution are of human and environmental concern. Phytoremediation is one of the suitable high-efficiency means to treat metal pollution. This study aims to observe the responses of *Pisum sativum* L. in its early life stage to three metals, arsenic (As), copper (Cu) and lead (Pb) in laboratory conditions. Seeds of *P. sativum* were treated with water containing 0, 50, and 500 µg/l of these metals over a period of 7 days. The results show that the germination of seeds is similar for the control and metal treatments, ranging from 90-100% after 4 days of watering. Shoot development of the seeds exposed to the metals and the control were not significantly different, except that the samples which had undergone the treatment with 500 µg Pb/l had longer shoots. Our results evidence a high capacity for metal tolerance in this plant in the early stages of its life. Therefore, *P. sativum* may be a promising candidate for the phytoremediation of metal contamination and pollution.

Keywords: metals, phytoremediation, *Pisum sativum* L., tolerance.

Classification number: 2.3

Introduction

Heavy metals are wide distributed in many different habitats, such as the soil, atmosphere, and water, and have important functions in biota. The emission of heavy metals into the environment is due to two major causes, human activities and natural geology. Artificial metal emissions are mainly related to combustion, mining, and processing [1]. In addition, other applications such as fertilisers, pesticides, irrigation water, and atmospheric deposition also contribute to heavy metal emissions. Recently, there has recently been abundant proof of environmental pollution caused by trace metals, for example, in the Moon and Shi rivers in Thailand, which were polluted by cadmium (Cd) exceeding the WHO limit [2], and the Gali river in Malaysia, which was heavily polluted by iron (Fe) at a concentration of up to 14,400 µg/l [3]. In addition, heavy metal pollution in northern Vietnam, particularly in Hung Yen province, including Pb and Cd in the soil at concentrations of up to 3,809 µg/g of soil, exceeds the safety standards of Vietnam [4]. Additionally, soil in northern Vietnam is also contaminated with As at high concentrations of up to 31 µg/g [5]. The same authors recorded trace metals, such as As, Cu, Pb, Cd, and zinc (Zn), at high concentrations in the Red river. In southern Vietnam, enrichment by heavy metals, including Cd, chromium (Cr), Cu, Ni, Pb, and Zn, in Thi Vai river and Can Gio mangroves has been noted [6]. In particular, Cu, Pb, Cr, nickel (Ni), and Zn concentrations were in excess of the Vietnamese safety guideline values. In the Mekong delta region, groundwater was contaminated with high concentrations of As, over 500 µg/l [7], presenting a serious health risk to local people.

In order to cope with such heavy metal contamination, physical and chemical methods of treating heavy metals in water and soil have been considered and applied. On the other hand, one safe and inexpensive method is to use plants as a means of absorbing heavy metals from the environment, a process referred to as phytoremediation.

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Phytoremediation removes environmental pollutants by means of a variety of mechanisms. The two most reliable mechanisms are phytoextraction and phytostabilisation [8]. Many plants can tolerate the toxicity of metals and reduce the mobility and bioavailability of metals in the roots and stems. Phytoremediation depends on the structure of the plant genome, as well as on the level of pollution and climatic conditions [9].

Thus far, there have been a number of studies on using plants to treat for heavy metals. While the plant *Psoralea pinnata* can accumulate up to 68% of Cr and 55% of Fe in its mass [10], another one, *Syngonium podophyllum*, was used to remove As from the soil; the treatment efficiency was 2.6 mg/m² of soil after 90 days [11]. In addition, the treatment of soil contaminated with 1,400 mg/kg of As with the fern (*Pteris vittata*) reached 18% after 6 months [12].

Green peas, *Pisum sativum*, are a member of the vine family, and can reach up to 2.7 m in length [13]. Green peas are grown around the world, the largest producers of green peas being China, India, Russia, France, and the United States [14]. The plant can thrive in many types of soil; however, the most suitable soil type is fertile and well-drained soil. The green pea plant can tolerate high heat amplitudes, withstand temperature from 12-25°C and develop in soil with a pH of 5.5-7 [13].

Metal pollution is becoming a serious problem in the world in general, and in Vietnam in particular. Studies have been conducted to counter this situation, and phytoremediation has been shown to be an efficient treatment model, showing feasibility with some plants. However, to our knowledge, no studies have been conducted with green peas. Hence, this study was conducted to investigate the germination, growth ability, and potential resistance of *Pisum sativum* in an environment exposed to As, Cu, and Pb.

Materials and methods

The seeds of *Pisum sativum* L. used for the investigation were purchased from Trang Nong Store, located in District 6, Ho Chi Minh city, Vietnam. The experiment was implemented in the Ecotoxicology Module, Laboratory of Environmental Analysis, Ho Chi Minh city University of Technology. The metals As, Pb, and Cd (for ICP/MS, Merck, Germany) used for the test were in stock solution of 1,000 mg/l.

For the experiment, the seeds were exposed to metals (As, Pb, and Cu) at concentrations of 0 (control), 50 µg/l, and 500 µg/l. The metal concentrations in the experiments were selected based on the Vietnamese regulation 39:2011/MONRE - a national technical regulation on the quality of water used for irrigation [15]. For each concentration of

exposure, 10 seeds were laid on tissue paper in a plastic container and three replicates (n=3) for each treatment were prepared at the start of the tests. The seeds were watered daily (~ 6 ml) with distilled water only (control) or water containing trace metals at the concentrations mentioned above. The tests lasted for 7 days. During the first four days of the experiment, the germination of the seeds in each exposure was observed and recorded. When the tests terminated, the seedling in each treatment was weighed, and its shoots were measured exactly with a ruler, to 0.1 mm.

The Kruskal-Wallis test, Sigmaplot version 12, was used for evaluating the significant differences on in the fresh weight (FW) and shoot length of the control and metal-exposed seedlings.

Results and discussion

Effects of metals on the germination rate of *Pisum sativum*

The results demonstrated that the germination rate of *Pisum sativum* in the control sample reached 100% after 4 days of incubation. In addition, the rate of germination of the peas was relatively high in all the exposure samples in the same period of time. Specifically, in the first four days, 97% of the seeds sprouted in the As50 plot and 94% in the As500 plot (Table 1). For those exposed to Cu, the peas' germination rate was 91% and 93%, respectively, in Cu50 and Cu500 (Table 1). Finally, in the plots exposed to Pb, the germination rate was 90% in the Pb50 and - notably - 100% in the Pb500 (Table 1).

Table 1. Seed germination ratio (%) of *Pisum sativum* after 4 days of incubation.

Control	As50	As500	Cu50	Cu500	Pb50	Pb500
100	97	94	91	93	90	100

Pisum sativum had similar germination rate when exposed to all three metals. In a study by Kunjam, et al., the rate of germination of *P. sativum* exposed to Cu at 20,000 µg/l still reached 100%, and there were only certain negative effects when the Cu concentration exceeded 60,000 µg/l [16]. Unfortunately, the data on the germination rates of seeds exposed to Pb and As could not be compared to academic references due to the lack of published studies. In excess concentrations, as in this study, the *P. sativum* germination rate conclusively indicated normal germination. It was also demonstrated that the resistance of *P. sativum* in the first four days was extremely stable for the individually exposed concentrations of As, Cu, and Pb.

Effects of metals on the fresh weight of *Pisum sativum*

Regarding the fresh weight of the peas after 7 days

of exposure to the three metals, the results showed no statistically significant differences, although there was generally a slight decrease in the fresh weight of the exposed peas compared to the beans in control plot. In the control plot, the mean fresh weight of the beans was 0.69 g, while the mean fresh weight of the beans in the As50 exposure plot was 0.62 g, and in the As500 exposure plot it was 0.63 g (Fig. 1). For the plots exposed to Cu, the mean fresh weight of the peas was 0.62 g and 0.59 g for the plots of Cu50 and Cu500, respectively (Fig. 1). The beans exposed to Pb had a mean fresh weight of 0.61g in the Pb50 plot and 0.68 g in the Pb500 plot (Fig. 1). Compared to the control plots, the p values of these fresh weight mean values was always greater than 0.05; as a result, there is no statistically significant difference.

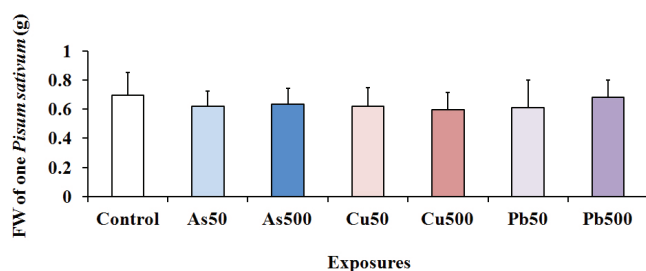


Fig. 1. Fresh weight of *Pisum sativum* after 7 days of incubation.

The results also showed that when distilled water and metal-exposed water was used, there was no significant difference in the harvesting parameters of the fresh weight of peas at 50 and 500 $\mu\text{g/l}$ exposure concentrations. This leads to the conclusion that after a week of development, *P. sativum* shoots had an appreciable resistance to all three heavy metals. On the other hand, the resistance revealed the potential absorption of these metals into the shoot, which requires further investigation. It is important to note that studies of the fresh weight of *P. sativum* exposed to As, Cu, and Pb are not very popular, consequently there is no specific source reference.

Effects of the metals on the development of shoot length

The shoot length of *P. sativum* after one week of incubation showed a significant difference in the Pb500 plot ($p < 0.05$), though there was no statistically significant difference in the other plots compared to the control. Specifically, the control plot resulted in a mean shoot length of 28.3 mm, which was not much different from the values of 28.2 mm and 25.9 mm in the As50 and As500 plots, respectively (Fig. 2). Where *P. sativum* was exposed to Cu, for the Cu50 plot, the average shoot length of the beans was 28.857 mm; while in the Cu500 plot, the value was 31.7 mm, which was slightly longer (Fig. 2).

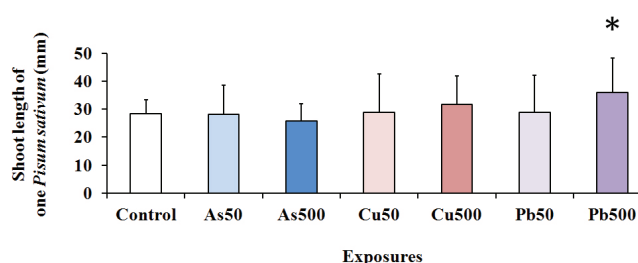


Fig. 2. Shoot length of *Pisum sativum* after 7 days of incubation. Asterisk indicates the significant difference between the control and exposures by means of the Kruskal-Wallis test ($*p < 0.05$).

With regard to shoot development, previous studies on other plants such as *L. leucocephala*, *B. oleracea*, and *A. esculentus* have demonstrated that shoot growth was more or less influenced by metal exposure [17, 18]. However, for *P. sativum*, the toxic effects of all three heavy metals did not affect shoot prolongation. This is different from the results of another study of shoot extension, which showed that exposure to Cu at concentrations as high as 20,000 and 40,000 $\mu\text{g/l}$ resulted on shoot stimulation [16]. The most likely causes for this may be due to the bound to active sites of enzymes, cell structure metabolism, or the cell division mechanism of *P. sativum* being highly adaptable to As, Cu, and Pb contaminants. It was also demonstrated the shoot development stimulation in the exposure to Pb500. To better understand this, investigations of the mechanisms of heavy metal absorption and processing in *P. sativum* are highly recommended. Moreover, further research ought to be conducted on the likelihood of metals and their concentrations stimulating the growth of shoots.

During the early stage of life, plants are usually highly sensitive to contaminants. The results showed that, at the concentration of three metals used in this study, there was almost no negative effect on the subjects. Therefore, it can be deduced that this plant is tolerant of these three metals, and thus can be considered potential resource for reducing metallic environmental pollution. Therefore, further research and empirical work on this are highly recommended, especially to explore the exploitation of the ability of *P. sativum* to overcome pollutants from contaminated sites by means of the phytoextraction mechanism. More specifically, future studies should include the exploration of the tolerance of *P. sativum* to high concentrations of metals, heavy biomass, and metal accumulation, as well as its ability to grow rapidly and its profuse root system.

Conclusions

We found that the three metals, As, Cu and Pb, at concentrations of up to 500 $\mu\text{g/l}$ did not negatively affect the germination and shoot development of *Pisum sativum* over a period of 7 days. This demonstrates the high capacity for

tolerance of this plant to the metals even in its early stage of life. Therefore, the plant shows good potential for use as a candidate for the phytoremediation of metal contamination and pollution. Further investigations of the responses of this plant to a combination of metals are suggested.

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The authors declare that there is no conflict of interest regarding the publication of this article.

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Temperate fruits in the northern mountainous region: scientific results achieved and solutions for development in coming years

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Abstract:

Perennial fruit crops generally and temperate fruits in particular make an important contribution to the sustainable development of the socio-economy in the northern mountainous region of Vietnam where the living standard of local farmers - 40% of whom are minorities from 30 groups - is still low (about 70% of the average of the country as a whole), though significant assistance has been made by the government and a slowly developed socio-economy has been recorded. It is obvious that proper temperate fruit production not only benefits local growers but also protects the environment in a significant way. Recent results concerning varietal selection and cultivation technologies for temperate fruits are summarized in this paper and some of the principal solutions, including proper planning, breeding programme enhancement, the establishing of production and value chains, and the training of skillful technicians and producers that is aimed at promoting production in the future, are also proposed.

Keywords: chilling units, northern mountainous region, temperate fruits.

Classification number: 3.1

The role and necessity of temperate fruit development in the northern mountainous region of Vietnam

The fourteen provinces in the northern mountainous region of Vietnam cover an area of more than 10 million hectares - 31% of the country - and have a population of 13,291,000. Forty percent of these people belong to 30 minority groups. The northern mountainous region is considered to be the most difficult one, contributing only 9.6% to GDP. The living standard of the local people is only approximately 70% if that of the country as a whole. The topography of the region is complex and is divided into various ecological sub-regions that comprise diverse biological and zoological systems. The results of recent investigations of the frontier demonstrate that 542 plant species exist in the mountainous north of which 13 species are included in the world plant red book.

In recent years, the Vietnamese government has paid great attention to investment in the mountainous regions in general, and in the northern mountainous region in particular, with the aim of socio-economic improvement and poverty eradication. Many variously prioritized national programmes have undertaken investment for the exploitation of local advantages in order to increase the efficiency of land use and to improve agro-forestry production, sustainable economic development, and to protect significant natural resources in the region, among other goals. Primary achievements relating to commodity production enhancement and improving the living standards of local people have been obtained. It is, however, obvious that these achievements are not in proportion to the capacity of the region, and the potential advantages of the mountainous north - such as the availability of vast tracts of land, a large labour force, and a diverse biology and climate - have not been adequately exploited.

Up to now, the agricultural system, including the crop structure, in this region is still a strategic problem that needs to be reconsidered and settled in a sustainable way. Apart

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from forestry, tea, and medicinal plants in small cultivated areas are regarded as the main crops in the region. A variety of issues that have been raised in the cultivation of other leading crops - such as the correlation of maize with soil erosion, and the viability of coffee with the appearance of frost and rubber in relation to strong winds - must be taken into account.

Perennial fruit crops and temperate fruit crops particularly are quite diverse in the region and have some advantages compared to other annual crops, especially in terms of environmental protection and the benefits. The proper development of temperate fruits can both effectively exploit the available soil and significantly improve the living standards of local farmers whose lives currently meet with difficulties.

In fact, in line with a long-standing tradition of agricultural production, some specific cultivated areas for temperate fruits have been established and developed, for example, Sa Pa (peach and plum), Bac Ha (Tam Hoa plum), Moc Chau (plum), Ngan Son (pear), Nguyen Binh (pear), Ham Yen (king mandarin), Bac Quang (king mandarin and orange), Bac Son (mandarin), Doan Hung (pumelo), and Yen Chau (mango). It is, however, obvious that production of temperate fruits in the northern mountainous region encounters many difficulties. These can be summarized as follows:

- Local cultivars that are considered the leading ones in the current production of temperate fruits in the region have mostly degenerated and are characterized by low yield and quality, while a limited quantity of promising varieties that have been introduced are cultivated on a small scale (Table 1).

- Temperate fruit development is currently not adequately planned, says, species and varieties are not selected according to ecological requirements generally and chilling demand in particular, resulting in minimal benefits earned by producers and unsustainable production.

- Cultivating technologies to be disseminated to farmers for application in temperate fruit production are in many cases not suited to the actual conditions, particularly post-harvest treatment and processing.

In addition, the lack of close links between fruit producers and traders in production and value chains has also negatively affected temperate fruit development in the region.

In order to improve temperate fruit development in the mountainous northern region and meet increasing demands for local consumption, the study on varietal programmes and advanced technologies is necessary and should be given priority.

Table 1. Main cultivars of temperate fruits recently cultivated in the northern mountainous region [1].

No	Cultivars	Main characteristics				
		Fruit weight (g)	Yield (T/ha at 6 years old)	Harvest time	Taste of pulp	Fruit outside colour
I	Peach [<i>Prunus persica</i> (L.) Batsch.]					
1	SunRay	60-90	4.5-6.2	Apr-May	Crisp, sweet	Pink, hairless
2	Sun Wright	60-90	3.5-5.0	Apr-May	Crisp, sweet	Pink, hairless
3	MaRaVilHa	60-90	3.5-5.0	Apr-May	Soft, sweet	Pinkish yellow
4	Flordaprince	70-100	9.6-11.0	Apr-May	Soft, sweet	Pinkish yellow
5	HaKuTo	75-110	6.8-7.0	Apr-May	Soft, sweet	Whitish green
6	ViViAn	70-100	7.0-10	Apr-May	Acidulous	Whitish green
7	DCS1	70-100	9-12.0	Apr-May	Soft, sweet	Pinkish yellow
8	D1	70-100	6.8-7.0	Apr-May	Soft, sweet	Pinkish yellow
9	Yun Nan	75-120	9.6-11.0	May-Jun	Soft, sweet	Pinkish green
10	Local peach	50-70	10-12	Jun-Jul	Soft, sour	Pinkish
II	Persimmon (<i>Diospyros kaki</i> L.)					
1	Jiro	200-350	10-15	Aug-Sept	Crisp, sweet	Yellow
2	Fuyu	200-350	10-15	Sept-Oct	Crisp, sweet	Yellow
3	Unknown	200-350	15-20	Oct-Nov	Soft, sweet	Pink
4	Quan Ba	40-90	6-12	Sept-Oct	Crisp, sweet	Yellow
5	Luc Yen	60-120	7.5-12.5	Sept-Oct	Crisp, sweet	Yellow
III	Pear [<i>Pyrus pyrifolia</i> (Burm. f.)]					
1	Tai Nung	200-350	10 -15	Jun-Jul	Sweetish	Yellowish green
2	Heng shan	150-300	11-15	Jun-Jul	Acrid	Brown
3	Jin hua	100-300	2.5-3.0	Jun-Jul	Sweetish	Yellowish green
4	Shi suan	200-350	7.5-8.5	Jun-Jul	Sweet, juicy	Yellow
5	No.18-19	200-350	10-15	Jun-Jul	Sweetish	Brownish yellow
6	Su li	200-350	3.5-5.5	Jun-Jul	Sweetish	Brownish yellow
7	VRQ 01	200-250	6-9	Jun-Jul	Sweetish	Brownish yellow
8	VRQ 02	200-350	7-11	Jun-Jul	Sweetish	Brownish yellow
9	VRQ 03	200-250	7-10	Jun-Jul	Sweetish	Brownish yellow
10	DL19	220-420	8.5-9.4	Jun-Jul	Sweet	Brownish yellow
11	DL20	200-360	10-15	Jun-Jul	Sweet	Brownish yellow
12	DL21	200-360	10-15	Jun-Jul	Sweetish	Brownish yellow
13	Shang khe	350-800	4.2- 5.0	Jun-Jul	Sweetish, juicy	Brownish yellow
14	Huang hua	200-350	7.5-8.5	Jun-Jul	Sweetish	Greenish yellow
15	Jin suan	180-300	3.5-5.5	Jun-Jul	Sweetish	Yellowish green
16	SaPa	180-300	8 -10	Jun-Jul	Sweetish, acrid	Green
17	Shi suan 2	200-350	7.5- 8.0	Jun-Jul	Sweetish	Yellow
18	Tai Nung 06	200-350	10 -15	Jun-Jul	Sweetish	Yellowish green
19	Bac Ha macot	30- 50	10-15	Aug-Sept	Hard, acrid	Brown
20	SaPa macot	20 - 40	9-13	Aug-Sept	Hard, acrid	Brown
21	Ha Giang pear	250-450	10- 13	Jul-Aug	Hard, acrid	Brown
22	BV1	300-550	10-15	Jun-Jul	Hard, acrid	Greenish yellow
23	BV2	210-380	10-15	Jun-Jul	Hard, acrid	Yellowish green

Summarized results of recent studies on temperate fruits in the northern mountainous region

Studies on varietal selection and breeding programmes

For years, scientific research works have focused on the evaluation of the adaptability of local and introduced temperate fruit cultivars in different locations in the northern mountainous region, from which promising ones have been

selected for further screening before being cultivated at a large scale. The results of such studies on these aspects, as conducted by variously related institutions, can be summarized as follows:

Local and introduced varieties of temperate fruits collected and tested in the conditions of northern mountains are quite diverse. Some of the promising cultivars from these collections have been evaluated in-depth and screened for large-scale production in appropriate locations.

+ For pears: 18 varieties imported from China and Taiwan in 2001 and six introduced from Taiwan in 2013 have been tested in Lao Cai, Ha Giang, Son La, Bac Kan, and Lang Son, of which the BV1, BV2, and Heng Shan cultivars/varieties, primarily, are considered to be promising [2-5]. In addition, some local cultivars/varieties, such as Trang Dinh pears and Pho Bang pears were also studied and selected as acceptable.

+ For peaches: six local cultivars - Early Yunnan, Late Yunnan, Trau, Tuyet, Tay, and Thoc - and six introduced ones, including Early Grand, Flordaprince, Tropic beauty, Sunwright, and 90-3 NW, were evaluated. Of these, the Early Yunnan and Trau cultivars were considered to have good agricultural characteristics and were selected for further screening.

+ For plums: results from studies conducted on varietal selection showed that in the collection of seven local plum cultivars and three introduced ones, two local cultivars - Com (harvested early) and Tim (harvested late) - were considered to be promising in terms of their high yield, good quality, and insect resistance, *Dacus dorsalis*, while Simca and Blackamper (introduced cultivars) would not flower because of high chilling requirement.

+ For persimmons: although many varieties of local persimmon have been traditionally cultivated in small-scale farmer orchards in the northern mountainous region, none of these has been considered as a commercial cultivar since they are all astringent and the area for the cultivation of persimmons is very limited. As regards quality, some local cultivars, such as Hac Tri, Bac Kan, and Quan Ba have been selected for further screening. In addition, some non-astringent persimmon varieties that have been introduced from outside (e.g. from Japan and Taiwan), including Fuji and Jiro, have been evaluated of their adaptability to northern mountainous conditions; however, results have also been quite limited.

Studies of cultivating technologies

Compatibility of introduced cultivars grafted onto local rootstocks:

In accordance with the idea of importing suitable varieties from outside, the study of the compatibility of introduced cultivars used as scions and local rootstock cultivars should be carefully implemented. The information

that follows has been taken from the co-operative planting material exchange project involving Taiwan and Vietnam conducted by VAAS in 2013-2016 [4].

- For persimmons: when grafted on aged fruit trees, a high percentage of shoot survival, ranging from 77.5 to 100.0%, was recorded for all four introduced cultivars of persimmon. In terms of the ratio of surviving shoots, a short duration from grafting to bud emergence, and good growth of the shoots grafted, the best results were reported for Hiratanenashi and Tone Wase.

When grafted on the local rootstock cultivars Phu Tho trung persimmon and Sa Pa persimmon, a percentage of shoot survival for all four introduced varieties varying from 76.5 to 82.8% was recorded. Better results were observed for the Nishimura and Tone Wase cultivars.

- For peaches: when grafted on the local rootstock cultivars Thoc peach and Son La peach, good results were observed for all four introduced peach cultivars. The shoot survival ratio was 80% or more and no significant difference was recorded between cultivars and locations. For the grafting on seedlings, the rootstock cultivars mentioned above were used and the percentage of grafted shoot survival obtained was 84.5 to 88.7%. It is clear that all of the four introduced cultivars have good compatibility with the local rootstock. Tropic beauty gave the best results and A 2-2-39 ranked second, a bit better than Flodared and B115.

- For pears: when grafted on aged trees of the Tai Nung pear cultivar that had been budded from local rootstock seedlings, good compatibility indicated by high ratio of shoot survival was recorded for all of the introduced varieties. The best results were observed for the Mi Xue and Heng Shan cultivars (more than 95%) compared to the others (about 93% of shoots survived). For grafting on seedlings, the same situation was reported, with the good compatibility of the introduced varieties with the local ones Big fruit Macoc and Small fruit Macoc indicated by the high percentage of grafted shoot survival observed in both implementing locations, Moc Chau and Sa Pa (94.5% and more than 80%, respectively).

In other studies [6], the same results were obtained. Tran Thanh Toan, PPRI concluded that the local Thoc peach cultivar could be used as rootstock for introduced ones because of good compatibility and fast growth; while Ha Quang Thuong NORMAFI has stated that the local persimmon cultivar, Trung Lap Thach, is regarded as good rootstock cultivar for grafting with a range of scion varieties, both local and introduced ones.

Growth and productivity of grafted shoots of introduced cultivars:

Apart from the compatibility of scion-rootstock cultivars, the growth of introduced varieties was also evaluated according to the health of the grafted shoots, as indicated

by two principal criteria: the diameter, measured at a site close to grafting point, and its length. The datas presented in Table 2 can be summarized as demonstrating that all the varieties introduced from Taiwan showed relatively good growth and that no significant difference in the growth of the same cultivars tested in various locations was recorded.

Table 2. Diameter (D) and length (L) of grafted shoots of introduced cultivars in different locations (cm) [4].

Persimmon			Peach			Pear		
Cultivar	D	L	Cultivar	D	L	Cultivar	D	L
Bac Ha, Lao Cai								
Hiratone Nashi	0.34	29.6	B115			Mixue	2.23	94.5
Tone Wase	0.30	28.6	Flordared			Heng shan	2.16	90.5
NishimuraWase	0.39	31.7	Tropic Beauty			Ming Fu	2.01	88.2
Mackawa Jiro	0.41	33.5	A2-2-29			Jin xian	1.56	76.2
						Zhi Zi	1.73	82.2
						GaoQiang	1.85	87.9
Sa Pa, Lao Cai								
Hiratone Nashi	0.30	25.3	B115	4.45	140.2	Mixue	4.25	148.2
Tone Wase	0.30	24.1	Flordared	3.97	136.5	Heng shan	3.97	139.5
NishimuraWase	0.32	22.9	Tropic Beauty	4.93	148.6	Ming Fu	3.83	132.6
Mackawa Jiro	0.36	25.7	A2-2-29	4.82	152.5	Jin xian	3.47	118.5
						Zhi Zi	3.54	123.7
						GaoQiang	3.67	130.6
Dong Van, Ha Giang								
Hiratone Nashi	0.31	31.6	B115	4.23	143.6	Mixue	4.04	144.5
Tone Wase	0.30	29.6	Flordared	3.84	137.2	Heng shan	3.66	135.3
NishimuraWase	0.33	34.7	Tropic Beauty	4.71	146.1	Ming Fu	3.52	130.2
Mackawa Jiro	0.35	36.5	A2-2-29	4.56	155.4	Jin xian	3.28	115.6
						Zhi Zi	3.15	121.8
						GaoQiang	3.35	128.1
Ngan Son, Bac Kan								
Hiratone Nashi	0.34	28.5	B115	4.03	99.8	Mixue		
Tone Wase	0.34	27.1	Flordared	3.89	10.2	Heng shan		
NishimuraWase	0.36	25.8	Tropic Beauty	4.23	10.2	Ming Fu		
Mackawa Jiro	0.41	28.9	A2-2-29	4.41	9.6	Jin xian		
						Zhi Zi		
						GaoQiang		
Trang Dinh, Lang Son								
Hiratone Nashi	0.38	33.3	B115	3.98	93.4	Mixue	2.70	94.5
Tone Wase	0.34	32.2	Flordared	4.02	104.3	Heng shan	-	-
NishimuraWase	0.44	35.7	Tropic Beauty	4.11	101.2	Ming Fu	-	-
Mackawa Jiro	-	-	A2-2-29	4.27	98.7	Jin xian	2.02	83.3
						Zhi Zi	-	-
						GaoQiang	2.27	86.3
Moc Chau, Son La								
Hiratone Nashi	0.28	38.3	B115	3.98	63.13	Mixue	4.04	74.8
Tone Wase	0.29	35.1	Flordared	4.02	57.25	Heng shan	3.66	76.6
NishimuraWase	0.30	29.6	Tropic Beauty	4.11	5.5	Ming Fu	3.52	43.7
Mackawa Jiro	0.27	30.3	A2-2-29	4.27	6.63	Jin xian	3.28	34.6
						Zhi Zi	3.15	57.9
						GaoQiang	3.35	78.9
Don Duong, Lam Dong								
Hiratone Nashi	2.98	68.6	B115			Mixue		
Tone Wase	2.60	38.2	Flordared			Heng shan		
NishimuraWase	2.37	55.6	Tropic Beauty			Ming Fu		
Mackawa Jiro	2.98	-	A2-2-29			Jin xian		
						Zhi Zi		
						GaoQiang		

The yields (kg/tree) of the introduced cultivars presented in Table 3 were the means of the same cultivars grafted on 4-6 year old trees grown in two crops in different locations.

It should also be mentioned that the yield and quality of the perennial fruits, including persimmon, peach, and pear, should significantly improve with 10 to 12 years of further growth.

Table 3. Yields and fruit characteristics of introduced cultivars [4].

Cultivars	Yields (kg/tree)		Fruit characteristics (means)			
	Min	Max	Diameter (cm)	Length (cm)	Weight (g)	Brix (%)
Persimmon						
Hiratanenashi	12.8	19.3	6.1	6.5	128.0	19.3
Tone Wase	11.7	16.1	6.6	5.3	142.7	16.1
Nishimura Wase	14.7	20.2	5.8	4.6	94.6	20.2
Mackawa Jiro	15.6	21.3	7.2	4.6	254.7	21.3
Peach						
B115	6.6	8.4	5.2	4.7	71.3	11.8
Flordared	6.0	7.0	6.0	4.5	82.1	9.7
Tropic beauty	6.9	8.9	4.8	4.6	68.3	9.2
A2-2-39	6.6	8.9	5.7	5.3	91.2	11.5
Pear						
Mixue	6.2	10.2	8.1	7.4	295.2	13.2
Heng Shan	3.5	5.8	6.3	5.5	263.7	12.1
Ming Fu	-	-	-	-	-	-
Jin Xian	4.5	6.5	6.6	5.8	145.3	11.3
Zhi Zi	5.7	8.7	6.3	5.2	130.7	10.4
Gao Qiang	4.3	5.3	6.8	5.8	164.3	11.2

Based on the abovementioned criteria relating to the productivity and quality of the cultivars studied, general considerations can be summarized as follows: i) almost all the cultivars introduced from Taiwan, except Ming Fu (pear), can flower and fruit in the first and second year after top worked on aged fruit trees; and ii) of all the cultivars studied, Mackawa Jiro (persimmon), B115 and A2-2-39 (peach), and Mi Xue and Heng Shan (pear) can be considered the promising ones.

Training and pruning technologies:

In the past, local farmers cultivated temperate fruits without training or pruning, resulting in low quality products. This is the reason that these techniques have received great attention in recent research. Tables 4 and 5 below provide some examples that prove their impact on the growth and productivity of temperate fruits grown in northern mountainous region.

Table 4. Effects of training and pruning on the growth of pears [1].

Location	Treatment	Tree age	Trunk diameter (cm)	Canopy diameter (m)	Tree height (m)
Sa Pa, Lao Cai	T1	3	6.02	1.56	1.96
	T2	3	6.86	2.23	2.18
	T3	3	5.17	1.14	2.10
Dong Van, Ha Giang	T1	3	5.54	1.26	1.66
	T2	3	6.32	2.11	1.82
	T3	3	4.96	1.04	1.76
Sin Ho, Lai Chau	T1	3	5.32	1.12	1.52
	T2	3	6.17	2.04	1.72
	T3	3	4.82	0.98	1.67

T1: palmate training with three prunings per year; T2: open vase training with three prunings per year combined with bending; T3: (control) natural canopy without pruning.

Table 5. Effects of training and pruning on the productivity of pears [1].

Locations	Treatment	Tree age	Fruit length (cm)	Fruit diameter (cm)	Fruit weight (g)	Fruit quantity/ tree	Yield (kg/ tree)
Sa Pa, Lao Cai	T1	3	7.02	6.77	319.3	22.5	9.65
	T2	3	7.16	6.97	322.5	40.7	14.70
	T3	3	6.84	6.61	303.7	11.9	3.64
Dong Van, Ha Giang	T1	3	6.96	6.65	300.3	16.7	8.51
	T2	3	7.10	6.91	321.5	33.6	13.40
	T3	3	6.76	6.56	313.4	7.5	2.35
Sin Ho, Lai Chau	T1	3	7.05	6.69	311.3	17.6	7.28
	T2	3	7.03	6.87	318.5	31.1	12.50
	T3	3	6.78	6.58	303.7	8.2	2.50
<i>LSD_{0.05}</i>							3.1
<i>CV (%)</i>							10.8

Soil moisture management in temperate fruit orchards:

Water resources used for irrigation are one of main constraints for agricultural production, including for fruit crops. Technologies that save water and limit soil evaporation have, therefore, received significant attention. In the case of perennial crops, intercropping and mulching are considered effective and feasible (Tables 6 and 7). The examples in the tables (one for pears and one for persimmons) demonstrate the impact of these techniques on temperate fruit growth and productivity.

Table 6. Effects of mulching and intercropping on the yield of pears [1].

Location	Treatment	Tree age	Fruit length (cm)	Fruit diameter (cm)	Fruit weight (g)	Fruit quantity/ tree	Yield (kg/ tree)
Sa Pa, Lao Cai	T1	3	7.22	6.95	327.5	51.3	16.8
	T2	3	7.04	6.87	320.3	48.4	15.2
	T3	3	6.87	6.65	307.6	40.9	12.6
Dong Van, Ha Giang	T1	3	7.15	6.87	327.5	47.4	15.5
	T2	3	6.96	6.78	320.3	43.2	13.1
	T3	3	6.81	6.59	307.6	35.9	11.0
Sin Ho, Lai Chau	T1	3	7.09	6.85	327.5	45.6	14.9
	T2	3	6.95	6.76	320.3	41.3	13.2
	T3	3	6.75	6.57	307.6	32.1	9.9
<i>LSD_{0.05}</i>							1.2
<i>CV (%)</i>							5.6

Table 7. Effects of watering method, intercropping, and mulching on the yield of persimmons [5].

Location	Treatment	Fruit weight (g)	Fruit quantities/ tree	Yield (kg/tree)		% compared to control
				Calculated	Harvested	
Hac Tri	I	83.40a	278.50a	23.20b	20.97b	100.00
	II	90.74a	304.11a	27.60a	24.99a	119.18
	III	87.89a	297.05a	26.08ab	24.19a	115.36
	IV	89.82a	298.91a	26.79ab	24.80a	118.29
	<i>LSD_{0.05}</i>	6.92	36.32	3.27	2.33	-
	<i>CV%</i>	3.9	5.9	6.3	4.9	-
Quan Ba	I	33.14a	799.88b	26.49b	24.65b	100.00
	II	35.04a	861.20ab	30.18a	27.92a	113.27
	III	34.52a	848.32ab	29.24a	27.04a	109.69
	IV	34.99a	881.00a	30.83a	27.62a	112.04
	<i>LSD_{0.05}</i>	2.93	65.74	2.72	2.13	-
	<i>CV%</i>	4.3	3.9	4.7	4.0	-
Dien Bien	I	134.23b	191.21a	25.73b	23.60b	100.00
	II	143.66a	199.49a	28.66a	26.52a	112.36
	III	141.40ab	199.16a	28.13a	26.14a	110.77
	IV	143.01ab	198.71a	28.40a	26.25a	111.21
	<i>LSD_{0.05}</i>	9.36	18.39	2.27	1.98	-
	<i>CV%</i>	3.3	4.7	4.1	3.9	-

Notes: the values followed by different letters in the same columns indicate that the differences are significant at 5% probability. I (control): no irrigation, no mulching; II: irrigated at fixed stages: when flower buds emerged, 2-3 weeks after fruit setting, 2-4 weeks before harvest (40 litres/tree/one time); III: mulched with straw, dry leaves of 1.5 cm thickness; IV: intercropped with legume crops.

Scientific solutions to promote future temperate fruit development

In order to sustainably develop temperate fruits in the northern mountainous region, the following key issues should be taken into account:

- + Determining species and good varieties to be grown properly and effectively in various locations in the northern mountains, with attention paid to chilling requirements (Table 8).

- + Completing and developing suitable techniques for multiplication (both seedlings and TOP grafting) to be applied to leading cultivars of temperate fruits cultivated in the northern mountainous region.

- + Completing and developing advanced cultivation technologies that suit local conditions and the biological characteristics of varieties grown to meet increasing consumer demand. The bending technique should accordingly be considered and introduced into production [7].

- + Establishing small- and medium-scale pilot demonstrations of temperate fruit production to link scientists, producers, managers, and trader by means of production and value chains.

- + Training skilled technicians and local famers in advanced technologies for temperate fruit cultivation.

Table 8. Temperate fruits planned commercially in different locations in the northern mountainous regions.
CU: chilling unit.

No.	Location	Altitude (m)	Mean temp. of coldest month		CU	Species to be grown
			Max.	Min.		
1	Sa Pa, Lao Cai	1,580	12	6	616	Peach, pear, plum, persimmon
2	Bac Ha, Lao Cai	930	17	8	322	Peach, pear, plum, persimmon
3	Sin Ho, Lai Chau	1,530	14	6	522	Peach, pear, plum, persimmon
4	Tam Duong, Lai Chau	960	18	9	256	Peach, pear, plum, persimmon
5	Moc Chau, Son La	970	17	9	281	Peach, pear, plum, persimmon
6	Pha Din, Dien Bien	1,380	16	9	316	Peach, pear, plum, persimmon
7	Mu Cang Chai, Yen Bai	960	20	9	278	Peach, pear, plum
8	Dong Van, Ha Giang	1,480	17	7	568	Peach, pear, plum
9	Trung Khanh, Cao Bang	520	18	9	291	Peach, pear, plum, persimmon
10	Nguyen Binh, Cao Bang	490	19	10	237	Peach, pear, plum, persimmon
11	Ngan Son, Bac Kan	520	18	9	244	Peach, pear, persimmon
12	Bac Son, Lang Son	390	18	10	218	Pear

Conclusions

1. Temperate fruits in general and pears, peaches, plums, and persimmons in particular can be properly cultivated in the northern mountainous regions of Vietnam where land availability and climatic conditions are considered favourable for their requirements. It is worth mentioning that temperate fruits play an important role both in improving the living standards of local people in environmental protection.

2. The results of various studies indicate that suitable varieties of temperate fruits and cultivating technologies have been selected for application in large-scale production.

3. Accordingly, in order to develop temperate fruits in the northern mountains, the combination of good varieties and advanced techniques should be taken into account and applied appropriately.

The authors declare that there is no conflict of interest regarding the publication of this article.

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Generating *in vitro* solid tumor models on gelatin-alginate scaffolds

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Abstract:

In recent years, three-dimensional (3D) *in vitro* tumor models have become more common in cancer research across the world. Selecting scaffolds, seeding cells into the scaffolds, and culturing them are pivotal for the morphologies and proliferation of cancer cell lines. Therefore, this experience has developed a 3D tumor model based on co-culturing MCF-7 cells and fibroblasts (3:1) into gelatin-alginate scaffolds (3x3x5 mm³). Quantity, proliferation, and morphologies of tumors were evaluated through cell yield on a scaffold, enzymatic assay, cell number, hematoxylin and eosin stains (H&E), and scanning electron microscope (SEM). The results indicate that the complex of MCF-7 cell lines and fibroblasts in scaffolds proliferated and increased in terms of cell mass number and cell mass size. Their morphologies align with those of the *in vivo* figures demonstrated previously. This research facilitates new opportunities for the study of cancer biology and drug screening in Vietnam.

Keywords: co-culture, fibroblast, Gelatin-alginate scaffold, MCF-7 cell line, three-dimensional (3D) *in vitro*.

Classification number: 3.2

Introduction

Breast cancer is a leading cause of cancer deaths among women globally, following lung cancer [1]. Understanding cancer biology, definitively confirming diagnoses, and prescribing effective treatments are currently major challenges. Although it is common in cancer cell research, a monolayer culture system does not resemble characteristics of a typical tumor *in vivo*, such as adhesions and interactions among cells and extracellular matrices (ECM). Additionally,

cell proliferation and increases in gene expression, which are caused by a deposition of ECM, have also been demonstrated to decline dramatically in 2D cell culture [2]. In addition, although the organ culture remains in terms of properties such as interactions among cells, matrices, and interstitial fluids, which results in acquiring extraordinary abilities in cellular differentiation, it encounters challenges in maintaining specimens and poor viability of the tissues [3]. The three-dimensional (3D) cell culture can overcome the obstacles of the 2D cell culture system *in vitro*, in the animal tumor, and cancer model. Firstly, ECM, stromal elements, and initial avascular regions support building the models and mimicking the tumors *in vivo*. Secondly, tumor-stromal interactions can create a more realistic environment, thereby eliciting an *in vivo* condition such as responses to cancer therapies. Thirdly, 3D cultures more closely resemble cell morphologies and their surroundings, which define the characteristics of these cells in the tumor mass [3, 4].

Gelatin is a natural polymer produced by the partial hydrolysis of collagen, which is the most common component in the connective tissues of mammals [5]. Because of its thermal sensitivity, gelatin can deposit multiple layers due to a process of gel transformation into sol [6]. Gelatin has been more commonly used than collagen across the globe; collagen presents challenges in terms of creating a concentrated collagen solution from its parent substance and is also more expensive than gelatin [5]. However, gelatin has poor mechanical properties, which limits its broader potential applications; using covalent crosslinking with alginate enhances the stiffness of the model [6]. Alginate is a naturally occurring nontoxic polysaccharide found in brown algae. Alginate is hydrophilic, biocompatible, and relatively economical [7]. While original alginate does not promote adherence and attachment among cells, gelatin does promote this [6]. Therefore, the mixing between gelatin and alginate help avoid their drawbacks.

MCF-7 (Michigan cancer foundation - 7) is the most

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commonly used breast cancer cell line in the world, with T-47D and MDA-MB-231 cell lines. MCF-7 is an ideal breast cancer cell line because it expresses Estrogen receptors (ER), which are sensitive to hormone response. Therefore, models that use MCF-7 have become common in recent years [8]. MCF-7 cultured in chitosan scaffold, collagen-alginate hydrogel, agarose [9-11] achieves effective results, but these disadvantages are the limits of the culture time (about eight days) due to the death of these cells.

To improve the probability of survival, researchers often combine MCF-7 with stromal cells, such as fibroblasts, adipose cells, etc. Therefore, this study aims to create 3D *in vitro* cancer models based on tissue engineering principles by combining MCF-7 cell lines with fibroblasts on gelatin-alginate scaffolds. Fibroblasts are used because they are the most abundant element in connective tissues. In addition, they secrete extracellular components to form a tissue scaffold [1]. Furthermore, fibroblasts have been demonstrated to be activated in cancer and create cancer-associated fibroblasts (CAF) [12], which facilitate the development, progression, invasion, and metastasis of breast cancer [1].

The connections between cell masses are the major advantage of this research; these are similar to the *in vivo* human tumors, while most three-dimensional models from previous research concerning gel often produce spherical spheres, which limit the development of tumor mass.

Materials and methods

Materials

MCF-7 cell lines, fibroblasts, and gelatin-alginate scaffolds were supplied by TEBM lab, University of Science, Vietnam National University, Ho Chi Minh city.

Trypsin/EDTA, DMEM/F12, FBS, penicillin-streptomycin, gelatin, and alginate were purchased from Sigma-Aldrich (St. Louis, MO).

Cell maintenance

Human mammary epithelial cells MCF-7 and fibroblast were cultured in Dulbecco's Modified Eagle Medium and Nutrient Mixture F-12 containing 10% FBS and 1% penicillin or streptomycin in plastic flasks to help maintain and expand them. Cells were incubated at 37°C and 5% CO₂. A sub-confluent culture (80-90% confluence) was utilized in the following experiments.

Preparation of gelatin or alginate sponges (GA sponges) as scaffolds for cells

Gelatin or alginate sponges were prepared based on the previously established protocol. Gelatin and alginate (Sigma) were completely dissolved in distilled water at 50°C to create 1% gelatin and 1% alginate solutions. These

solutions were then mixed with a ratio of 8 gelatin per 2 alginates in terms of volume and incubated at -80°C for 24 hours. These frozen blocks were then incubated in 0.3% EDC for 24 hours at 4°C in dark conditions and freeze-dried. Finally, GA sponges were sterilized by irradiation in 25 kGy and cut into small pieces of 5x5x3 mm³.

The cell culture on GA scaffold

MCF-7 breast cancer cells and fibroblasts were maintained in a T-flask in an incubator at 37°C and 5% CO₂. We used an incubation method to inject cells into scaffolds. The inoculum concentration consisted of 2x10⁴ cells and 2x10⁵ cells for all experiments involving cell culture with the ratio of 1 fibroblast per 3 MCF-7. Fibroblasts (0.5x10⁴ cells) and scaffold complexes were cultured in a 96-well dish at 37°C and 5% CO₂ for 20 minutes. Then, 200 µl were added to the dish. After three days, MCF-7 (1.5x10⁴ cells) was injected into these scaffolds and maintained at 37°C and 5% CO₂. The procedure was similar to the sample of 2x10⁵ cells.

Cell seeding efficiency was determined by counting the cell density in the supernatant.

After seeding cells into scaffolds, shaking culture was maintained for four weeks. The culture medium was changed every two days.

Control samples were used without scaffolds. First, fibroblasts (0.5x10⁴ cells) were seeded into a 96-well dish before being supplemented with a 200 µl culture medium at 37°C and 5% CO₂ for 20 minutes. Second, MCF-7 cells (1.5x10⁴ cells) were seeded in a 96 well plate three days later. The procedure is similar to the sample of 2x10⁵ cells.

Cell Proliferation Assay

After two days, the supernatant media were collected for glucose estimation. Glucose concentration in the culture supernatants was determined by enzymatic assays; the changes in optical density were measured and compared with standard solutions.

Cell morphology analysis

Scanning electron microscopy (SEM, S-3000N) and hematoxylin and eosin (H&E) staining were used to evaluate the cell morphology in the GA scaffold. First, tumor masses were washed three times with cold D-PBS and fixed with 3% Glutaraldehyde for 24 hours. The cells were then dehydrated in a range of ethanol concentrations (50, 70, 80, 90, and 100%). For H&E staining, the samples were fixed in 10% formalin and embedded in paraffin. The specimens were cut into slices and stained by hematoxylin and eosin (H&E) to analyse the morphologies.

Immunocytochemistry staining

The specimen was stained with vimentin to determine

changes in the characteristics of the tumor mass.

Statistical analysis and image processing

Statistical analyses were conducted using GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA). ($P < 0.05$), which determined statistical significance.

Images were developed by ImageJ version 1.52e (National Institutes of Health, Bethesda, Maryland, U.S.).

Results

The method of seeding MCF-7 and fibroblasts into GA scaffolds

Incubation is regarded as a common method because cell adhesion is more stable on the scaffold relative to centrifugation (Fig. 1). Although simple, this method is overly tedious and is difficult to execute in low concentrations.

We have selected a 2×10^4 cells scaffold based on the research of Pan, et al. (2016), Zheng, et al. (2012), and Dhiman, et al. (2005) [6, 9, 10]. The purpose of the increase in the number of cells to a 2×10^5 cell scaffold is to assess the cell adhesion of these tumors and form the extraordinary tumor structures. The result is that a low concentration is fairly similar to a high concentration ($p > 0.005$). Therefore, we implemented both concentrations for the following experiments.

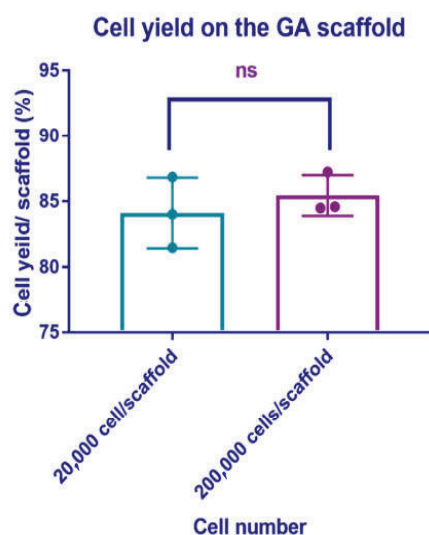


Fig. 1. The yield of cells on the GA scaffolds using a low concentration (2×10^4 cell scaffold) and a high concentration (2×10^5 cell scaffold).

The growth of MCF-7 cells on the GA scaffolds

Warburg et al. assume that tumor cells rapidly use the glucose and convert it into lactate. This is the most fundamental and enduring observation related to tumor

metabolism [6]. Therefore, the determination of the glucose concentration remaining in the solution after the culture is based on OD assay. Glucose concentration and proliferation are inversely correlated.

In the 2×10^4 cell scaffold samples (low-concentration samples), cell mass continuously proliferated to day 4 and was then stable from day 6 to day 18; it then decreased by day 22. Particularly after day 22, cell proliferation generally resurfaced but declined after day 26 (Fig. 2). This result aligns slightly with Dhiman's research, in which glucose concentration decreased dramatically by day 6 (rapid cell proliferation by day 6) [10]. In addition, Zheng, et al. (2012) have demonstrated that the decrease in glucose concentration by day 4 aligns with this experiment [9]. However, the decrease in proliferation from day 18 to 22 results from breaking through cancer cell masses and cell death, which results from long-term hypoxia in the tumor. The repeated proliferation clearly reveals that unequal nutrition distribution in the scaffold is responsible for a heterogeneous tumor in these scaffolds. Although loss occurs in some cell masses, the others begin to proliferate drastically. However, these masses decline rapidly without a stable stage. This is because of acid accumulation, which causes an unstable scaffold (weak mechanical property) and reduced cell adhesion with ECM. It has been that a GA scaffold supported adhesion and proliferation of the MCF-7 cell line in about 18 days.

The 2×10^5 cell scaffold samples (high-concentration samples) facilitated cell proliferation effectively on the GA scaffold as well; however, they did not do so more effectively than the low concentration samples. This is because the initial number of cells seeding into the scaffold was considerably excessive relative to the scaffold space and the culture medium. It is insufficient for cells to proliferate and grow; cells compete with each other, and these cells may be dead. Cell adherence to the scaffold then continuously proliferates and grows but is limited relative to the other.

The cancer cells slightly proliferated up to day 12. However, the proliferation of the tumor increased dramatically from day 12 to day 18 and had then declined by day 22. After day 22, its figure resembled a low concentration sample. The extremely high cell concentration caused a vast number of cells to not create masses, and the shortage of nutrition caused the cell death. After creating stable cell masses, it began to proliferate rapidly.

Relative to control samples, tumor proliferation in the scaffold was more significant after day 8; the scaffold supported the samples to allow them to be maintained and developed effectively.

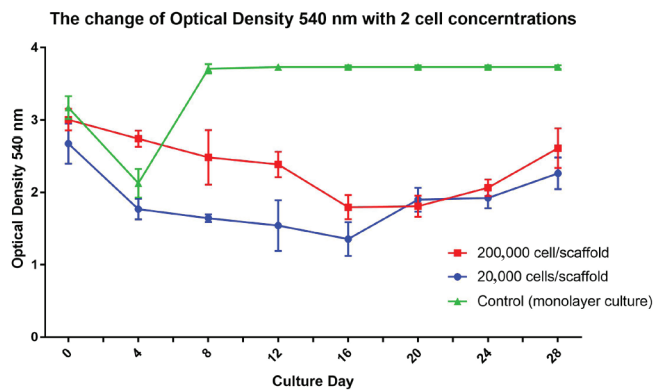


Fig. 2. Cell growth in 28 days. A blue line of the 2×10^4 cell sample, a red line of the 2×10^5 cell sample, and a green line of monolayer samples.

The cells adhered to the scaffold and proliferated after seeding into the scaffold. In the microscope slides, cells created clusters like spheroids, which were disseminated uniformly in the scaffold. It was observed that round-like cells gathered in the ‘tumour’ (Figs. 3A, 3B). This aligns with Remedios Castello-Cros’ research, which describes round and less-spread cancer cells on the scaffold [13].

While cells in the monolayer culture spread on the surface of TPCS dishes and exhibited sheet-like trigonal or polygonal morphologies, those on the scaffold displayed a spheroid shape (Fig. 3C). Cells and their interactions filled and formed a multi-layer tumor cell mass. The findings of Lei Chen, et al. (2012) [2] and Hongzhou Huang, et al. (2013) [14] have demonstrated similar outcomes.

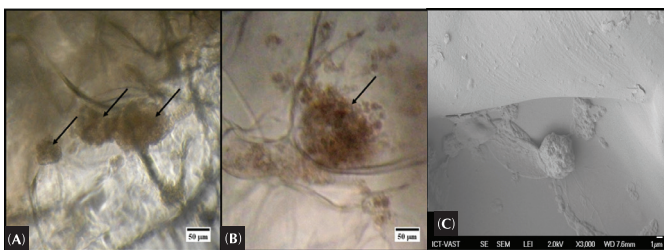


Fig. 3. Images of cell mass on the GA scaffold from the microscope. (A) 2×10^4 cell scaffold sample (20x), (B) 2×10^5 cell scaffold sample (20x). The black arrow indicates cell mass, (C) SEM image of the cancer cell mass on the GA scaffold (3000x).

With routine fibroblast H&E staining, only the highly thin, elongated nuclei of the cells are clearly visible [15]. In the tumor, the cancer cell was stained dark violet and had a round nuclei structure [2].

With H&E staining, cells in the 2×10^4 cell scaffold sample created round cell masses or were spheroid at day 0 (Figs. 4A, 4D). In addition, oblong cell masses appeared (Figs. 4B, 4C). These masses often had large round dark-violet-stained cell nucleus and fibroblast whose nuclei are spindle-

shaped. The nucleus is bigger than ordinary ones because DNA nuclear is double in dozens of time. Particularly, Fig. 4D displays many cells whose nuclei were elongated and stained darker than other fibroblasts (black arrows).

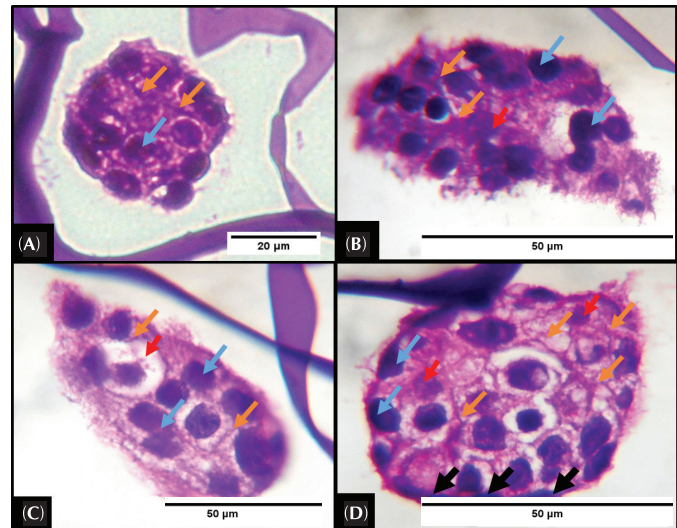


Fig. 4. Images of cancer cell masses with a 2×10^4 cell scaffold at day 0. Orange arrows indicate cell membranes, blue arrows indicate nuclei of cancer cells, red arrows indicate necrosis area, and black arrows indicate elongated nuclei.

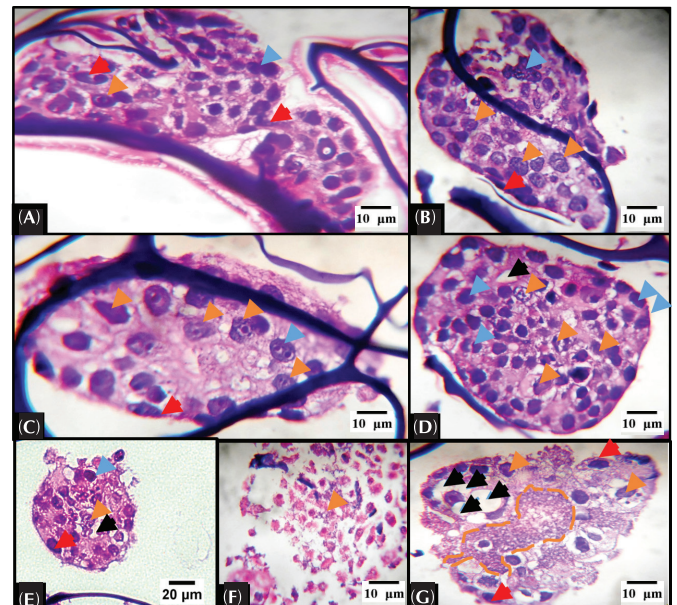


Fig. 5. Images of cancer cell mass of a 2×10^4 cell scaffold at different culture times.

(A+B+C+D) Day 7; (E+F) Day 14; (G) Day 21. Blue arrows indicate normal nuclei of cancer cells (particularly cell division at image B and image C), orange arrows indicate necrotic cells, red arrows indicate the elongated nuclei (image A displays the bridge between two masses), and black arrows indicate lumen in tumors.

Cell morphology had changed dramatically by day 7; the nuclei were paler than on sample day 0 and were multi-lobed (some granules) (Fig. 5). Fig. 5A illustrates the connection of two cell masses; this is a reason why there were larger tumors. These masses' nuclei were the same as a sample at day 0; therefore, combining masses helped the cells to develop better. A particular structure in Fig. 5B had nuclei which were divided into two or four lobes.

Tumors tended to break up into smaller cell masses after two weeks (Fig. 5E); the cancer cell masses were not as dense and solid as at day 0. Xiong, et al. cultured an MDA-MB-231 cell line on the bacterial cellulose. Xiong, et al. described the lumina of the cell mass, which is the space inside of a tubular structure, as being covered by one or two cell layers [16]. At day 21, tumor masses have a fibroblast area which strains denser than other areas (an orange circle at Fig. 5G). This may be an area which contains CAF that has interacted with a normal cancer cell.

For the 1.5×10^5 cell scaffold sample, the structure of the cell mass is similar to the 1.5×10^4 cell scaffold sample. However, the structure of cell mass at the former is less solid than the latter at day 7 and day 14.

According to the 2×10^5 cell scaffold sample, cells were dead at day 0 because of extremely high density (Fig. 6A). Dead cells were rejected after eliminating old mediums and supplying new ones every two days. As with the low concentration sample, after the cell masses were broken apart, cells at day 7 tended to create a larger mass. In addition, cell masses had many extraordinary structures, such as pyknosis, karyorrhexis, karyolysis, etc. (Fig. 6B).

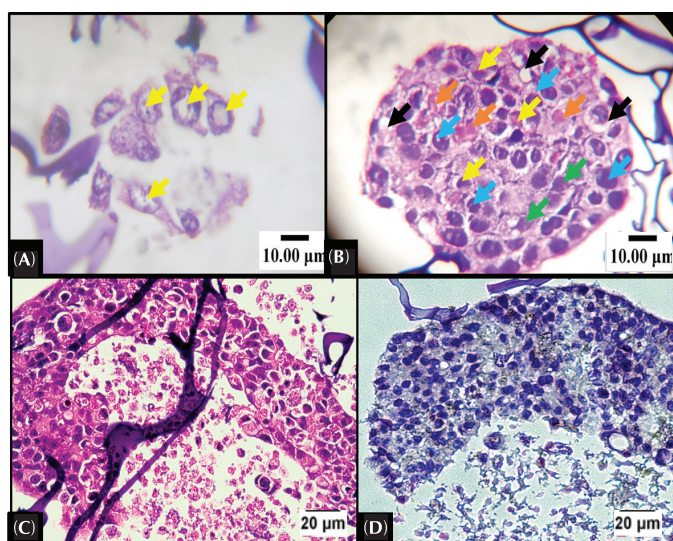


Fig. 6. Images of cancer cell masses with a 2×10^5 cell scaffold at different culture times. (A) Day 0; (B) Day 7; (C) Day 28; (D) Day 28 (vimentin stain). Necrosis cell: green arrows (pyknosis), yellow arrows (karyorrhexis), and orange arrows (karyolysis). Blue arrows indicate the normal nuclei of cancer cells, and black arrows indicate lumen in tumors.

At days 14 and 21, no cell masses were recorded in the scaffold, but at day 28, we observed the special structure, which had non-necrosis areas and necrosis areas (Figs. 6C, 6D). When staining with vimentin, the substance confirmed the mesenchymal origin of some tumors; this was expressed in the tumor masses (Fig. 6D).

Discussion

Although numerous cells proliferated drastically in the shake culture condition, other cells which were located deeper inside the tumor tended to lack nutrients and oxygen (hypoxia). Evidence suggests that tissue hypoxia is a crucial element in initiating tumor necrosis during tumor progression because it contributes to tumors' rapid growth and the inadequate supply of blood [17]. Tumor hypoxia is an important feature of aggressive cancers [18]. Cancer cells in necrotic areas can express different characteristics, such as dedifferentiation and malignancy. This causes specific features of the tumor, such as high-grade structure and resistance to radiation and chemotherapy [19].

Nuclear changes assume one of three patterns, which all result from the breakdown of DNA and chromatin. The first pattern is pyknosis, which is characterised by nuclear shrinkage and increased basophilia, in which the DNA condenses into a dark shrunken mass. In karyorrhexis, the pyknotic nucleus can undergo fragmentation (Figs. 5D, 6B). Lastly, the basophilia fades due to fragment DNA by DNase activity (karyolysis). In one to two days, the nucleus in a dead cell completely disappears (Fig. 6B) [17].

The changes in glucose consumption were suitable for the tumor proliferation indicated by histological images. For example, breakdowns in tumor mass are correlated with decreasing glucose consumption after day 18 and the creation of new clusters after day 21, which caused an increase in glucose consumption at day 22 in the low concentration sample. After breaking down the cancer cell mass on day 14 at a low concentration sample and on day 0 at a high concentration sample, new tumors were formed with extraordinary structures. This demonstrates that the tumor changed its properties, which may be the primary reason for drug resistance and tumor aggression (Figs. 5G, 6C).

Notably, at higher concentrations, there were masses at day 28, although they were observed at days 14 and 21. Cells in broken masses could perhaps gather to create new masses. In addition, the result of the vimentin strain (Fig. 6D) demonstrated that these cells produced a mesenchymal characteristic, which rendered the tumor masses more malignant.

The optimal duration of the development of cells on the GA scaffold was about seven days. To increase the productivity of the tumors, cells were grown in the rotating cell culture system (RCCS). It was rotated in a gentle fluid orbit so that they could make contact with the others, form

complex 3D structures, and attain a tissue-like phenotype. Therefore, cells in the RCCS functionally resembled tissues in the human body [3].

Tumor tissues have three zones, including the tumor, interface, and normal ones. Normal tissues contained intermingled fibro-fatty tissue, which is an interface area composed of altered fibroblast and a tumor sector characterised by an invasive ductal type of carcinoma [20]. A sample day 21, we recorded the altered fibroblast area (orange area), which contained CAF with darker staining relative to the surrounding areas (Fig. 5G). The appearance of lobular ducts indicated that these fibroblasts are altered into breast cancer-specific ones.

Through SEM images, scaffold structures with multiple layers indicated that the formation of masses was caused by the connection of cell clusters and adhesive elements which these cells secrete. The development and acid secretion of tumor masses imposed pressure on the GA scaffold, which spurred the collapse into the layers of the scaffold network. The gelatin and alginate provided the micro-environment in which cells could stably settle.

Because of the lower number of cells, the morphology of the MCF-7 line was spheroid, while there were many morphologies for a higher number of cells (luminal, spheroid). Subsequently, 3D models in previous research often formed separate spheroids. The advantages of this model include the cell-cell interactions, as well as tumorid-tumorid interactions, which mimic breast tumors *in vivo*.

The tumor microenvironment is regarded as a temporal and spatial 3D structure with distinctly physiochemical and histopathological properties, which are pivotal in cell molecular signaling, morphology, motility, differentiation and proliferation [4]. However, the technique applied in this research is not simple, and the morphology of cells is heterogeneous; this causes problems research in terms of the effect of drugs on cells.

Conclusions

Seeding MCF-7 cells and fibroblasts into the scaffold through incubation at 37°C and 5% CO₂ in 20 minutes with a 2x10⁴ cell scaffold and a 2x10⁵ cell scaffold enables cells to grow and proliferate on the scaffold.

The development of the tumor in this research is similar to the tumor mass *in vivo*. The appearance of hypoxia necrosis is an indispensable tendency and helps cancer invade and metastasis.

The cell-scaffold complex created through incubation has a form which is similar to human breast tumor masses.

The authors declare that there is no conflict of interest regarding the publication of this article.

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Establishing a molecular protocol for detection of *EGFR* mutations in patients with non-small cell lung cancer

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Abstract:

Lung cancer is one of the quickest and most fatal diseases and is divided into two types: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Approximately 50% of NSCLC patients harbour mutations in the epidermal growth factor receptor (*EGFR*) gene, spanning from exon 18 to exon 21, which are responsible for the tyrosine kinase domain of the *EGFR* protein. Currently, gefitinib and erlotinib are two drugs inhibiting *EGFR* which are used in the treatment of lung cancer. The indication for use of these drugs depends on the mutation status of *EGFR*. Resistance to treatment with these two drugs has been found with mutations in exon 20; however, mutations in exon 18, 19, 21 benefited from the same treatment. Therefore, we built a molecular protocol for detecting mutations in exon 21 of *EGFR* in NSCLC patients in Vietnam which, in this study, is based on the real-time polymerase chain reaction high resolution melting (PCR HRM) technique. We successfully designed pairs of primers for the real-time PCR HRM technique to detect mutations in exon 21 and for the Sanger sequencing method to confirm the real-time PCR HRM results. We also investigated the optimal concentration of $MgCl_2$ for the real-time PCR HRM protocol. Performance characteristics of the real-time PCR HRM protocol were evaluated in terms of specificity, limit of detection, repeatability, and it showed good results. Finally, we applied the molecular protocol to 172 clinical samples and compared these with the Sanger nucleotide sequencing method and the peptide nucleic acid (PNA) clamping method. The results of the comparison demonstrate that the molecular protocol constructed for detection of exon 21 mutations can be used in the treatment of NSCLC patients.

Keywords: *EGFR*, exon 21, mutations, PNA Clamp.

Classification numbers: 3.2, 3.5

Introduction

Lung cancer is the leading cause of cancer mortality worldwide. This is especially the case in Vietnam. Lung cancer comprises two types: NSCLC, which accounts for approximately 80-85% of lung cancer patients, and SCLC, which accounts for approximately 15-20% of lung cancer patients [1]. NSCLC has a poor prognosis if it is diagnosed at an advanced stage.

EGFR is a member of the ErbB receptor tyrosine kinase family; it has an extracellular ligand-binding domain, a transmembrane domain, and an intracellular tyrosine kinase. *EGFR* (also called ErbB1, Her1) is a transmembrane receptor tyrosine kinase that transduces signals that are

critical for cell proliferation, differentiation, and apoptosis [2]. Overexpression of *EGFR* has been observed in tumours in more than 60% of metastatic NSCLC patients and is correlated with a poor prognosis [3]. Overexpression or mutational activation of *EGFR* is associated with the development and progression of numerous human malignancies. Drugs as tyrosine kinase inhibitors (TKIs) that target the ATP-binding cleft of *EGFR* have been developed for the treatment of NSCLC patients with *EGFR* mutations [4].

EGFR is located on chromosome 7 at the 7p12 locus and it is in the proto-oncogene group. *EGFR* is 110 kb long, comprising 28 exons [5]. *EGFR* mutations associated with

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NSCLC belong to four exons, from 18 to 21. Mutations in *EGFR* cause the *EGFR* protein to exist in a non-ligand-activated state that increases the sensitivity of the tumour or resistance to *EGFR* TKIs.

In the last few years, *EGFR* receptor inhibitor drugs such as erlotinib (Tarceva) and gefitinib (Iressa) have been used for the treatment of NSCLC and have been shown to have encouraging effects [6]. These two drugs inhibit *EGFR* autophosphorylation by inhibiting ATP binding to *EGFR* and reducing the affinity between *EGFR* and its ligand. Patients treated with gefitinib and erlotinib show good progression, such as significant tumour size reduction, longer life duration, and fewer side effects than conventional chemotherapy or radiation. The level of treatment response to the drug depends on the presence or absence of mutations in *EGFR*; patients should be tested for *EGFR* mutations prior to the administration of targeted therapies.

It has been reported that the frequency of *EGFR* mutations in NSCLC patients is dependent on ethnicity, with a rate of 54.1% found in Asian populations [7]. Vietnam is one of the countries with a high rate of *EGFR* mutations and therefore *EGFR* mutation assessment before treatment is essential for successful treatment. In Vietnam, *EGFR* mutations are detected using Sanger sequencing or commercialized molecular kits. The sequencing method is not appropriate to detect gene mutations with a low frequency. On the other hand, commercialized kits are expensive and involve a complicated process. For example, the PNA clamping kit costs 70,000,000 VND for 50 reactions (about 1,400,000 VND per reaction), while the chemical reagents for the real-time PCR HRM assay cost approximately 120,000 VND per reaction. According to the manual, 'PNAclamp™ *EGFR* Mutation Detection Kit Ver. 2', the PNA clamping kit involves a complicated process. Real-time PCR HRM is a powerful technique in molecular biology for the detection of mutations, polymorphisms, and epigenetic differences in double-stranded DNA samples [8]. It is a simple, quick, and accurate method for detecting genetic modifications. In addition, it is more cost effective than other methods such as nucleotide sequencing, real-time PCR with *Taqman* probes, and DNA hybridization techniques with specific probes. However, it requires that the primer pair be well-designed so that homologous DNAs that differ in terms of only one nucleotide can be differentiated by melting curves. For these reasons, we established a molecular protocol based on the real-time PCR HRM technique to detect mutations in *EGFR*'s exon 21. This protocol will be useful for the treatment with gefitinib and erlotinib of NSCLC patients bearing *EGFR* mutations.

Materials and methods

Samples

A total of 172 lung cancer biopsy specimens was collected from Hanoi Oncology Hospital and Cho Ray Hospital. These patients were recommended for *EGFR* mutation testing by physicians due to clinical features associated with *EGFR* mutations. Of the 172 samples, 140 were diagnosed as being adenocarcinoma, and 32 were of other or unknown histology. The tissues were stained using the hematoxylin-eosin (HE) method. The tumour-rich areas were identified by geneticists with the aid of a microscope to ensure that sufficient material was available for the real-time PCR HRM assay. The bacterial strains, including *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* were provided by Gia Dinh People's Hospital.

Reagents

All the chemical reagents for DNA extraction, PCR, real-time PCR HRM, and agarose gel electrophoresis were purchased from Qiagen, Kapa Biosystems, Merck, and Sigma. The nucleotide sequencing kit was supplied by Applied Biosystems. The primers were synthesized and supplied by Phu Sa Biochem. The PNAclamp™ *EGFR* Mutation Detection kit Ver. 2 was purchased from Panagene, Korea.

DNA extraction from formalin-fixed paraffin-embedded (FFPE) tissue

The biopsy specimen was fixed with formalin and was embedded in paraffin. The specimens were then stained with HE dye and examined under a microscope. The location of the suspected cancer cells was zoned and compared to the cell region on the tissue. A sharp knife was used to cut the tumour-rich areas, including a portion of the surrounding cells. The tissue was washed several times in xylene to dissolve the paraffin wax and then the xylene was removed by washing the tissue several times with ethanol before DNA was extracted. Genomic DNA was extracted using the QIAamp DNA FFPE Tissue kit (Qiagen, Germany) according to the manufacturer's protocol. The extracted DNA was then checked by means of the spectrophotometry method and maintained at 4°C until use.

Design of the HRM primers

In order to ensure the best performance for mutation detection using HRM, primer pairs were designed to create PCR products with sizes ranging from 50 to 120 bp. The primers were designed with Primer3Plus software. Each amplicon was analyzed using Umelt software online to ensure that it contained only a single melting peak. The primers were checked with OligoAnalyzer software and the Blast tool for their *in-silico* performance characteristics.

HRM assays

The real-time PCR HRM reaction was performed in a 48-well Eco Plate on the Eco™ Real-time PCR system (Illumina, USA). The 20 µl final volume of the reaction mixture contained: 1X KAPA HRM Fast Master mix, 3 mM MgCl₂, 100 nM of 21F1, 100 nM of 21R1, 10 ng of genomic DNA, and PCR-grade water. The KAPA HRM Fast Master mix contained an optimized concentration of EvaGreen dye and a highly engineered version of *Taq* DNA polymerase. The cycling and melting conditions were as follows: one cycle at 95°C for 3 min; 45 cycles at 95°C for 30s, 66°C for 30s, one cycle of 95°C for 1 min, and a melt from 70°C to 95°C, rising 0.2°C per second. HRM analysis was performed using Eco Software (Ver. 4.1). The normalized graph and the difference graph were used to analyze the data.

DNA sequencing

The amplification reaction of the PCR product for nucleotide sequencing contained the following components: 1X KAPA 2G master mix, 3.5 mM MgCl₂, 500 nM of 21F2 and 21R2, 30-50 ng of genomic DNA (if possible), and PCR-grade water. The PCR reaction was performed using the following conditions: initial denaturation at 95°C for 5 min; 40 cycles at 95°C for 20s, 60°C for 30s, 72°C for 30s, and one cycle at 72°C for 7 min.

The PCR product was generated by means of agarose gel electrophoresis. The band of interest was then cut into the 1.5 ml eppendorf to prepare the DNA purification. The PCR products were purified using the Zymo Gel Recovery kit (Zymo Research, USA). Next, the purified product was sequenced using the Big Dye Terminator v3.1 kit (Applied Biosystems, USA), in accordance with the kit manual. Following the Big Dye reaction, the product was cleaned with a sequencing clean kit. The sequencing products were run on a 3100 Genetic Analyzer (Applied Biosystems). The sequencing data was visualized using sequencing analysis v5.3.

PNA Clamp

All samples were set up using the PNA Clamp™ *EGFR* Mutation Detection kit (Ver. 2, Panagene, Korea). These samples were set up according to the manufacturer's instructions. The PNA Clamp data was visualized using 7500 software Ver. 2.3.

Results

Identification of the target DNA sequence of exon 21 of *EGFR*

Exon 21 of *EGFR* encodes the tyrosine kinase region of *EGFR*; its length is 156 bp. The sequence of exon 21 was loaded from GenBank and in Fig. 1, it is shown which mutations (if any) occur in codon L858R and codon L861Q.

```
ggcatgaactacttgaggaccgtcgttgggcaccgcgacctggcagcaggaacgtactggtgaaacaccgcagcatgtc
aagatcacagattttgggctggccaaacctgctgggtgcggaagagaagaataccatgcagaaggaggcaaa
```

Fig. 1. The exon 21 sequence loaded from GenBank. The two nucleotides t, in bold, occur from left to right at codon 858 and codon 861.

Oligonucleotide design

We designed the 21F1-21R1 primer pair for amplification of exon 21 by means of real-time PCR HRM and the 21F2-21R2 primer pair for the nucleotide sequencing of this exon. The nucleotide sequences of the primers were as follows:

21F1: 5' CGC AGC ATG TCA AGA TCA CAG 3' -
21R1: 5' GGT ATT CTT TCT CTT CCG CAC C 3'

21F2: 5' TGG CAT GAA CAT GAC CCT GAA 3' -
21R2: 5' CAG CCT GGT CCC TGG TGT C 3'

The 21F1-21R1 primer pair was designed to generate a PCR product of 66 bp in size. The small size of 66 bp helps to avoid other SNPs in the proximity of the L858R and L861Q mutations. In contrast, the 21F2-21R2 primer pair was designed to generate a PCR product of 295 bp which is suitable for sequencing by means of the Sanger technique.

In the next step, we checked the technical parameters of the primers such as T_m, % GC, and the free energy of the secondary structures using OligoAnalyzer software. The results are presented in Table 1.

Table 1. Technical parameters of the designed primers.

Parameters	Primer			
	21F1	21R1	21F2	21R2
Nucleotide	21	22	21	19
GC content (%)	52.4	50	47.6	68.4
T _m (°C)	56.5	55.6	56.9	60.6
Hairpin (kcal/mole)	0.04	1	-1.55	0.25
Self-dimer (kcal/mole)	-5.38	-4.41	-5.38	-3.55
Hetero-dimer (kcal/mole)	-5.12		-6.21	

The results in Table 1 show that the four primers met the specific requirements for working well in PCR. Finally, we tested the theoretical specificity of these primers using Blast software. The results show that the primers match the human DNA in the *EGFR* gene on chromosome 7 (data not shown).

In addition, using the Umelt software, we predicted the melting curve of 21F1-21R1 primers for wild and mutant forms (of both L858R and L861Q mutants). The melting curve peaks were clearly separated at T_m levels from 0.2°C to 0.7°C (data not shown). In conclusion, the designed primers were suitable for the subsequent experiments.

Building the real-time PCR HRM for exon 21 EGFR mutation detection

We set up a real-time PCR HRM reaction with the 21F1-21R1 primer pair on a few human DNA samples. All of them were positive in the real-time PCR. When analyzed by means of HRM, they showed different melting curves corresponding to the L858R and L861Q mutations, as shown in Fig. 2.

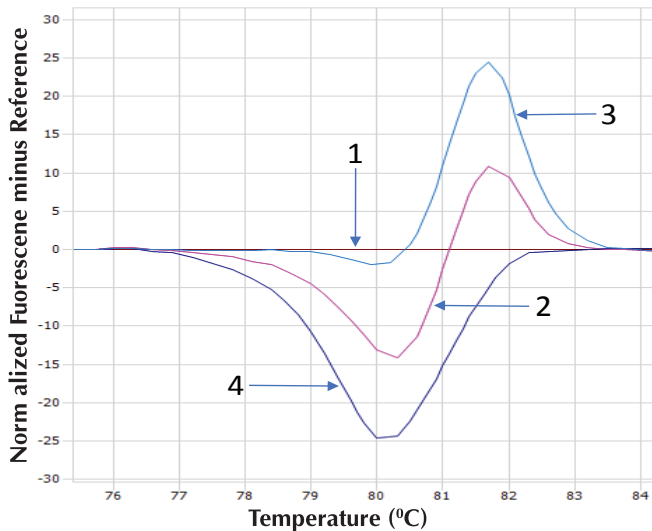


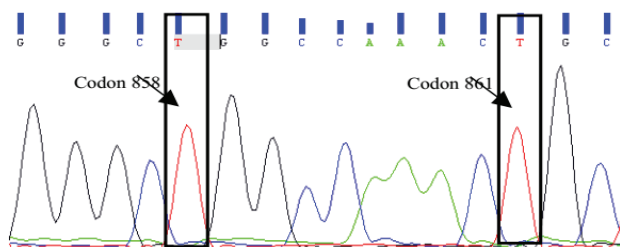
Fig. 2. The real-time PCR HRM results of exon 21 mutation detection. 1: the wild type, 2: the L858R heterozygous mutation, 3: the L858R homozygous mutation, 4: the L861Q heterozygous mutation.

According to the prediction of the U melt software, there were four different melting curves which corresponded to the expected curves of the wild type (curve 1), the L858R heterozygous mutation (curve 2), the L858R homozygous mutation (curve 3), and the L861Q heterozygous mutation (curve 4). The expected curve of the L861 homozygous mutation was not found in HRM results.

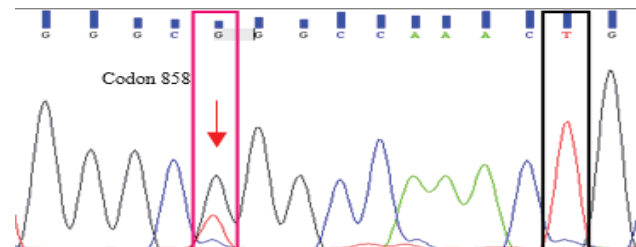
To verify the results of the real-time PCR HRM, we ran Sanger nucleotide sequencing on these four samples. The results of the nucleotide sequencing of the four samples are shown in Fig. 3.

The nucleotide sequencing results show that there were two peaks of thymine at codon 858 and codon 861 in the wild-type sample. There were two peaks of guanine and thymine at codon 858 in the L858R heterozygous mutation sample. The L858R homozygous mutation sample had one peak of guanine at codon 858. The L861Q heterozygous mutation sample had one peak of adenine and thymine at codon 861. Thus, the Sanger nucleotide sequencing confirmed that the mutation detection results using real-time PCR HRM were correct.

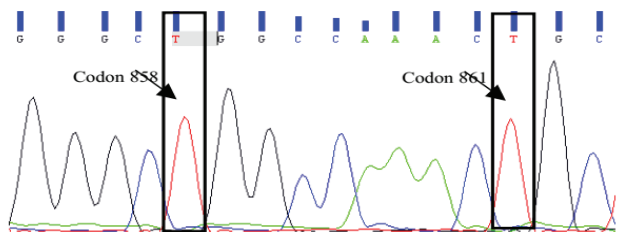
With these four samples, we performed the optimization of $MgCl_2$ concentration for the real-time PCR HRM.



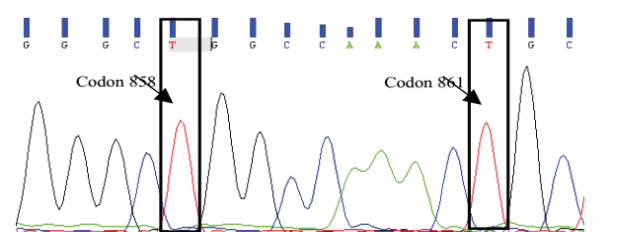
The wild-type sample



The L858R heterozygous mutation sample



The L858R homozygous mutation sample



The L861Q heterozygous mutation sample

Fig. 3. Results of the Sanger nucleotide sequencing of four samples of wild type, L858R heterozygous mutation, L858R homozygous mutation, and L861Q heterozygous mutation.

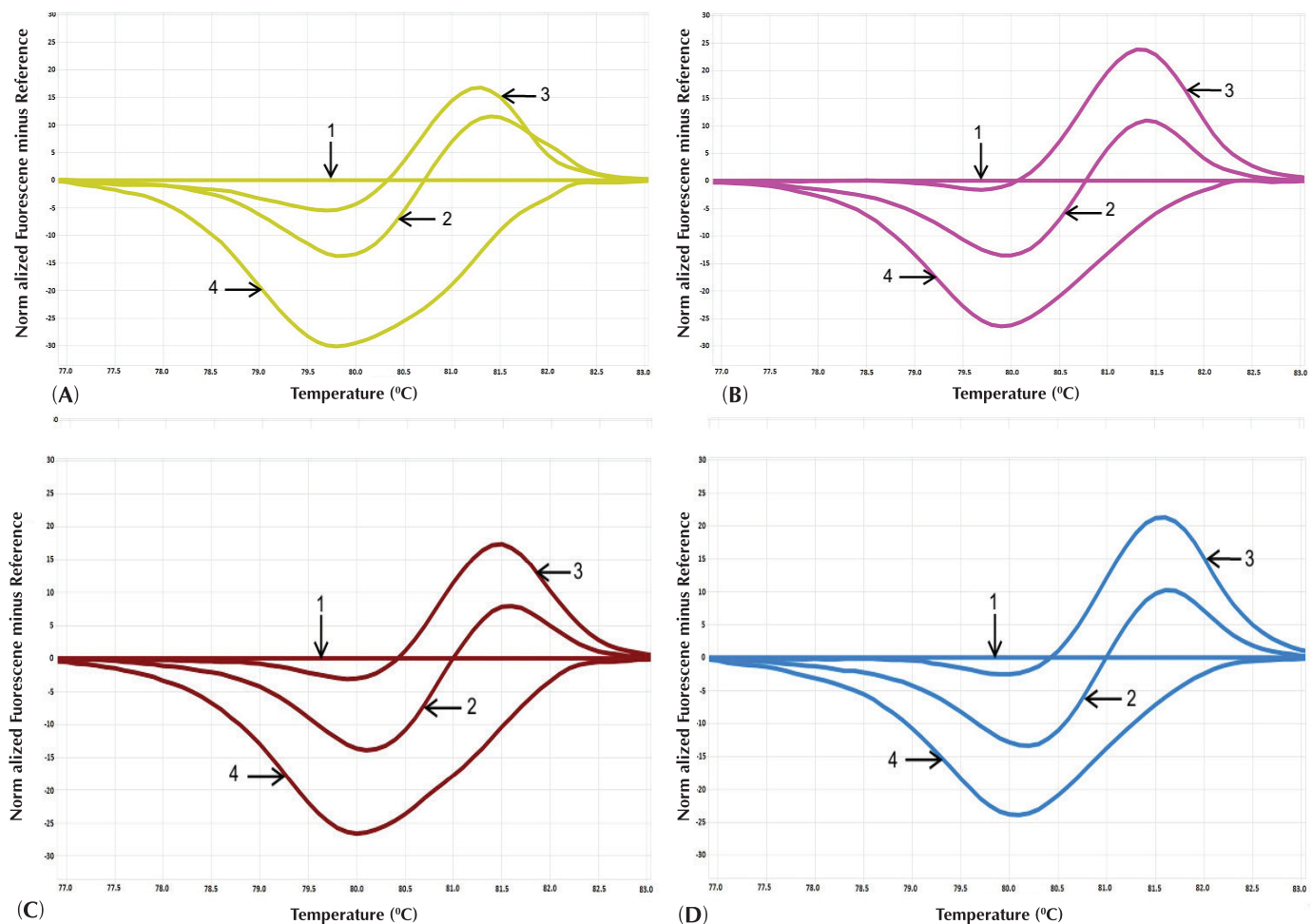


Fig. 4. Examination of the optimal MgCl_2 concentration for the real-time PCR HRM. (A) 1.5 mM, (B) 2 mM, (C) 2.5 mM, and (D) 3 mM.

Optimization of MgCl_2 concentration

MgCl_2 is the major component that influences the melting temperature of PCR products when analyzed using HRM. Therefore, we examined the optimum MgCl_2 concentration for distinguishing different melting curve types corresponding to the mutations at codons 858 and 861 of exon 21. We investigated the MgCl_2 concentrations of 1.5 mM, 2 mM, 2.5 mM, 3 mM, and 3.5 mM. The results of the optimization of MgCl_2 concentration are shown in Fig. 4.

The results in Fig. 4 show that the four melting curve genes corresponding to the wild type, the L858R heterozygous mutation, the L858R homozygous mutation, and the L861Q heterozygous mutation were more clearly distinguished when the MgCl_2 concentrations were increased. At the MgCl_2 concentration of 3.5 mM, a negative control has an amplified signal (data not shown). Thus, we selected the MgCl_2 concentration of 3 mM for the real-time PCR HRM.

Analytical specificity of the real-time PCR HRM protocol

Analytical specificity of the real-time PCR HRM protocol was demonstrated by means of the selective amplification of the human DNA region containing exon

21 of *EGFR* by means of the 21F1-21R1 primer pair. To effect this, we first performed restriction enzyme analysis of the PCR product of the 21F1-21R1 primer pair because this PCR product is too small to be analyzed using Sanger nucleotide sequencing. The restriction enzymes used in this experiment were the *Mbo*I enzyme, which cuts the target product into two fragments of 14 bp and 52 bp, and the *Dpn*I, which cuts the target product into two fragments of 16 bp and 50 bp. The results of the restriction analysis are shown in Fig. 5.

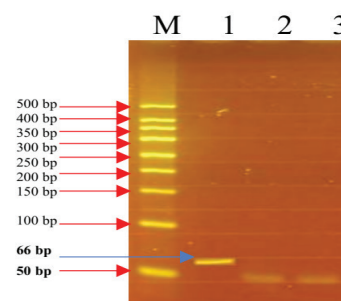


Fig. 5. Restriction analysis of the PCR product of 21F1-21R1. M: DNA ladder, 1: the untreated PCR product, 2: the PCR product treated with *Mbo*I, 3: the PCR product treated with *Dpn*I.

The results in Fig. 5 show that the restriction products from the treatment of the 21F1-21R1 PCR product with *Mbo*I and *Dpn*I had the expected sizes when compared to the DNA ladder. DNA bands of approximate 50 bp appeared in lane 2 and lane 3, while the bands of 12 bp and 14 bp were too small and they were run out of the gel. This result confirmed that the 21F1-21R1 amplified the target region in exon 21 of *EGFR* containing the L858R and L861Q mutations.

We also investigated the selective amplification of the 21F1-21R1 primer pair on the genetic material of various agents, including human and human pathogenic bacteria (*Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*) in the real-time PCR. We selected these bacteria for the selective amplification experiment because they can coexist in humans as a result of infection and the total DNA extracted from human samples can contain bacterial DNAs. The results of the selective amplification of the 21F1-21R1 primer pair are presented in Fig. 6.

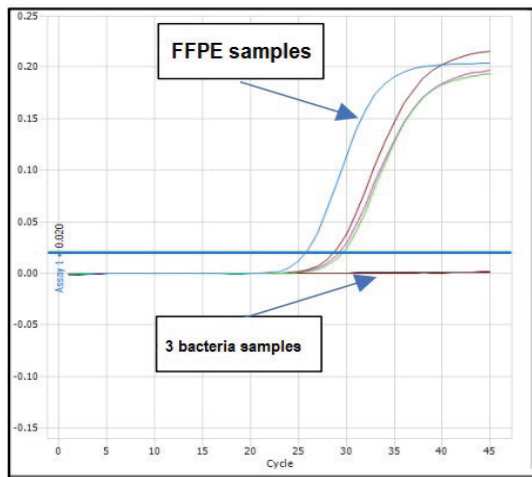


Fig. 6. Selective amplification of the 21F1-21R1 primer pair on various genetic materials from humans and bacteria.

The results in Fig. 6 show that only the human DNA samples generated a positive result in the real-time PCR reaction with the 21F1-21R1 primer pair; DNA samples from the bacteria produced negative results in the reaction with the same primer pair. To confirm that the negative results in the real-time PCR with the 21F1-21R1 primer pair on the bacterial DNA samples were truly negative, we performed a PCR with the 8F-534R primer pair on these DNA samples. This is a primer pair specific to the 16S rRNA gene of all eubacteria. The results showed that the DNA samples from *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* were positive for the PCR with the 8F-534R primer pair (data not shown). These results confirmed that the negative results of the real-time PCR with the 21F1-21R1 primer pair on the bacterial DNA samples were truly negative. Thus, the real-time PCR HRM protocol with the 21F1-21R1 primer pair was specifically designed for the detection of the L858R and L861Q mutations in exon 21 of *EGFR*.

Limit of detection of the HRM method

Most of the FFPE tissue samples of NSCLC cases obtained for this study were small in size. Therefore, the amount of DNA extracted from these samples were too small for analysis. To check the limit of detection of the real-time PCR HRM protocol to detect the L858R and L861Q mutations, we performed the real-time PCR HRM on a range of amounts of DNA, from 1 to 15 ng, with four samples of the wild-type sample, the L858R homozygous mutation sample, the L858R heterozygous mutation sample, and the L861Q heterozygous mutation sample. The results of the limit of detection of the HRM method are shown in Table 2.

Table 2. Results of the limit of detection of the HRM method (in four replicates).

Sample	1 ng	5 ng	7 ng	10 ng	15 ng
Detections (normal + mutation)/total run					
The wild-type sample	4/4	4/4	4/4	4/4	4/4
The L858R homozygous mutation sample	4/4	4/4	4/4	4/4	4/4
The L858R heterozygous mutation sample	4/4	4/4	4/4	4/4	4/4
The L861Q heterozygous mutation sample	4/4	4/4	4/4	4/4	4/4

The results in Table 2 show that the molecular protocol could detect DNA concentrations at 1 ng. At 1 ng DNA levels, all the samples were amplified and the melting curve analysis showed the distinction between the wild type and mutant.

Repeatability

We detected the mutations in exon 21 of *EGFR* using the real-time PCR HRM protocol on four samples of the wild-type sample, the L858R homozygous mutation sample, the L858R heterozygous mutation sample, and the L861Q heterozygous mutation sample five times in the same experiment batch to measure the repeatability of the protocol. The repeatability results were expressed by the coefficient of variation (CV) and the CV values were calculated as a percentage. The results are shown in Table 3.

Table 3. Repeatability calculation of the real-time PCR HRM for exon 21 mutation detection.

Repeat	Wild-type sample	L858R homozygous mutation sample		L858R heterozygous mutation sample		L861Q heterozygous mutation sample
1	81	79.8	81.6	79.5	81.2	81.6
2	81.1	79.7	81.6	79.4	81.2	81.7
3	81.2	79.9	81.7	79.5	81.2	81.6
4	81.1	79.8	81.7	79.4	81	81.7
5	81.2	79.8	81.7	79.4	81.2	81.8
Average	81.13	79.80	81.66	79.44	81.16	81.68
Standard deviation	0.08367	0.07071	0.05477	0.05477	0.0894	0.08367
% CV	0.00103	0.00089	0.00067	0.00069	0.0011	0.00102
Total % CV	0.00090					

According to Table 3, the CV value of the repeatability test was 0.00090%. This CV proves the high precision of the real-time PCR HRM protocol for the detection of mutations in exon 21 of *EGFR*.

Evaluating the real-time PCR HRM protocol on 172 human DNA samples

We evaluated the performance of the real-time PCR HRM protocol for detection of the mutations in exon 21 of *EGFR* on 172 human DNA samples which were provided by Hanoi Oncology Hospital and Cho Ray Hospital. The detection of these mutations was also performed using the Sanger nucleotide sequencing and the PNAclamp™ *EGFR* Mutation Detection kit. The results of mutation detection in exon 21 of *EGFR* are shown in Table 4.

Table 4. Results of *EGFR* mutation detection by real-time PCR HRM, nucleotide sequencing, and the PNA clamping method.

Results/methods	HRM	SEQ	PNA Clamp
L858R mutation	45	40	44
L861Q mutation	2	2	2
No mutation	125	126	124
Sample invalid/suspected	0	4	2
Total sample with results/total sample	172/172	168/172	170/172

HRM: high resolution melting; SEQ: sequencing; PNA Clamp: peptide nucleic acid clamping.

The number of samples successfully analyzed by means of the three methods were 172, 168, and 170 for the real-time PCR HRM protocol, the Sanger nucleotide sequencing, and the PNAclamp™ *EGFR* Mutation Detection kit, respectively. In these 172 samples, 166 (96.5%) showed identical results for all three methods. The real-time PCR HRM protocol detected 45 samples with the L858R mutation while only 40 and 44 samples with this mutation were detected by the Sanger nucleotide sequencing method and by the PNAclamp™ *EGFR* Mutation Detection kit, respectively. Six cases that did not match among three methods were samples 19, 72, 24, 34, 171, and 172. The results of these samples using the three methods are shown in Table 5.

Table 5. The results of the samples using the three methods.

Sample	HRM	GTT	PNA Clamp
19	Mutation L858R heterozygous	Wild type	Mutation L858R
72	Mutation L858R heterozygous	Wild type	Mutation L858R
24	Mutation L858R heterozygous	Suspected wild type	Mutation L858R
34	Mutation L858R heterozygous	Suspected wild type	Mutation L858R
171	Wild type	Invalid	Invalid
172	Mutation L858R heterozygous	Invalid	Invalid

As can be seen in Table 5, the real-time PCR HRM protocol and the PNAclamp™ *EGFR* Mutation Detection kit produced identical results for four samples (19, 72, 24, and 34) while the Sanger nucleotide sequencing produced different results for the same samples. Moreover, the real-time PCR HRM protocol was successful for the last two samples (171 and 172)

while the PNAclamp™ *EGFR* Mutation Detection kit and the Sanger nucleotide sequencing produced invalid results. Further experiments need to be conducted to confirm the results of these six samples.

Discussion

The appearance of the targeted therapy made the cancer treatment procedure more efficient and less toxic than traditional radiotherapy/chemotherapy. Tyrosine kinase inhibitors of *EGFR* are good examples of targeted therapy for NSCLC patients with *EGFR* mutations. Among *EGFR* mutations, L858R and L861Q in exon 21 were proven to confer selective sensitivity to treatment with some TKIs such as erlotinib and gefitinib [6]. Thus, a molecular protocol to detect these beneficial mutations would be useful for the treatment of NSCLC patients with *EGFR* mutations.

With the L858R mutation, the leucine amino acid (L) is replaced by the amino acid arginine (R), meaning that the nucleotide T is replaced by the nucleotide G. Therefore, with L858R there will theoretically be three forms: T/T (wild type), T/G (heterozygous), and G/G (homozygous). With the L861Q mutation, the leucine amino acid (L) is replaced by glutamine amino acid (Q), meaning that the nucleotide T is replaced by the nucleotide A. Hence, there are three forms: T/T (wild type), T/A (heterozygous), and A/A (homozygous). It is interesting to note that we did not find any references which mentioned that the two mutations (L858R and L861Q) are concomitant, which means that a molecular protocol based on the real-time PCR HRM technique can be applied to detect the L858R and L861Q mutations. In fact, we did not find any sample out of the 172 from NSCLC patients which had the concurrent L858R and L861Q mutations in this study. As mentioned above, as real-time PCR HRM is a powerful technique for the detection of mutations with simple manual, we selected this technique to establish a molecular protocol for detection of the L858R and L861Q mutations in *EGFR*.

The PCR products for HRM analysis often have a size of 100-300 bp; however, in this study, we designed the primer pair to amplify the DNA region of 66 bp. The small size of the PCR product enhances the differentiation of homologous DNA sequences that differ only at the L858R and L861Q positions. Moreover, the size of 66 bp helps to avoid SNPs in the proximity of these two mutations. In fact, there are many SNPs in the region surrounding L858R and L861Q, some of which can be listed here: rs1433831615, rs104886012, rs148934350, rs397517134, rs764700695, and rs1471553524. These SNPs may interfere with the melting curves generated by the primer pair in this study. When theoretically analyzed by means of HRM, the 66 bp PCR product generates five melting curve patterns corresponding to the wild type, the L858R heterozygous mutation, the L858R homozygous mutation, the L861Q heterozygous mutation, and the L861Q homozygous mutation. In fact, we obtained four melting curves corresponding to the wild type, the L858R heterozygous mutation, the L858R homozygous mutation, and the L861Q heterozygous mutation using the real-time PCR HRM protocol on

20 human DNA samples. These mutations detected by the real-time PCR HRM protocol were confirmed by the Sanger nucleotide sequencing method.

The 21F1-21R1 primer pair used for the real-time PCR HRM protocol had characteristics suitable to working properly in the real-time PCR HRM reaction [9]. The size of the primers was 21-22 bp, with % GC in the range from 40-60%. There are no more than three guanine or cytosine at the 3' end. The T_m difference between the two primers was not more than 5°C. The free energy of the secondary structures (hairpin, homodimer, and heterodimer) of the primers did not exceed the experimental parameter of -9 kcal/mol which may interfere with the binding to the target DNA strands during PCR. The Blast results showed that the two primers annealed to the human DNA sequences. In the experiment, the 21F1-21R1 primer pair amplified the target region of 66 bp, which was then confirmed by restriction analysis with *MboI* and *DpnI*. These two REs were capable of cutting the target 66 bp sequence at their recognition sites to produce specific DNA fragments. *MboI* cut the target product into two fragments of 14 bp and 52 bp at 5'- $\wedge$ GATC-3'. *DpnI* cut the target product into two fragments of 16 bp and 50 bp at 5'-GA $\wedge$ TC-3'. In addition, the 21F1-21R1 primer pair demonstrated its ability to selectively amplify human DNA among several other genetic materials. These results demonstrate the high analytical specificity of the real-time PCR HRM protocol. Other performance characteristics of the real-time PCR HRM protocol concerning limit of detection and repeatability were good, with a limit of detection of 1 ng of total DNA, and CV of 0.0009%.

Finally, we evaluated the molecular protocol for the detection of L858R and L861Q in 172 samples of human DNA collected from hospitals where NSCLC patients were being treated. We also detected the L858R and L861Q mutations in these 172 samples using two other methods: Sanger nucleotide sequencing and the method using the PNAclap™ *EGFR* Mutation Detection kit. All three methods showed identical results for 166 of the 172 (96.5%) samples. Discrepancies occurred in the results of the three methods with 6 of the 172 (3.5%) samples. The real-time PCR HRM protocol successfully detected mutations in all of the 172 samples, while the number of samples that were invalid or were suspected to be invalid was four for Sanger nucleotide sequencing and two for the PNAclap™ kit. We could not reperform the detection of the mutations using Sanger nucleotide sequencing and the PNAclap™ kit on the discrepant samples due to the limits of the study; however, further experiments could be undertaken to show the utility of the real-time PCR HRM protocol in comparison with other methods of detecting L858R and L861Q mutations.

Conclusions

In this study, we established the molecular protocol based on the real-time PCR HRM technique for the detection of the L858R and L861Q mutations in exon 21 of *EGFR*. The performance characteristics of the genotyping protocol, in terms of analytical specificity, limit of detection, and repeatability, were good. Application of this mutation detection protocol to 172 human DNA samples showed good results in comparison with Sanger

nucleotide sequencing and the PNA clamping method. These results demonstrate the utility of the real-time PCR HRM protocol for the detection of exon 21 mutations in clinical samples, which is essential for the treatment of NSCLC patients with *EGFR* mutations.

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The authors declare that there is no conflict of interest regarding the publication of this article.

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Species composition and distribution of brachyuran crabs in Duyen Hai town, Tra Vinh province

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Abstract:

Brachyuran crabs are the most diverse group of crustaceans. They are found in most marine habitats such as coral reefs, sandy beaches, rocky beaches, mangroves, and seagrass meadows. In this study, we investigated the species composition of brachyuran crabs at 10 sampling locations belonging to three habitats (mangrove forest, artificial dykes, sandy beaches) at Duyen Hai town on August (rainy season) and December (dry season) in 2017. We found that there were 22 species belonging to 10 genera of six families present in the research area. The Ocypodidae family is the most abundant, with 45.5% of the species, following by the Grapsidae (22.7%), Sesarmidae (13.6%), Portunidae (9.1%), Matutidae (4.5%), and Xanthidae (4.5%). The densities of brachyuran crabs ranged from 2.0 ± 1.1 (inds/m²) to 17.2 ± 4.3 (inds/m²). The mangrove forest habitat showed a greater diversity of species than the sandy beach and artificial dyke habitat. The rainy season supported greater diversity than the dry season.

Keywords: brachyuran crabs, Duyen Hai town, mangrove forest, species composition.

Classification number: 3.4

Introduction

Crabs are a common group of the crustaceans. They have diverse species composition and widespread distribution. Currently there are about 7,000 species of crabs belonging to 98 families in maritime, freshwater, and terrestrial environments [1]. Brachyuran crabs are the dominant group of the macrobenthic communities in mangrove ecosystems [2]. They exist in greater species numbers, are more abundant, and have greater biomass than many other animals in most mangrove habitats [3]. Brachyuran crabs depend directly on mangrove areas for survival [4]. Their food resources in mangrove forests include benthic microalgae, phytoplankton, and sediment organic matter [5]. They are also the most important group for the mangrove ecosystem as they make 80% of faunal biomass [6] and are key engineers of the ecosystem [3]. The species composition and distribution of brachyuran crabs depend on their habitats and the environmental conditions. Researches on crab communities often are studies of their composition, number of species, and abundance [7]. In Vietnam, brachyuran crabs have been investigated by a number of researchers, such as Nhuong (2003) [8], Nhuong and Khac (2004, 2006) [9, 10], My, et al. (2012) [11], Chertoprud, et al. (2012) [12], and Tong, et al. (2016) [13]. However, these studies generated only preliminary data on brachyuran crabs in northern and central Vietnam and in the Can Gio district of Ho Chi Minh city. Brachyuran crabs in the Mekong river delta and Duyen Hai town of Tra Vinh province have not previously been studied. The present study was conducted to provide the information on their species composition, density, and distribution in Duyen Hai town in Tra Vinh province.

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Materials and methods

Study area

This study was conducted in Duyen Hai town, Tra Vinh province. This is located in the north-west of the Vietnamese Mekong delta and is between the Co Chien and Hau rivers [13]. It has monsoon climate with two seasons (dry and wet). The investigations were undertaken in August (wet season) and December (dry season) in 2017. For the study, samples were collected at 10 sampling sites in three different habitats: artificial dykes (DH1, DH4, DH7), mangrove forests (DH2, DH8, DH9, DH10), and sandy beaches (DH3, DH5, DH6) (Fig. 1).

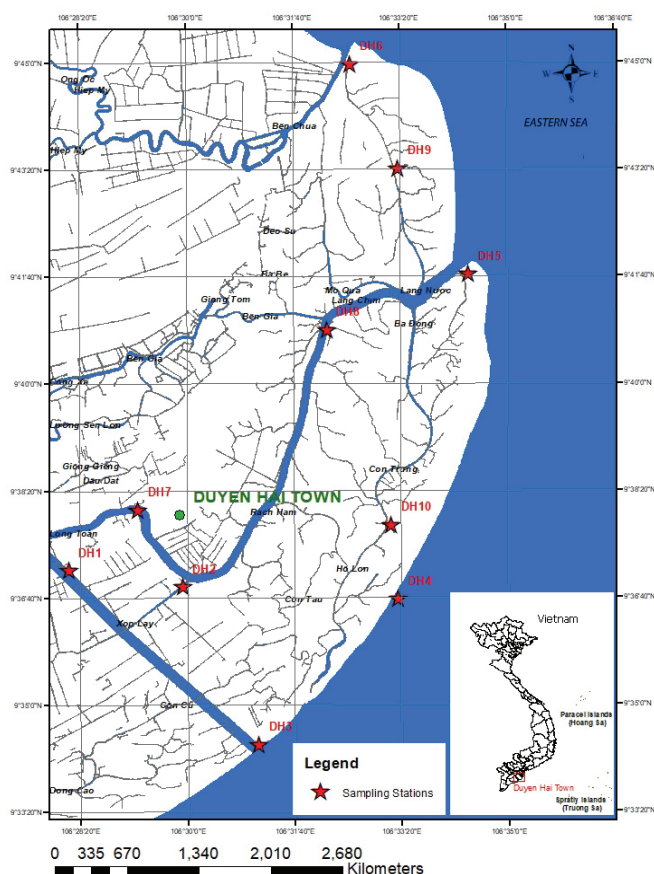


Fig. 1. Sampling stations for brachyura crabs in the study area.

Field sampling

The specimens of brachyuran crabs were collected using the method of Trivedi, et al. (2012) [14]. The qualitative samplings were recorded along a transect 50 metres in length. The quantitative samplings were collected in a quadrat area of 1 square metre [9]. The crabs were collected by hand, digging, or by the pumping out of holes and were photographed for noteworthy characteristics in the

field. The samples were then washed in water and placed in jars. The specimens were conveyed to the laboratory in an icebox and preserved in 90% alcohol.

Laboratory analysis

In the laboratory, the crab specimens were identified using a Olympus SZ-ST (Olympus, Tokyo, Japan) dissecting microscope and by making use of the following literature: Crane (1975) [15], FAO (1998) [16], Ng, et al. (2001) [17], Ng and Davie (2002) [18], Ng, et al. (2008) [19], Shih, et al. (2010) [20], and Davie (2012) [21]. The samples of brachyuran crabs are kept at Marine Joint Laboratory of Vietnam - Korea at the Institute of Tropical Biology.

Data analysis

The data on the number of species and abundance of brachyuran crabs were checked to ascertain whether they fulfilled the assumptions of homogeneity of Levene's test. One-way analysis of variance was applied to examine the significance of the differences based on the habitats and the seasons. The analysis was completed using Tukey's HSD test. All statistical analyses were performed using SPSS v.20.0 (IBM Corp., Armonk, NY, USA). The degree of similarity of the crab communities across habitats was calculated using the Bray-Curtis similarity coefficient in the PRIMER VI analytical package developed by Plymouth Marine Laboratory, UK.

Results

A total of 22 species of brachyuran crabs were recorded in Duyen Hai town. They belonged to 10 genera and six families. The six families of brachyuran crabs were the Ocypodidae, Grapsidae, Sesamididae, Portunidae, Matutidae, and Xanthidae. Of these, the Ocypodidae had the highest number of species, 10 (45.5% of total), followed by the Grapsidae (5 species, 22.7%). The Sesamididae were present as three species (13.6%). Two species (9.1%) belonged to the Portunidae while the Matutidae and Xanthidae were present as only one species each (4.5%) (Fig. 2). The genus *Uca* had the highest number of species while the rest of the genera had 1-2 species/genus each.

Metopograpsus latifrons (Grapsidae) was the most dominant brachyuran crab species and was recorded at seven different sampling sites. In contrast, *Grapsus albolineatus* (Grapsidae) occurred only at site DH4. The highest number of species were detected at DH8 site (14 species) followed by sites DH2 and DH9 (12 species), DH10 (11 species), and DH1 (four species). Sites DH3, DH4, DH5, and DH6 had three species each, while site DH7 had the lowest number, with only two species.

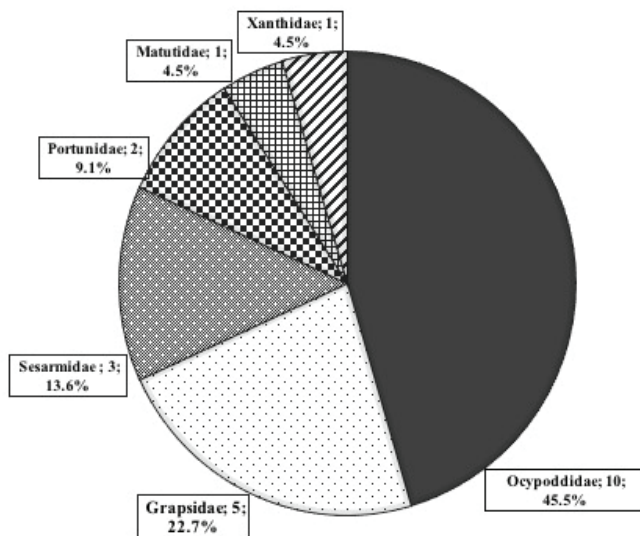


Fig. 2. Relative contribution by family of crabs in Duyen Hai town, Tra Vinh province.

The sampling stations fell into three habitat types: artificial dykes at three sampling sites (DH1, DH4, DH7), mangrove forests at four sampling sites (DH2, DH8, DH9, DH10), and sandy beaches at three sampling sites (DH3, DH5, DH6). The habitat assignment of the brachyuran crabs was based on the location where each species was found most frequently. Fig. 3 shows that the brachyuran crabs at the sampling stations in Duyen Hai town belonged to three groups equivalent to the three habitat types.

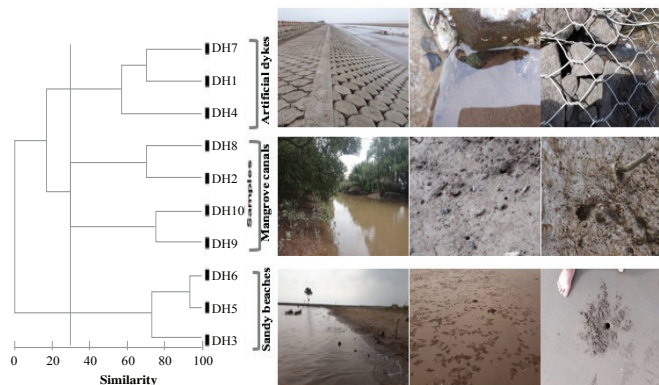


Fig. 3. Dendrogram of brachyuran crabs recorded at the stations and their habitat types.

Of the three habitats, the mangrove forest had the highest number of species, 17, while at the artificial dykes and sandy beaches only three species were recorded for each habitat (Table 1). The species of the families Grapsidae and Ocypodidae were the most dominant in the study area. While *Metopograpsus latifrons* (Grapsidae) was dominant at the artificial dykes, *Dotilla wichmanni* (Ocypodidae) was the dominant species on the sandy beaches, and *Parasesarma plicatum* (Grapsidae), *Uca crassipes*, and *Uca*

annulipes (Ocypodidae) were dominant in the mangrove forest.

Table 1. The species composition of brachyuran crabs in the three habitat types in Duyen Hai town.

No.	Species and Families	Habitat types		
		Artificial dykes	Mangrove forest	Sandy beaches
Family Grapsidae				
1	<i>Metopograpsus latifrons</i> (White, 1847)	X	X	
2	<i>Metopograpsus</i> sp.	X		
3	<i>Grapsus albolineatus</i> (Lamarck, 1818)	X		
4	<i>Parasesarma plicatum</i> (Latreille, 1806)		X	
5	<i>Parasesarma unguatum</i> (H. Milne Edwards, 1853)		X	
Family Matutidae				
6	<i>Ashtoret lunaris</i> (Forskål, 1775)			X
Family Ocypodidae				
7	<i>Ocypode cerathophthalmus</i> (Pallas, 1772)			X
8	<i>Dotilla wichmanni</i> De Man, 1892			X
9	<i>Uca annulipes</i> (H. Milne-Edwards, 1837)		X	
10	<i>Uca crassipes</i> (White, 1847)		X	
11	<i>Uca</i> sp.1		X	
12	<i>Uca (Gelasimus) borealis</i> Crane, 1975		X	
13	<i>Uca lactea</i> (De Haan, 1835)		X	
14	<i>Uca dussumieri</i> (Milne Edwards, 1852)		X	
15	<i>Uca flammula</i> (Crane, 1975)		X	
16	<i>Uca paradussumieri</i> Bott, 1973		X	
Family Portunidae				
17	<i>Scylla serrata</i> (Forskål, 1775)		X	
18	<i>Scylla paramamosain</i> (Estampador, 1949)		X	
Family Sesarmidae				
19	<i>Episesarma versicolor</i> (Tweedie, 1940)		X	
20	<i>Episesarma singaporensis</i> (Tweedie, 1936)		X	
21	<i>Perisesarma eumolpe</i> (De Man, 1895)		X	
Family Xanthidae				
22	<i>Platypodia granulosa</i> (Ruppell, 1830)		X	
Total species		3	17	3

Figure 4 shows the density of brachyuran crabs at the sampling stations and in the different habitats. There was a distinct seasonal variation in the abundance of crabs ($p=0.004<0.05$), with a high density in the rainy season and low values in the dry season. The greatest abundance of brachyuran crabs (17.2 ± 4.3 inds/m² at site DH3) was observed in the rainy season while the lowest (2.0 ± 1.1

inds/m² at site DH4) was found in dry season. There was a clear, significant ($p < 0.05$) difference in crab abundance. The highest density occurred on the sandy beaches (ranging from 7.6 ± 1.3 inds/m² to 17.2 ± 4.3 inds/m²), followed by the mangrove forests (ranging from 7.0 ± 1.1 inds/m² to 15.8 ± 1.9 inds/m²), while the lowest densities occurred in the artificial dykes (ranging from 2.0 ± 1.1 inds/m² to 5.4 ± 1.0 inds/m²) (Fig. 4).

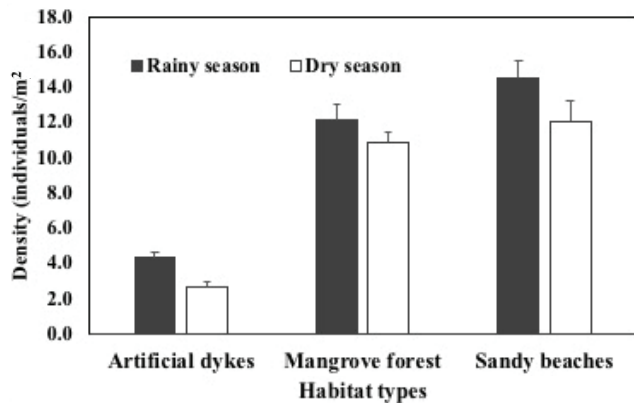


Fig. 4. Densities (mean±SD) of brachyuran crabs in the habitats studied.

Cluster analysis illustrated that the species composition of crab in the study area was divided into three groups (Fig. 5). Group 1 exclusively comprised climber crab assemblages present in the artificial dyke habitat. Group 2 comprised mangrove crabs found in the mangrove forest habitat. Group 3 comprised only sandy assemblages distributed in the sandy beach habitat.

At the artificial dykes, three species, *Metopograpsus latifrons*, *Grapsus albolineatus*, and *Metopograpsus* sp., belonging to family Grapsidae were recorded. *Grapsus albolineatus* and *Metopograpsus* sp. were exclusively present at the dykes (as group 1 in Fig. 5.), while *Metopograpsus latifrons* was found at both the dykes and in the mangrove forests. Species such as *Metopograpsus latifrons* and *Metopograpsus* sp. dominated and were detected at all the sampling stations in the artificial dyke habitat, while *Grapsus albolineatus* was present only at the sea dyke (DH4).

In the mangrove forest habitats, there were 17 species of brachyuran crabs in seven genera and five families. *Metopograpsus latifrons*, *Uca annulipes*, *Uca crassipes*, *Parasesarma plicatum* were dominant and were recorded at all the sampling stations, while *Parasesarma plicatum* and *Parasesarma unguatum* only appeared in small mangrove

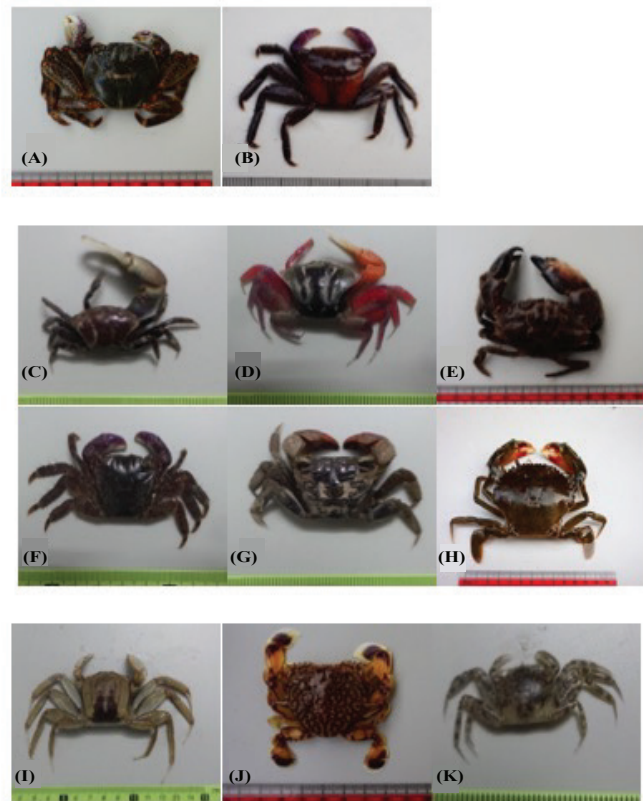
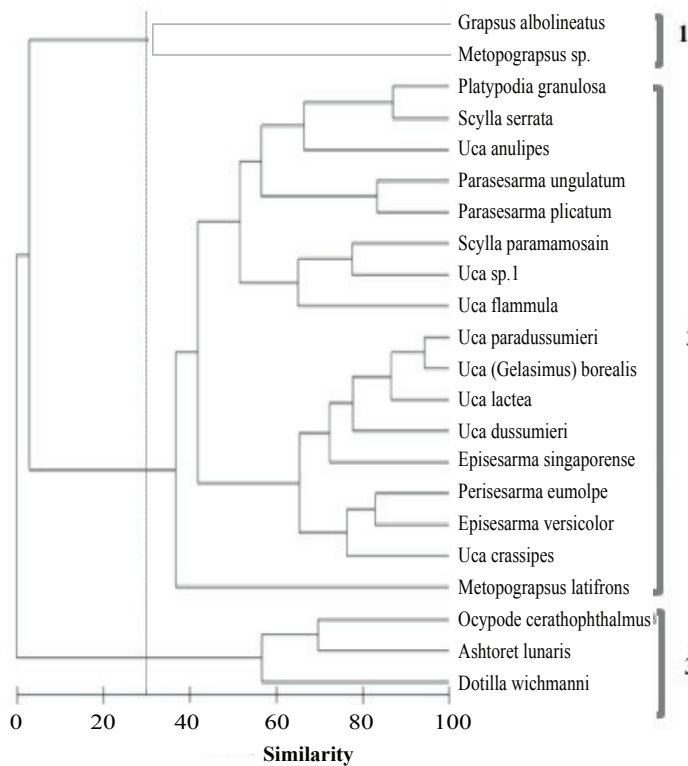


Fig. 5. Species clusters of brachyuran crabs in the habitat types, and some species. (A) *Grapsus albolineatus*; (B) *Metopograpsus* sp.; (C) *Uca paradoxumieri*; (D) *Uca annulipes*; (E) *Platypodia granulosa*; (F) *Metopograpsus latifrons*; (G) *Parasesarma plicatum*; (H) *Scylla serrata*; (I) *Ocypode ceratophthalmus*; (J) *Ashtoret lunaris*; (K) *Dotilla wichmanni*.

canals (DH9 and DH10), and *Episesarma versicolor*, *Episesarma singaporense*, and *Perisesarma eumolpe* occurred exclusively in large mangrove canals (DH2 and DH8). The families Sesarmidae (three species), Portunidae (three species), Xanthidae (one species) and almost all Ocypodidae species were exclusively distributed in the mangrove forest habitats (as group 2 in Fig. 5).

On the sandy beaches, three species belonging to the families Matutidae (one species) and Ocypodidae (one species) were found. *Ocypode cerathophthalmus* and *Dotilla wichmanni* were the dominant species and were recorded at all the sampling sites in the sandy beach habitat, while *Ashtoret lunaris* was detected only at site DH3 (as group 3 in Fig. 5).

Discussion

Mangrove forests have been well-studied for brachyuran crab diversity [22]. More than 100 species of brachyuran crabs in mangrove forests have been reported in Malaysia and Singapore [23]. Brachyuran crabs are a prominent faunal component and play important ecological roles in mangrove forests [24]. Twenty-two species of brachyuran crabs were found in three different habitats in Duyen Hai. Seventeen species were recorded in mangrove forest habitats, while three species were found at artificial dykes and on sandy beaches. The number of species of brachyuran crabs is dependent on the mangrove vegetation and hydrobiological factors [15]. Mangrove forests with vegetation, low temperatures, and a wet surface are a suitable habitat for brachyuran crabs; hence, they had high species richness [25].

The species composition of brachyuran crabs in Duyen Hai included grapsids, ocypodids, sesarmids, and portunids. These species are commonly distributed in the coastal areas [10] and mangrove forests [3] in Vietnam. Portunid crabs belonging to the genus *Scylla* are of high value and are an important source of income for local fishers [26]. Ten ocypodid species were recorded in Duyen Hai; these comprise the preliminary data on them in Tra Vinh province. As in other areas in Vietnam, the most abundant species in the study area belong to the Ocypodidae and Grapsidae families [8]. The genus *Uca* are a group of crabs common to most tropical and subtropical coastal areas [27]. They are largely diurnal, active at low tide, often sympatric, and always gregarious [15]. They are distributed on substrates ranging from mud to sand with a minimum of silt, and in mangroves, such as mangrove forest habitat in Duyen Hai, or in other forms of vegetation [15]. Hence, they had the highest number of species in the study area.

The distribution features of brachyuran crabs are

dependent on the characteristics of different biotopes, vegetation structures, food availability, and on the nature of the substrate [9, 28]. In the sandy beach habitat (DH3, HH5, and DH6) sand bubbler crabs (*Dotilla*) and sand crabs (*Ocypode cerathophthalmus*) were also found [9]. The hard surfaces of the dyke habitat (DH1, HH4, and DH7) are suitable for purple climber crabs (*Metopograpsus*) and sally lightfoot crabs (*Grapsus albolineatus*) [25]. In the mangrove forest habitat (DH2, DH8, DH9, DH10), mangrove crabs such as sesarmids, grapsids, ocypodids, ucidids, and portunids were also found. These families occur across the mid- and low-intertidal zones in the mangroves [24]. The Grapsidae family is also found in the interior of the mangrove forest, while the genus *Uca* dominates the perimeter of mangrove forest, and the genus *Sesarma* is prevalent on the mangrove ground [9]. Water salinity varied significantly across sampling sites, which also accounts for the varied distribution of crabs [28]. Some species, such as *Ocypode cerathophthalmus*, *Ashtoret lunaris*, and *Dotilla wichmanni* were recorded in coastal areas where the water salinity is high, as with sites DH3, DH4, DH5, and DH6. In contrast, the grapsids and sesarmids were found in mangrove forests where the water salinity is low, as with sites DH2, DH8, DH9, and DH10.

Conclusions

The present study provides the first data on the composition, density, and distribution of brachyuran crabs in Duyen Hai town. In three habitats, 22 species belonging to 10 genera and six families were recorded. The number of species was highest for the Ocypodidae family and lowest for the Matutidae and Xanthidae families. The species of the Grapsidae and Ocypodidae families were the most dominant species. Of the three habitats, the mangrove forest habitat had the highest number of species, while the sandy beaches and artificial dykes had the lowest. The density of brachyuran crabs ranged from 2.0 ± 1.1 (inds/m²) to 17.2 ± 4.3 (inds/m²). The density was highest on the sandy beaches and lowest at the artificial dykes. The density was higher in the rainy season than in the dry season.

The authors declare that there is no conflict of interest regarding the publication of this article.

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A case study of phytoplankton used as a biological index for water quality assessment of Nhu Y river, Thua Thien - Hue

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Abstract:

Studies of the composition and density of phytoplankton and the water quality of Nhu Y river were conducted over six months (March to August 2011). Phytoplankton samples were collected by filtration and immediately preserved in Lugol's solution. The phytoplankton species composition recorded 117 species belonging to six divisions: Cyanobacteria (24 taxa), Bacillariophyta (14 taxa), Chlorophyta (45 taxa), Euglenophyta (31 taxa), Cryptophyta (2 taxa), and Dinophyta (1 taxa), of which Chlorophyta and Euglenophyta were dominant in terms of species numbers. The total phytoplankton density fluctuated from 110,146 to 5,964x10³ individuals/litre and Cyanophyta were dominant in terms of individual density. The algal genus pollution index (Palmer index) ranged from 30 to 41, indicating that the water was highly organically polluted, and the Shannon-Weiner index results of 0.66-2.92 showed moderately to heavily polluted water. With values for the Diatomeae index of more than 0.2, the quality of the eight sites during the period of the study showed that the aquatic environment was eutrophic. Phytoplankton and their indexes are useful tools for assessing water environment quality.

Keywords: Nhu Y river, Palmer index, phytoplankton, Shannon-Weiner index, water quality.

Classification number: 3.4

Introduction

Phytoplankton are free-floating microscopic organisms with the potential to produce energy from photosynthesis. They play a significant role in their environment as primary producers and are the base of the food web in aquatic ecosystems. The algal groups are strongly sensitive to even a slight rise or fall in water quality. The composition and abundance of phytoplankton are extremely dependent on the environmental factors of their habitat, such as sunlight, dioxide, carbon, and nutrients. These conditions affect the density and distribution of algae throughout the water levels [1-5]. The presence of algae is important for assessing the resources and biodiversity of the water body. Evaluating the presence and distribution of phytoplankton contributes to clarifying the environmental characteristics and impact of changes in water quality on algal communities because of their high sensitivity to changing environmental conditions [6].

This article includes a status assessment of nutrient and organic pollution of Nhu Y river using the Palmer index [7], Shannon-Weiner diversity index [8], and Diatomeae index [9]. These contribute to quickly developing an environmental monitoring tool based on the distribution of phytoplankton in Nhu Y river.

Materials and methods

Study area and sample collection

Nhu Y river is located in the northeast of Hue city and is approximately 12 km in length. Generally, Nhu Y river plays an important role in the daily life and productive activities of Hue citizens, such as the supply household water, irrigation, and agriculture. Currently, Nhu Y river receives a volume of wastewater from the processes associated with living, farming, agriculture and the traditional craft activities of

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human beings.

Nhu Y river is an artificial river and is separated from Huong river by Dap Da dam. The water volume of the Nhu Y river receives a small flow from Loi Nong river as well as wastewater from the surrounding residents and fields. Moreover, Nhu Y river flows slowly and therefore its aquatic ecosystem is similar to the aquatic ecosystems of standing waterbodies.

The qualitative and quantitative samples were collected monthly from March to August in 2011 at eight sites (Table 1, Fig. 1). Phytoplankton samples were collected by means of a pyramid-shaped phytoplankton net that was 0.9 m long, 0.3 m in diameter, and had a mesh size of 20 μm ; they were preserved in a solution of Lugol in the field. These samples were kept on ice until they were analysed in the laboratory.

Table 1. The sampling sites along Nhu Y river.

Sampling sites	Local names	Latitude	Longitude
Y1	Dap Da bridge	16°28.400' N	107°35.711' E
Y2	Vi Da bridge	16°28.285' N	107°36.039' E
Y3	Van Duong village, Xuan Phu commune, Hue city	16°28.461' N	107°36.411' E
Y4	Tung Thien Vuong bridge	16°28.944' N	107°36.258' E
Y5	Chiet Bi village, Phu Thuong commune, Phu Vang district	16°29.330' N	107°36.649' E
Y6	Cong Luong village, Thuy Van commune, Huong Thuy town	16°29.464' N	107°37.330' E
Y7	Sam bridge	16°28.894' N	107°38.961' E
Y8	Next to rice fields, Thuy Thanh commune	16°28.321' N	107°38.521' E

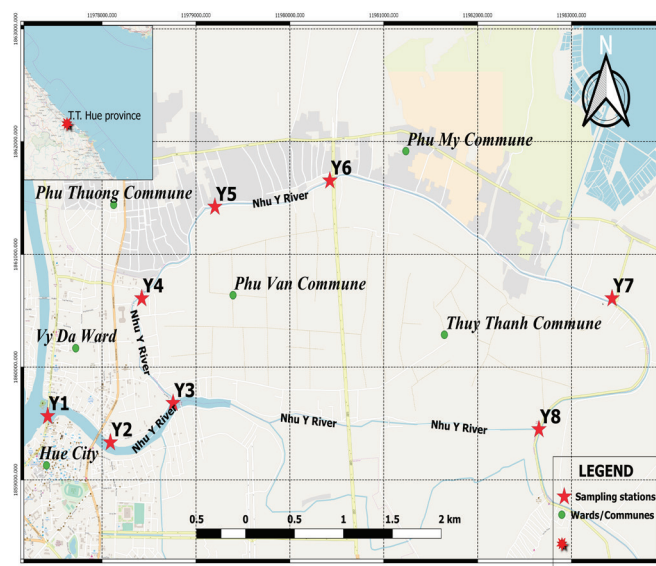


Fig. 1. Sampling sites in Nhu Y river.

Phytoplankton analyses

Phytoplankton were observed under at 200-400X magnification (Olympus BX51 microscope). Species identification was based on morphology according to studies such as Shirota (1966) [10]; Fukuyo, et al. (1990) [11]; Komarek and Anagnostidis (1999; 2005) [12, 13]; Yamagishi and Akiyama (1995) [14]; Canter-Lund and Lund (1995) [15]; Nguyen (2003) [9]; Nguyen, et al. (2007) [16]; and Duong and Vo (1997) [17]. A Sedgewick Rafter counting chamber was used to determine phytoplankton density.

Calculation of indexes

Palmer index:

The Palmer index is based on the presence of algal genera, which have the organic pollution tolerance in water bodies. The score was fixed in a range from 1 to 5 depending on the genus, the larger number indicating greater pollution. Algal genera that are less tolerant to organic pollution were assigned a lower number. Algal genera that are highly tolerant of organic pollution were assigned a higher number (Table 2).

Table 2. List of algal genera of tolerant organic-pollution according to Palmer [7].

Genus	Pollution index	Genus	Pollution index
1. <i>Anacystis</i>	1	11. <i>Micractinium</i>	1
2. <i>Ankistrodesmus</i>	2	12. <i>Navicula</i>	3
3. <i>Chlamydomonas</i>	4	13. <i>Nitzschia</i>	3
4. <i>Chlorella</i>	3	14. <i>Oscillatoria</i>	5
5. <i>Closterium</i>	1	15. <i>Pandorina</i>	1
6. <i>Cyclotella</i>	1	16. <i>Phacus</i>	2
7. <i>Euglena</i>	5	17. <i>Phormidium</i>	1
8. <i>Gomphonema</i>	1	18. <i>Scenedesmus</i>	4
9. <i>Lepocinclis</i>	1	19. <i>Stigeoclonium</i>	2
10. <i>Melosira</i>	1	20. <i>Synedra</i>	2

After confirming the presence of these algal genera in the sample, the pollution index factors of the algae present were calculated. A pollution index score ≥ 20 was considered to indicate high organic pollution; a score from 15 to 19 indicated probable organic pollution. Lower scores indicated less organic pollution.

The Shannon-Wiener diversity and Diatomeae indexes were calculated following Wilhm (1975) [8] and Nguyen (2003) [9], respectively.

Shannon-Wiener diversity index (H'):

$$H' = - \sum_{i=1}^n \frac{n_i}{N} \log_2 \frac{n_i}{N}$$

where H' : diversity index; N : the number of individuals in the samples; and n_i : the number of individuals in the i^{th} [8].

Diatomeae index:

$$\text{Diatomeae index} = C \cdot P^{-1}$$

in which: C : number of species of Centrales; and P : number of species of Pennales [9].

Results

Species composition and density of phytoplankton

Phytoplankton from the eight monitoring sites on Nhu Y river comprised 117 species distributed among six divisions (Table 3). Among the phytoplankton groups, the Chlorophyta phylum dominated with 45 species, 38.5% of the total. This was followed by the Euglenophyta phylum with 31 species (26.5%). There were 24 species of Cyanobacteria (20.5%); 14 species of diatoms (11.9%), two species of the Cryptophyta phylum (1.7%), and only one species of the Dinophyta phylum (0.9%). In general, the number of phytoplankton species was higher in June, July, and August, and lower in March, April, and May. The number of species of phytoplankton fluctuated from 76 (March) to 113 (August) in each survey (Table 4).

Table 3. Structure of phytoplankton communities from Nhu Y river during the period of the study.

Phylum	March	April	May	June	July	August	Total	%
Cyanobacteria	4	9	15	21	23	23	24	20.5
Bacillariophyta	13	12	12	13	11	14	14	11.9
Chlorophyta	32	36	41	39	43	43	45	38.5
Euglenophyta	24	19	26	29	30	30	31	26.5
Cryptophyta	2	2	2	2	2	2	2	1.7
Dinophyta	1	1	1	1	1	1	1	0.9
Total species	76	79	97	105	110	113	117	100

Almost all the phytoplankton species and genera present in Nhu Y river were those found in fresh water, such as *Anabaena*, *Anthrospira*, *Microcystis*, *Oscillatoria*, *Pandorina*, *Scenedesmus*, *Ankistrodesmus*, *Pediastrum*, *Crucigenia*, *Coelastrum*, *Chlorella*, *Actinastrum*, *Euglena*, *Phacus*, *Trachelomonas*, *Melosira*, *Cyclotella*, *Rhodomonas*, and *Cryptomonas* (Fig. 2). Hence, the aquatic ecosystem in the area studied was mostly influenced by fresh water from the hinterland.

Table 4. List of algal species present at Nhu Y river.

No.	Taxa	March	April	May	June	July	August
Cyanobacteria							
1	<i>Anabaena affinis</i> Lemmermann, 1898			+	+	+	+
2	<i>Anabaena circinalis</i> Rabenhorst ex Bornet and Flahault, 1886			+	+	+	+
3	<i>Anabaena spiroides</i> Klebahn, 1895		+	+	+	+	+
4	<i>Anabaena</i> sp.				+	+	+
5	<i>Anthrospira platensis</i> Gomont, 1892		+	+	+	+	+
6	<i>Anthrospira</i> sp.				+	+	+
7	<i>Aphanocapsa delicatissima</i> West and West, 1912			+	+	+	+
8	<i>Aphanizomenon aphanizomenoides</i> (Forti) Horecká and Komárek, 1979				+	+	
9	<i>Aphanizomenon</i> sp.			+	+	+	+
10	<i>Jaaginema</i> sp.		+	+	+	+	+
11	<i>Limnithrix planctonica</i> (Woloszynski) Meffert, 1988		+	+	+	+	+
12	<i>Merismopedia punctata</i> Meyen, 1839	+	+	+	+	+	+
13	<i>Merismopedia tenuissima</i> Lemmermann, 1898	+	+	+	+	+	+
14	<i>Microcystis aeruginosa</i> (Kützinger) Kützinger, 1846			+	+	+	+
15	<i>Microcystis flosaquae</i> (Wittrock) Kirchner, 1898					+	+
16	<i>Microcystis protocystis</i> Crow, 1923				+		+
17	<i>Microcystis wesenbergii</i> (Komárek) Komárek, 1968			+	+	+	+
18	<i>Oscillatoria curvipes</i> Agardh and Gomont, 1892					+	+
19	<i>Oscillatoria limosa</i> Agardh ex Gomont, 1892	+	+		+	+	+
20	<i>Oscillatoria perornata</i> Skuja, 1949			+	+	+	+
21	<i>Oscillatoria agardhii</i> Gomont, 1892	+	+	+	+	+	+
22	<i>Phormidium</i> sp.		+		+	+	+
23	<i>Raphidiopsis curvata</i> Fritsch and Rich, 1930			+	+	+	+
24	<i>Spirulina princeps</i> West and West, 1902					+	+
Bacillariophyta							
25	<i>Cyclotella comta</i> (Ehrenberg) Kützinger, 1849	+	+	+	+		+
26	<i>Cyclotella</i> sp1.	+	+	+	+	+	+
27	<i>Cyclotella</i> sp2.	+	+	+	+	+	+
28	<i>Gomphonema</i> sp.	+	+		+		+
29	<i>Gyrosigma</i> sp.	+	+	+	+	+	+
30	<i>Melosira granulata</i> (Ehrenberg) Ralfs, 1861	+	+	+	+	+	+
31	<i>Melosira</i> sp.	+	+	+	+	+	+
32	<i>Navicula</i> sp.	+	+	+	+	+	+
33	<i>Nitzschia closterium</i> (Ehrenberg) W. Smith, 1853	+	+	+	+	+	+
34	<i>Nitzschia</i> sp.	+	+	+	+	+	+
35	<i>Pimularia</i> sp.	+	+		+		+
36	<i>Surirella tenera</i> Gregory, 1856			+	+	+	+
37	<i>Synedra ulna</i> (Nitzsch) Ehrenberg, 1832	+	+	+	+	+	+
38	<i>Synedra</i> sp.	+	+	+		+	+
Chlorophyta							
39	<i>Actinastrum hantzschii</i> Lagerheim, 1882	+	+	+	+	+	+
40	<i>Ankistrodesmus acicularis</i> (Braun) Korshikov, 1953	+	+	+	+	+	+
41	<i>Ankistrodesmus arcuatus</i> Korshikov, 1953	+	+	+	+	+	+
42	<i>Ankistrodesmus falcatus</i> (Corda) Ralfs, 1848					+	+
43	<i>Ankistrodesmus gracilis</i> (Reinsch) Korshikov, 1953	+	+	+	+	+	+
44	<i>Ankistrodesmus longissimus</i> (Lemmermann) Wille, 1909	+	+	+	+	+	+
45	<i>Chlamydomonas</i> sp.	+	+	+	+	+	+
46	<i>Chlorella</i> sp.	+	+	+	+	+	+
47	<i>Closterium gracile</i> Brébisson ex Ralfs, 1848		+	+	+	+	+
48	<i>Closterium</i> sp.	+	+	+	+	+	+
49	<i>Coelastrum sphaericum</i> Nägeli, 1849	+	+	+	+	+	+
50	<i>Coelastrum microporum</i> Nägeli, 1855	+	+	+	+	+	+
51	<i>Crucigenia lauterbornii</i> Schmidle, 1900	+	+	+	+	+	+
52	<i>Crucigeniella rectangularis</i> (Nägeli) Komárek, 1974	+	+	+	+	+	+
53	<i>Dictyosphaerium ehrenbergianum</i> Nägeli, 1849		+	+	+	+	+
54	<i>Eudorina elegans</i> Ehrenberg, 1832	+		+	+	+	+
55	<i>Gonium quadratum</i> Pringsheim ex Nozaki, 1990			+	+	+	+
56	<i>Micractinium pusillum</i> Fresenius, 1858		+	+	+	+	+
57	<i>Micractinium quadrisetum</i> (Lemmermann) Smith, 1916	+	+	+	+	+	+
58	<i>Oocystis borgei</i> J. Snow, 1903		+	+	+	+	+
59	<i>Pandorina morum</i> (Müller) Bory de Saint-Vincent, 1824	+	+	+	+	+	+

60	<i>Pandorina</i> sp1.	+	+	+	+	+	+
61	<i>Pandorina</i> sp2.	+	+	+	+		
62	<i>Pediastrum biradiatum</i> Meyen, 1829	+	+	+		+	+
63	<i>Pediastrum duplex</i> Meyen, 1829	+	+	+	+	+	+
64	<i>Pediastrum simplex</i> Meyen, 1829					+	+
65	<i>Pediastrum tetras</i> (Ehrenberg) Ralfs, 1845	+	+	+	+	+	+
66	<i>Scenedesmus acuminatus</i> (Lagerheim) Chodat, 1902	+	+	+	+	+	+
67	<i>Scenedesmus arcuatus</i> (Lemmermann) Lemmermann 1899	+	+	+	+	+	+
68	<i>Scenedesmus bicaudatus</i> Dedusenko, 1925	+		+	+	+	+
69	<i>Scenedesmus curvatus</i> Bohlin, 1897	+	+	+			
70	<i>Scenedesmus denticulatus</i> Lagerheim, 1882	+	+	+	+	+	+
71	<i>Scenedesmus ellipsoideus</i> Chodat, 1926	+	+			+	+
72	<i>Scenedesmus obliquus</i> (Turpin) Kützing, 1833		+	+	+	+	+
73	<i>Scenedesmus perforatus</i> Lemmermann, 1903			+	+	+	+
74	<i>Scenedesmus protuberans</i> Fritsch and Rich, 1929	+	+	+	+	+	+
75	<i>Scenedesmus quadricauda</i> (Turpin) Brébisson, 1835	+	+	+	+	+	+
76	<i>Schroederia setigera</i> (Schroder) Lemmermann, 1898		+	+	+	+	+
77	<i>Staurastrum dickiei</i> Ralfs, 1848	+	+	+	+	+	+
78	<i>Staurastrum natator</i> West, 1892					+	+
79	<i>Tetraedron constrictum</i> Smith, 1916			+	+	+	+
80	<i>Tetraedron incus</i> (Teiling) Smith, 1926	+	+	+	+	+	+
81	<i>Tetraedron trigonum</i> (Nägeli) Hansgirg, 1888	+	+	+	+	+	+
82	<i>Tetrastrum heteracanthum</i> (Nordstedt) Chodat, 1895			+	+	+	+
83	<i>Treubaria triappendiculata</i> Bernard, 1908	+	+	+	+	+	+
Euglenophyta							
84	<i>Euglena acus</i> Ehrenberg, 1830	+	+	+	+	+	+
85	<i>Euglena caudata</i> Hübner, 1886				+	+	+
86	<i>Euglena elongata</i> Schewiakoff, 1892	+			+	+	+
87	<i>Euglena gracilis</i> Klebs, 1883	+	+	+	+	+	+
88	<i>Euglena oxyuris</i> Schmarda, 1846	+	+	+	+	+	+
89	<i>Euglena rostrifera</i> Johnson, 1944	+	+	+	+	+	+
90	<i>Euglena sociabilis</i> Dangeard, 1902	+		+	+	+	+
91	<i>Euglena spirogyra</i> Ehrenberg, 1832	+	+	+	+	+	+
92	<i>Euglena viridis</i> Ehrenberg, 1830	+	+	+	+	+	+
93	<i>Lepocinclis fusiformis</i> (Carter) Lemmermann, 1901			+	+	+	+
94	<i>Lepocinclis ovum</i> (Ehrenberg) Lemmermann, 1901	+	+	+	+	+	+
95	<i>Lepocinclis reewykiana</i> Conrad, 1934					+	+
96	<i>Lepocinclis salina</i> Fritsch, 1914	+	+	+	+	+	+
97	<i>Phacus anomalus</i> Fritsch and Rich, 1929	+	+	+	+	+	+
98	<i>Phacus comortus</i> Bourrelly, 1952				+	+	+
99	<i>Phacus helikoides</i> Pochmann, 1942				+	+	+
100	<i>Phacus longicauda</i> (Ehrenberg) Dujardin, 1841	+	+	+	+	+	+
101	<i>Phacus orbicularis</i> Hübner, 1886	+			+	+	+
102	<i>Phacus pleuronectes</i> (Müller) Dujardin, 1841	+	+	+	+	+	+
103	<i>Phacus</i> sp.	+	+	+	+	+	+
104	<i>Phacus tortus</i> (Lemmermann) Skvortzov, 1928	+	+	+	+	+	+
105	<i>Phacus trapezoides</i> Stawinski, 1969	+	+	+	+	+	+
106	<i>Strombomonas australica</i> (Playfair) Deflandre, 1930						+
107	<i>Strombomonas longicauda</i> (Swirensko) Deflandre, 1930	+		+		+	+
108	<i>Strombomonas napiformis</i> (Playfair) Deflandre, 1930	+	+	+	+	+	+
109	<i>Trachelomonas armata</i> (Ehrenberg) Stein, 1878		+	+	+	+	+
110	<i>Trachelomonas intermedia</i> Dangeard, 1902	+	+	+	+	+	+
111	<i>Trachelomonas hispida</i> (Perty) Stein, 1878	+	+	+	+	+	+
112	<i>Trachelomonas nova</i> Drezepolski, 1925				+	+	+
113	<i>Trachelomonas ovalis</i> Daday, 1913	+	+	+	+	+	+
114	<i>Trachelomonas</i> sp.				+	+	+
Cryptophyta							
115	<i>Cryptomonas</i> sp.	+	+	+	+	+	+
116	<i>Rhodomonas</i> sp.	+	+	+	+	+	+
Dinophyta							
117	<i>Peridinium</i> sp.	+	+	+	+	+	+
Total Species		76	79	97	105	110	113

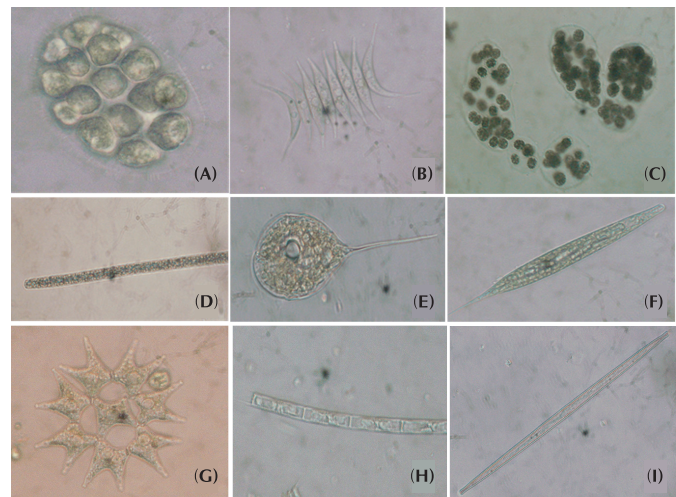


Fig. 2. Some widespread genera of algae in Nhu Y river. (A) *Pandorina*, (B) *Scenedesmus*, (C) *Microcystis*, (D) *Oscillatoria*, (E) *Phacus*, (F) *Euglena*, (G) *Pediastrum*; (H) *Melosira*; (I) *Synedra*. Scale bars = 20 µm.

Phytoplankton densities were high, ranging from 110,146 to 5,964x10³ individuals/litre, and the values were the highest at the sampling site 2 (Vi Da bridge) and lowest at the site 8 (Thuy Thanh commune) (Fig. 3). The dominant species in the zone of study were *Oscillatoria agardhii*, *Arthrospira platensis*, *Jaaginema* sp., *Microcystis wesenbergii*, *Pandorina* sp2., *Cryptomonas* sp., *Rhodomonas* sp. Among the dominant species, *Oscillatoria agardhii* occurred in most of the studied area.

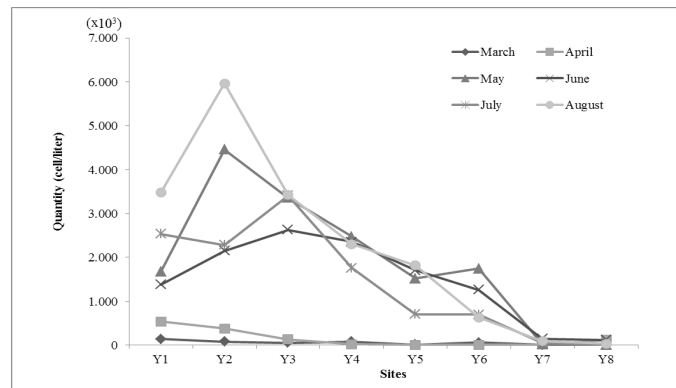


Fig. 3. Phytoplankton density in Nhu Y river during period of study.

Application of bio-indexes to assess the water quality in Nhu Y river

Palmer index:

Eighteen of the 20 genera in Palmer's algal genus list were present in Nhu Y river at the six monitoring times (Table 5). Many genera, such as *Ankistrodesmus*, *Chlamydomonas*, *Chlorella*, *Cyclotella*, *Melosira*, *Euglena*, *Oscillatoria*, *Pandorina*, *Phacus*, and *Scenedesmus* appeared on all six occasions.

Table 5. Algal genus tolerant of organic pollution in Nhu Y river.

No.	Genus	Sampling sites							
		Y1	Y2	Y3	Y4	Y5	Y6	Y7	Y8
1	<i>Anacystis</i>	-	-	-	-	-	-	-	-
2	<i>Ankistrodesmus</i>	A	A	A	A	A	A	A	A
3	<i>Chlamydomonas</i>	A	A	A	A	A	A	A	B
4	<i>Chlorella</i>	A	A	A	A	A	A	A	B
5	<i>Closterium</i>	D	C	C	B	B	C	A	A
6	<i>Cyclotella</i>	A	A	A	A	A	A	A	A
7	<i>Euglena</i>	A	A	A	A	A	A	A	A
8	<i>Gomphonema</i>	-	E	D	E	D	B	B	A
9	<i>Lepocinclis</i>	B	B	A	B	B	A	A	A
10	<i>Melosira</i>	A	A	A	A	A	A	A	A
11	<i>Micractinium</i>	A	B	A	B	B	B	D	D
12	<i>Navicula</i>	D	B	B	C	D	B	A	A
13	<i>Nitzschia</i>	B	B	B	B	A	A	A	A
14	<i>Oscillatoria</i>	A	A	A	A	A	A	A	A
15	<i>Pandorina</i>	A	A	A	A	A	A	A	A
16	<i>Phacus</i>	A	A	A	A	A	A	A	A
17	<i>Phormidium</i>	E	F	F	E	C	E	D	D
18	<i>Scenedesmus</i>	A	A	A	A	A	A	A	A
19	<i>Stigeoclonium</i>	-	-	-	-	-	-	-	-
20	<i>Synedra</i>	B	B	B	B	A	A	A	A

Note: (-): species were not present; (A): species were present during all six monitoring periods; (B): species were present during five monitoring periods; (C): species were present during four monitoring periods; (D): species were present during three monitoring periods; (E): species were present during two monitoring periods; (F): species were present during one monitoring period.

The value of algal genus pollution index during the monitoring in 2011 was relatively high, ranging from 30 to 41. In Nhu Y river, the Palmer's index value was generally higher in April and August than in March, May, June and July. Furthermore, the index value was the highest (41) at sites Y5 and Y6 (in April) and at Y3, Y7, and Y8 (in August) (Table 6).

Shannon-Weiner diversity index:

The phytoplankton diversity index values in this survey fluctuated from 0.66 to 2.92 (Table 6). In Nhu Y river, the diversity index values of all sites were less than 1 in May (except at Y7 and Y8), while the index value of all sites ranged from 1 to 2 in July and August (except at Y7 and Y8 in July, and Y8 in August). In addition, the values of the algal diversity index were greater than 2 at all sites in March, April (except Y8), and June.

Diatomeae index:

The values of the Diatomeae index in this study are presented in Table 6. In general, the Diatomeae index values were greater than 0.2 at most stations during the survey period.

Table 6. The values of the Palmer pollution index, Shannon-Wiener diversity index (H'), and Diatomeae index of phytoplankton in Nhu Y river.

Sites	Mar	Apr	May	Jun	Jul	Aug
Palmer index						
Y1	31	38	38	39	36	36
Y2	34	39	36	39	38	39
Y3	33	40	38	39	36	41
Y4	30	39	35	39	37	40
Y5	36	41	39	39	37	38
Y6	40	41	39	38	38	40
Y7	39	40	35	39	41	41
Y8	39	40	39	37	37	41
Shannon-Wiener index (H')						
Y1	2.51	2.46	0.88	2.49	1.60	1.70
Y2	2.52	2.31	0.97	2.53	1.64	1.67
Y3	2.60	2.57	0.66	2.36	1.78	1.61
Y4	2.84	2.19	0.76	2.39	1.67	1.18
Y5	2.90	2.93	0.86	2.47	1.83	1.67
Y6	2.16	2.88	0.79	2.47	1.90	1.92
Y7	2.79	2.65	2.87	2.83	2.30	1.69
Y8	2.30	1.54	2.74	2.65	2.79	2.92
Diatomeae index						
Y1	-	1.3	1.0	1.7	2.0	1.0
Y2	2.5	1.3	1.3	1.3	0.8	1.0
Y3	5	1.3	1.3	1.0	1.0	0.8
Y4	-	1.3	1.0	1.3	1.5	0.8
Y5	1.3	0.6	0.6	1.7	1.0	1.0
Y6	1.0	0.8	1.0	1.0	0.6	0.6
Y7	0.6	0.6	0.4	0.8	0.4	0.8
Y8	0.2	1.0	0.6	0.6	0.3	0.6

Discussion

Some investigations of phytoplankton in rivers of Vietnam have been performed and published. Luong and Phan (2014) [6] recorded 280 species of phytoplankton in Huong river systems in which Chlorophyta contributed the highest number of species. In a study of phytoplankton in La Nga river, 202 algae species were identified of which Chlorophyta were also the greatest number [18]. In Thi Vai river, 98 taxa were recorded, of which Bacillariophyta contributed the greatest number [19]. During the monitoring of the current study, Chlorophyta were dominant in terms of species numbers. The distribution of the number of species in Nhu Y river (117 taxa recorded) is considered average compared to some other rivers. However, the structure of phytoplankton communities in the rivers cited and in Nhu Y river comprised similar algal phyla, such as Cyanobacteria, Chlorophyta, Bacillariophyta, Euglenophyta, and Dinophyta.

Generally, the phytoplankton densities in this survey were very high, with over 10^6 individuals/litre, with strong growth of the *Oscillatoria agardhii* species. In addition, Nhu Y river was experiencing eutrophication during the

monitoring because it was affected by domestic wastewater, construction, irrigation, and agricultural activities.

According to Palmer (1969) [7], the quality of the water in Nhu Y river was characterised by highly organically polluted conditions because the index values were over 20 at all sites during the monitoring in 2011. Genera such as *Anabaena*, *Microcystis*, *Oscillatoria*, *Euglena*, *Phacus*, *Scenedesmus*, *Chlamydomonas*, *Navicula*, *Chlorella*, *Nitzschia* and *Ankistrodesmus* were found in organically polluted water, an assertion that was supported by Ratnasabapathy (1975) [20]; Gunale and Balakrishnan (1981) [21]; Jafari and Gunale (2006) [22]; Shams, et al. (2012) [23]; and Shams and Karimian (2017) [24]. Similar genera were recorded in the present investigation. *Oscillatoria* species, which were found to be the most active participants at all stations, may be good indicators of contaminated water bodies as similar observations were recorded by Sanjib, et al. (2007) [25] and Rai, et al. (2008) [26].

The Shannon-Wiener diversity index has been widely applied Wilhm (1975) [8] proposed three water quality categories for the Shannon-Weiner diversity index. A high H' value suggests a more vigorous ecosystem and, in contrast, a low H' value suggests meagre diversity in a structured community and a less healthy ecosystem. In the current study, the range of the H' value in Nhu Y river was 0.66-2.92 (Table 6). Nhu Y water sources can only be classified into categories II and III, indicating moderate and heavy pollution, respectively [8]. Similar results were recorded at the Mae Moh power plant [27] and the two waterbodies at Tiruvannamalai [28].

According to Nguyen (2003) [9], the values of the Diatomeae index at the survey sites in the Nhu Y river during the period of study were greater than 0.2, indicating that the water quality there was eutrophic.

Conclusions

During the survey period of March to August in 2011, 117 algal species belonging to six phyla were recorded in Nhu Y river, including Cyanobacteria, Chlorophyta, Bacillariophyta, Euglenophyta, Cryptophyta, and Dinophyta, of which the Chlorophyta and Euglenophyta phyla were dominant in terms of species numbers. In general, the algal density recorded in the present study was very high and reached millions of individuals/litre.

We identified 18 algal genera in Palmer's list (1969) [7] that are tolerant of organic pollution. The values of the Palmer index, the Shannon-Weiner diversity index and the Diatomeae index reflect the organic pollution and the eutrophic condition of Nhu Y river. Studies of phytoplankton are very important because in their habitat phytoplankton

play a crucial role as primary producers in the food web. Apart from physicochemical methods, phytoplankton indexes such as the Palmer, algal biodiversity, and Diatomeae indexes are useful tools for assessing the water quality of Nhu Y river.

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The authors declare that there is no conflict of interest regarding the publication of this article.

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Developmental and bone development indexes of medaka fish at 11 and 16 days of age

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Abstract:

The teleost fish medaka (*Oryzias latipes*) is currently used by laboratories worldwide as a model for many human diseases, including bone diseases. Our research group focuses on a medaka model for osteoporosis. To characterize the bone indexes of diseased animals, the bone development indexes of healthy wild-type fish are required for comparative analysis. Thus, this study examined the developmental and bone development indexes of wild-type medaka larvae at 11 and 16 days of age, the two developmental stages selected for the analysis of diseased patterns in osteoporosis-induced animals. The assessed parameters included the total body length of live larvae, number of caudal fin rays, vertebrae, and neural and hemal arches, as well as the total areas of mineralized vertebrae and lengths of mineralized arches. The obtained data are important for subsequent studies on bones using this fish model.

Keywords: bone development indexes, medaka, mineralized bone, vertebrae.

Classification number: 3.4

Introduction

Medaka fish (*Oryzias latipes*) has recently become a valuable and favourable *in vivo* model to study human diseases [1]. Native to flooded rice fields in Japan, Taiwan and other areas of Southeast Asia, this small, egg-laying teleost has been brought to laboratory and examined for a long time [1, 2]. The biology of medaka fish indicates numerous advantages for a model organism: simple rearing, low-cost maintenance, short generation time, transparent embryo, and especially, easy techniques for live *in vivo* imaging, as well as for transgenesis and genetic manipulations [1-4]. More importantly, medaka shares a remarkable similarity, particularly at the cellular and molecular levels, with humans in mechanisms underlying biological processes [5]. Thus, medaka has been used as models in a wide variety of human diseases such as mental illnesses, neurodegenerative ailments [6, 7], cancer [8] and metabolism disorders [1, 9, 10], including bone diseases [11-13].

Osteoporosis, a common bone disease featured by reduced bone mass, destructed bone structures and high risk of bone fractures, is an important public health concern [14]. Finding better drugs and treatments for the disease is always of great interest to researchers [14, 15]. In our laboratory, we have used a transgenic medaka model for osteoporosis [13] and established methods and procedures for evaluating the anti-osteoporosis effect of tested substances [16]. In one of these methods, the bone indexes of experimental fish at 11 and 16 days of age have been protocolized for analysis. Therefore, we conducted this study to provide data on bone developmental hallmarks and characteristics for the fish at these two developmental stages that can be used as reference for further research. The analysed indexes included the total body length of live larvae, number of bone structures, including caudal fin rays, vertebrae and neural and hemal arches, as well as the areas of mineralized vertebrae and

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lengths of mineralized neural and hemal arches.

Materials and methods

Chemicals

The main chemicals used in this study were NaCl, KCl, Na_2HPO_4 , KH_2PO_4 , MgCl_2 (Sigma), PFA (paraformaldehyde Sigma P6148) and Alizarin red (Sigma A5533).

Fish lines

In this study, we used wild-type medaka fish originally provided by the Winkler's group from the National University of Singapore (NUS). The fish were raised and maintained at the Laboratory at the Department of Physiology and Human Biology, Faculty of Biology, VNU University of Science.

Fish maintenance and husbandry

Fish were raised in a small fish facility with temperature set at 26 to 28°C and a 14-h/10-h light/dark cycle to induce spawning. For the synchronous development of fish in the study, embryos were kept at a density of 20 individuals in 1×E3 medium in a 12-cm Petri dish and incubated in an incubator set at 30°C. Fish larvae were also raised at the same density at 30°C during experiments.

Live imaging of medaka larvae for the measurement of total length

The total length or body length of the analysed fish was measured on their live images. Live wild-type fish of 11 and 16 days post-fertilization (dpf) were immobilized by anaesthetizing with tricaine 0.01% and mounted in 3% methyl cellulose on a microscope slide. The images of live fish were obtained by an Optika B5 camera under a Zeiss Stemi 2000-C stereoscope (Carl Zeiss AG). Total length was measured on the obtained images using the “line function” of ImageJ software and defined as the length of the line drawn from the tip of the snout along the midline to the posterior edge of the caudal fin ray (see details in the results).

Alizarin red bone staining of PFA-fixed larvae

Fish were fixed and stained by Alizarin red that visualizes mineralized bone in purple following standard protocols as previously described [13, 17].

Imaging and measurement of the mineralization indexes of bone structures

Pictures were taken on Alizarin red-stained fish of 11 and 16 dpf as previously described [13] using an Axioplan Z microscope equipped with an Optika B5 camera. Bone

mineralization indexes need to be determined, including the length of neural arches (na), length of hemal arches (ha) and area of vertebral body (vb). Measurement was performed on images of Alizarin red-stained larvae using ImageJ software (<https://imagej.nih.gov/ij/>).

Statistical analysis

Statistical analysis was conducted using GraphPad software v5 for t-test to determine the significant difference in bone indexes between experimental fish groups.

Results and discussion

Total length of 11 dpf and 16 dpf fish

Total length or body length is an indicator for the development in fish. It is defined as the length of the line measured from the tip of the snout along the midline to the posterior edge of the caudal fin ray [18] (dark blue lines in Fig. 1A).

Total length measurement was performed for two live fish groups of 11 dpf and 16 dpf with over 30 individuals each ($n=37$ for 11 dpf and $n=49$ for 16 dpf group). The mean values of the total length between two groups were calculated and statistically compared by t-test (Fig. 1B).

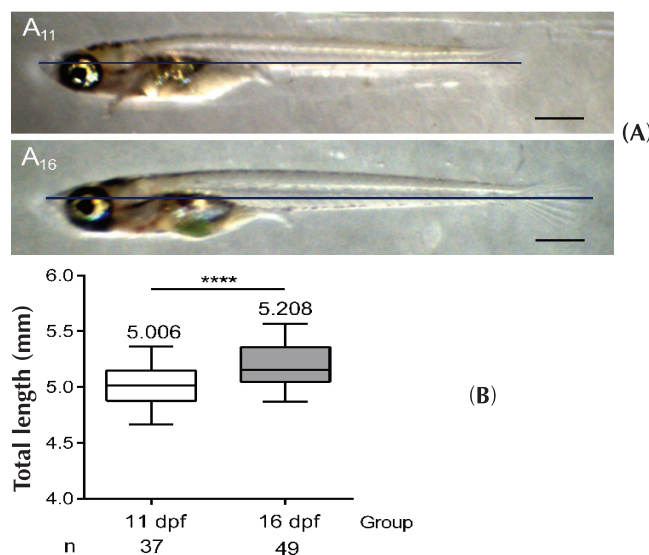


Fig. 1. Total length of fish at 11 dpf and 16 dpf: (A) Live images of fish at 11 dpf (A_{11}) and 16 dpf (A_{16}) and the measurement of their total lengths. Live larvae were mounted in methyl cellulose and imaged under a stereoscope with 1.5×magnification; dark blue lines drawn from the tip of the snout along the midline to the posterior edge of the caudal fin ray indicate the total/body length of the corresponding fish. Scale bar: 0.5 mm; (B) The mean values of the total length of fish from the two groups. Data are expressed as the mean values of total length, **** $p < 0.0001$. Bars indicate standard deviation.

Representative images (Fig. 1A) illustrate the increase in the total length of 16 dpf compared to 11 dpf fish. The mean values of the total length were 5.006 ± 0.027 mm and 5.208 ± 0.029 mm for 11 dpf and 16 dpf fish, respectively; the difference between these two values was statistically extremely significant ($p < 0.0001$). Thus, from 11 to 16 dpf, the total length of fish increased approximately 0.2 mm; in other words, the fish grew roughly 4% in length during five days of development.

Several studies have used total length as a developmental hallmark for staging fish [19, 20]. Chatani, et al. reported the total body lengths of medaka from day 3 to day 22 post-hatching (dph) as a developmental indicator for assessing the appearance and development of osteoclasts in the TRAP-GFP transgenic medaka larva [19]. In this study, the total length of the 3 dph transgenic larva was roughly 5 mm [19], similar to the length of our 11 dpf wild-type fish. However, the hatching time of the fish in Chatani's study was not reported [19]; moreover, as the developmental rate of fish can vary significantly depending on rearing conditions, especially on the culturing temperature [21], our data can be referenced by research that raises the fish with similar protocols (see details in the methods).

Mineralized bone structures of the fish

The mineralized bone structures of 11 dpf and 16 dpf fish were visualized by Alizarin red staining on PFA-fixed larvae (Fig. 2). In both of these fish groups, mineralized structures were observed in the head, trunk and tail regions, namely, parasphenoid (ps), operculum (op), cleithrum (cl), anterior basicranial commissure (abc), supraoccipital (soc), basibranchial plt. (bp) in the head of 11 dpf (Figs. 2A, 2A'), and additionally dentary (den), quadrate (qu), hyosymplectic (hys), ceratobranchial (cb) in the head of 16 dpf fish (Figs. 2B, 2B'); a vertebral column with a number of forming vertebrae each, if fully appeared, consisting of a vertebral body (vb) with a pair of forming neural (na) and hemal arches (ha) (Figs. 2A, 2B, 2A'', 2B'') in the trunk; and a number of caudal fin rays (fr) in the tail (Figs. 2A, 2B).

We previously reported the mineralized bone structures of 10 dpf and 15 dpf fish [22] compared to 11 dpf and 16 dpf fish, respectively, in this study; we did not observe any difference in the appearance of mineralized components (neither between 10 and 11 dpf nor between 15 and 16 dpf).

We subsequently quantified the indexes of some bone structures. First, we evaluated the number of certain types of mineralized structures that appeared in multiple units,

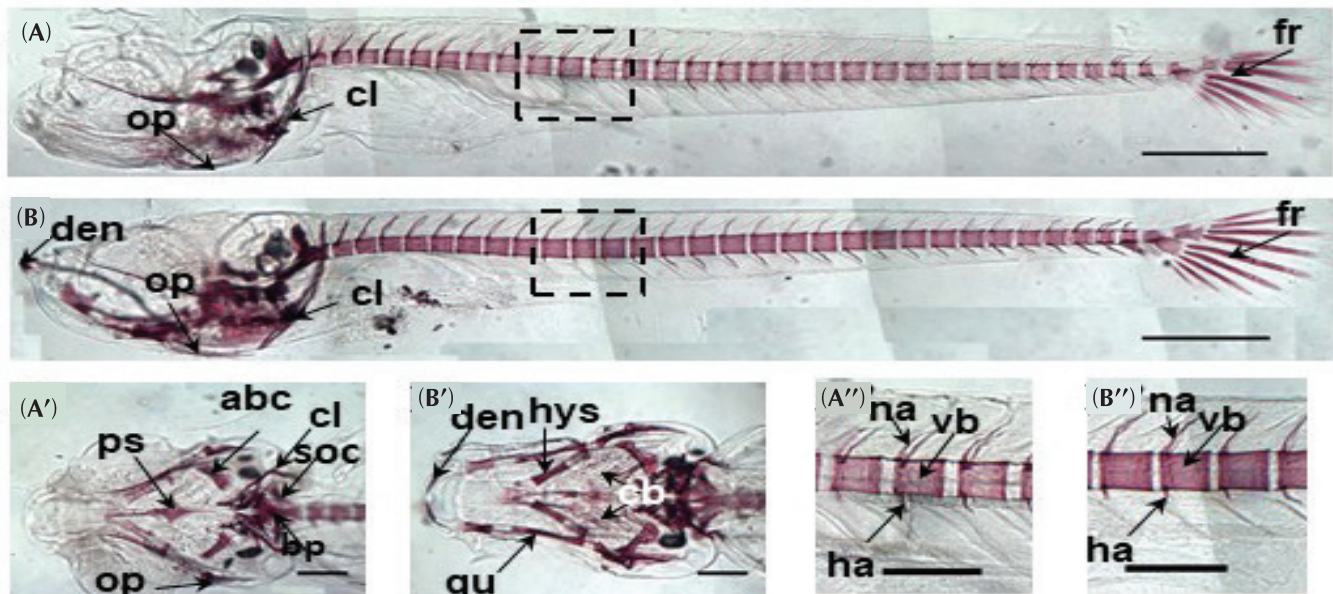


Fig. 2. Mineralized bone structures of fish larvae at 11 dpf and 16 dpf. Images of Alizarin red-stained mineralized bone structures of medaka fish at 11 dpf (A) and 16 dpf (B). (A'), (B') ventral images of the head of 11 dpf and 16 dpf fish, respectively. (A''), (B'') zoomed-in images boxed in (A), (B), respectively; parasphenoid (ps), operculum (op), cleithrum (cl), anterior basicranial commissure (abc), supraoccipital (soc), basibranchial plt. (bp), dentary (den), quadrate (qu), hyosymplectic (hys), ceratobranchial (cb); a vertebra consists of a vertebral body (vb), neural arch (na) and hemal arch (ha), fin ray (fr). Scale bars in A, B: 0.5 mm; in A'-B'': 0.2 mm.

including vertebrae with their vertebral bodies, neural arches, hemal arches and caudal fin rays. Results in Fig 3 indicated that the number of units of these bones varied to different extents in both fish groups. For the same bone structure, the number of units of 11 dpf fish had a wider variation than that of 16 dpf fish. The widest variation was observed in the number of hemal arches of the 11 dpf fish group that ranged from 0 to 26 units, and 50% of fish in the group (n=37) had the number of this bone ranging from 3 to 22 (median number was 13). This variation was reduced in 16 dpf fish to a range of 17 to 26 (median number was 23, and 50% of fish in the group (n=49) had 22 to 24 hemal arches). The increase in the number of bone units was also

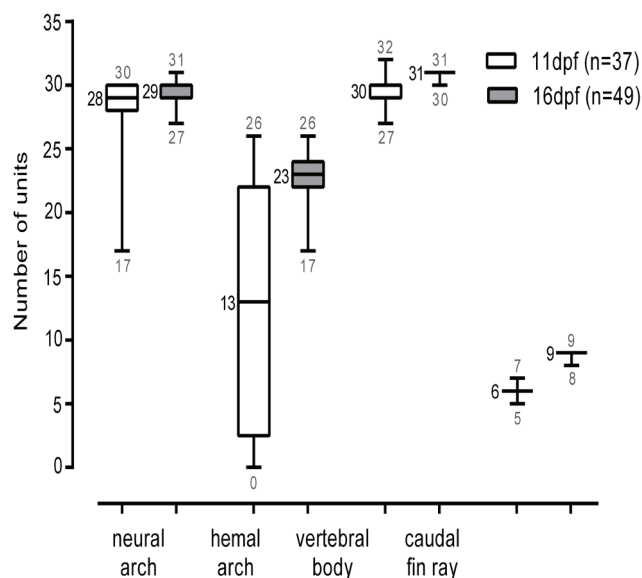


Fig. 3. The number of units of the analysed mineralized bone structures of 11 dpf and 16 dpf fish. Boxplot with whiskers from minimum to maximum and bands inside the box indicate medians (numbers denote the maximum, median and minimum median values of the corresponding groups). Boxes are interquartile ranges (IQR) or middle 50%.

most obvious for hemal arches (Fig. 3), from 13 in 11 dpf to 23 in 16 dpf fish. The variation and the increase in the number of neural arches of fish from 11 to 16 dpf could be observed at a lesser extent compared to those of hemal arches (Fig. 3).

High consistency in the number of units could be observed in the caudal fin rays and vertebral bodies of both fish groups, ranging from 5 to 7 with a median number of

6 fin rays for 11 dpf (30 in total 37 fish) and from 8 to 9 with a median number of 9 fin rays for 16 dpf fish (43 in total 49 fish). Moreover, the number of neural arches and vertebral bodies did not obviously increase (only 1 neural arch and 1 vertebral centrum were newly mineralized in 16 dpf compared to 11 dpf fish). This result demonstrated that in this period of development, the mineralized pattern classified by the number of neural arches and vertebral bodies was stabilized, whereas hemal arches were still highly mineralized and increased in number.

As we were searching for an indicator for the developmental stage of fish, based on these data, the number of caudal fin rays seemed to be the most reliable hallmark because it varied least among fish at the same age but differed significantly between 11 dpf and 16 dpf fish. Compared to the number of caudal fin rays, the number of vertebrae also did not considerably vary among fish at the same age, but the difference in quantity between the observed ages caused difficulty in differentiating one from the other as the number of vertebrae in fish can vary between individuals [23, 24]. Our results supported some previous studies that used the number of caudal fin rays as an indicator for medaka developmental staging [25].

Quantification of the mineralization of some bone structures

In our research, we selected vertebral column as the representative bone structure to be assessed for the level of mineralization; we therefore quantified the mineralization of bone components belonging to vertebrae, including the lengths of mineralized neural and hemal arches and the areas of mineralized vertebral bodies.

The mineralization level of the neural arches (I_{mn}) of a fish was defined as the total value of lengths of its mineralized neural arches (drawn by white lines in Fig. 4A) measured by ImageJ software. The total length of mineralized hemal arches (drawn by white lines in Fig. 4B) expressed the level of mineralization of these bone structures of one fish and denoted as I_{mh} . I_{ma} is the total value of all vertebral body areas of one fish (areas drawn by white lines in Fig. 4C). Measurement was performed for all fish of 11 dpf and 16 dpf groups. The mean values of I_{mn} , I_{mh} and I_{ma} of these two groups were calculated and statistically compared by t-test (Fig. 4D).

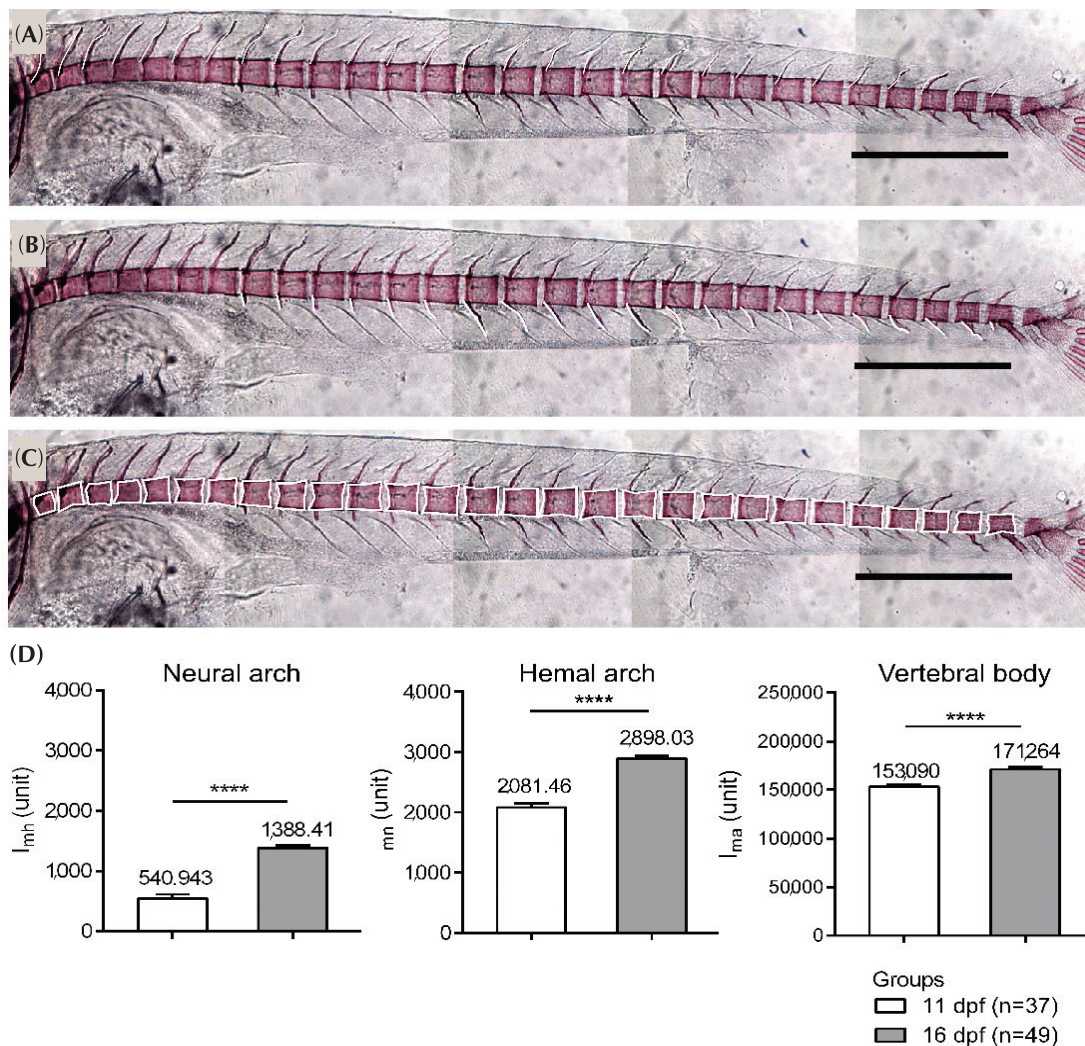


Fig. 4. Mineralization levels of neural bodies, neural and hemal arches of the fish. Neural (A) and hemal (B) arches are indicated by white lines for their length measurement (I_{mn} and I_{mh} indexes, respectively), vertebral bodies (C) are bordered by white lines for their area measurement (I_{ma} index). Lines and boxes are drawn and measured by ImageJ software. Scale bars: 0.5 mm, (D) mean values of I_{mh} , I_{mn} and I_{ma} of the 11 dpf and 16 dpf fish groups. ****p<0.0001.

The values of I_{mn} obtained are 2,081.46 and 2,898.03 for 11 dpf and 16 dpf fish, respectively. Thus, during five days of development, from day 11 to day 16 of age, the mineralization level of the neural arches (I_{mn}) of the fish increased about 39%. As the number of the neural arches increases only one (Fig. 3), the increase in the I_{mn} value was mainly due to the increase in their lengths and appropriate to indicate the development of the fish.

The results also suggested a sharp increase in the value of I_{mh} (Fig. 4D) (from 540.94 at 11 dpf to 1,388.41 at 16 dpf). Medaka fish gained an increase of 156% in the total length of hemal arches during five days of development, from day 11 to day 16 of age. As previously described, during this development period, hemal arches were rapidly

mineralized, mostly by the formation of new ones in the posterior vertebrae of the fish. Along with the rise in number, the forming hemal arches also increased in length, thus sharply increasing the total length of these bones. The mineralized hemal arches are known to appear in chronological order after the mineralization of vertebral centrums and neural arches [26, 27]. Remarkable increases in lengths and number of mineralized hemal arches in the fish from day 11 to day 16 suggest for the use of hemal arches as sites or models to be assessed for studies on new or *de novo* mineralization, as occurred in research to evaluate the effects of bone anabolic substances.

Further mineralization was observed in the vertebral bodies of 16 dpf compared to 11 dpf fish; the mean values

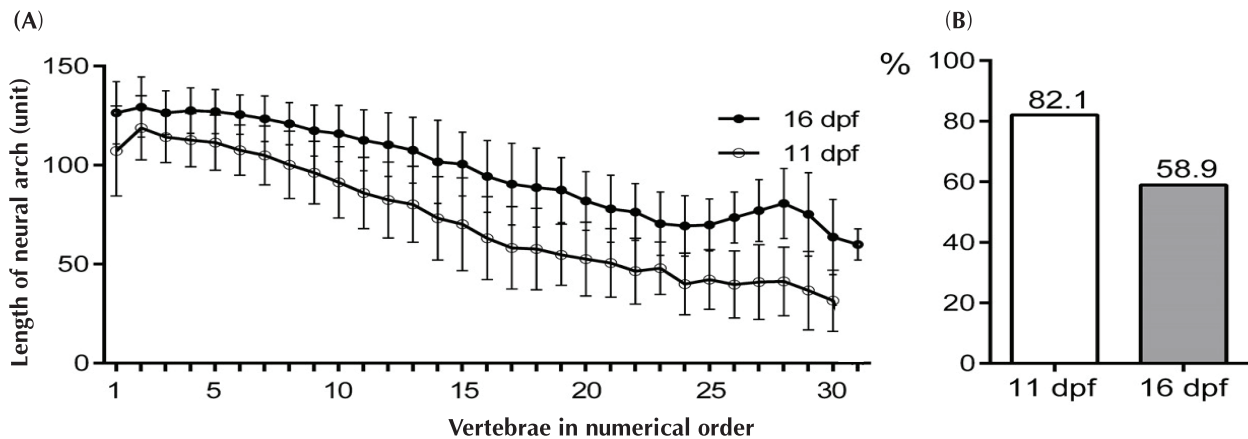


Fig. 5. Mean length values of individual mineralized neural arches in numeric order of 11 dpf and 16 dpf fish. (A) Mean values of neural arches of each vertebra; (B) $I_{ma} 15$ ratio: the ratio of the total length of the 15 first neural arches to the total length of all vertebral neural arches in 11 dpf and 16 dpf fish, (11 dpf: $n=37$ and 16 dpf: $n=49$). Bars indicate standard deviations, **** $p<0.0001$.

of the I_{ma} of 11 dpf and 16 dpf groups were 153,090 and 171,264 units, respectively, denoting an increase of 11.8% after five days. This result, together with the evidence of little increase in the number of vertebral bodies, suggest independent regulating processes for the mineralization of vertebral arches and bodies, which were also reported [11, 13].

Length of the 15 first neural arches relatively reflects total mineralization in 11 dpf fish

In recent years, our laboratory has been intensively screening for bioactive substances that have anti-osteoporosis potential. For this purpose, a medaka model for osteoporosis having damage in mineralized neural arches has been used [16, 28]. We therefore intended to evaluate the lengths of mineralized neural arches in normal wild-type fish to determine the possibility of using this bone structure as representative to assess the mineralization level of a fish (Fig. 5).

In both 11 dpf and 16 dpf fish groups, neural arches evidently shortened gradually along the anterior-posterior axis of the fish, except that the second neural arch was slightly longer than the first one (Fig. 5A). Moreover, as indicated in the data on 11 dpf fish, the total length of the 15 first neural arches contributed to 71% of the total length of all these bones; by contrast, this percentage in 16 dpf fish was merely 61% ($I_{ma} 15$ ratios are 0.71 and 0.61 for 11 dpf and 16 dpf fish, respectively, Fig. 5B). These data suggest the establishment of a methodology for quantifying the level of mineralization in the osteoporosis fish model at 11 dpf by taking the value of the total lengths of 15 first neural arches as an index to indicate the mineralization level of the fish.

Conclusions

This study analysed the bone and developmental indexes in medaka larvae at 11 and 16 days of age, including the total body length of live larvae, number of mineralized bone structures, as well as the area of mineralized vertebrae and length of mineralized neural and hemal arches. Data obtained suggest the use of the number of caudal fin rays as a larva developmental hallmark and the length of the 15 first neural arches as an index that relatively indicates the level of mineralization of 11 dpf fish. These data are important for further research on bones in medaka.

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The authors declare that there is no conflict of interest regarding the publication of this article.

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Influence of inoculum size, CO₂ concentration and LEDs on the growth of green microalgae *Haematococcus pluvialis* Flotow

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Abstract:

Green microalgae *Haematococcus pluvialis* is best known for astaxanthin production. The cultivation of *H. pluvialis* involves two main phases, namely green vegetative stage and cyst stage with astaxanthin accumulation. In fact, the growth of *H. pluvialis* in the vegetative stage is one of the most important parts in the entire cultivation process. The aim of the study is to investigate the influence of temperature, inoculum size, CO₂ concentration and light emitting diodes (LEDs) on the specific growth rate, cell density and dry weight of *H. pluvialis* in the vegetative stage. Results indicated that the temperature from 25 to 28°C and the inoculum size from 3×10^4 cells.ml⁻¹ were suitable for the growth of the studied strain. Illumination with red light LEDs (630 nm) at 80 μE.m⁻².s⁻¹, the highest specific growth rate (μ) was 0.197 day⁻¹ and the maximal density was of 2.87×10^5 cells.ml⁻¹. A concentration of 5% CO₂ (v/v) was the optimal dose for the growth of this strain. Under the condition of both 5% CO₂ and illumination with red light LEDs at 80 μE.m⁻².s⁻¹, the specific growth rate was 0.242 day⁻¹ and the cell density was 4.28×10^5 cells.ml⁻¹. Illumination with only blue LEDs (430 nm) at 120 μE.m⁻².s⁻¹ stimulated the astaxanthin accumulation with a maximum content at 2.36 μg.ml⁻¹.

Keywords: cell density, dry weight, growth rate, *Haematococcus pluvialis* Flotow.

Classification number: 3.4

Introduction

Astaxanthin, a red-orange carotenoid pigment, is a powerful biological antioxidant that occurs naturally in a wide variety of living organisms, especially in green microalgae *Haematococcus pluvialis* [1-3]. Astaxanthin is widely used in various areas such as as feed supplements for aquatic animals, cosmetics, nutraceutical and pharmaceutical industries [4-7]. The life cycle of *H. pluvialis* consists of four cell stages: vegetative growth, encystment, maturation or red stage, and germination. In the production of astaxanthin, *H. pluvialis* is cultivated mainly in the vegetative growth and the red stages [1, 8]. In facts, the vegetative growth is the most important stage in the entire cultivation process of *H. pluvialis*. In the phototrophic cultivation, aside from nutritional components, temperature, lighting condition and CO₂ concentration are essential factors effecting photosynthesis and cell growth [9]. Recently, LEDs have emerged as a replacement for traditional light sources, and they have rapidly expanded to multiple areas including cultivation of microalgae [10, 11]. The physiological effects of light with a specific range of wavelength on photosynthetic cells have been extensively studied by using combinations of special light sources [12, 13]. Although many reports have mentioned the conditions for cultivating *H. pluvialis* for astaxanthin [12-16], no standard procedure can be applied due to the complexity of cell life cycle, different strains, cultivation conditions and technical parameters. This study investigates the combination effect of inoculum size, CO₂ concentration and illumination condition for the cultivation of the green microalgae *H. pluvialis* Flotow. In addition, the study also attempts to determine the suitable conditions for the improvement of cell density, biomass and astaxanthin content for the large-scale production of this strain.

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Material and methods

Microalgal strain and stock culture preparation

Haematococcus pluvialis Flotow was obtained from the culture collection at the University of Göttingen (Germany). The stock culture was prepared by cultivating the purified microalgae in a 100-ml basal medium consisting of $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 15.0 mg; KNO_3 , 10.0 mg; $\beta\text{-Na}_2\text{glycerophosphate}$, 5.0 mg; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 4.0 mg; vitamin B12, 0.01 μg ; biotin 0.01 μg ; thiamine HCl, 1.0 μg ; PIV metals, 0.3 ml; Tris aminomethane, 50.0 mg; and distilled water, 99.7 ml. The PIV metals consisted of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 19.6 mg; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 3.6 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 2.2 mg; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.4 mg; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.25 mg; $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$, 100 mg; and distilled water, 100 ml. The medium was adjusted to pH 7.5 and autoclaved at 121°C for 15 min [2]. *H. pluvialis* was cultured at room temperature from 25 to 28°C and light intensity of roughly 50 $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ from fluorescent light with a light/dark cycle of 16:8 h. Agitation was completed by manually shaking the flasks thrice a day.

Specific growth rate and dry weight measurement

The specific growth rate was calculated in a duration of 48h in the logarithmic phase by using the following equation: $\mu = (\ln \text{DW}_t - \ln \text{DW}_0) / t$ [17]. Biomass productivity ($\text{g} \cdot \text{l}^{-1} \cdot \text{day}^{-1}$) was calculated as follows: $(\text{DW}_t - \text{DW}_0) / t$, where DW_t and DW_0 denote the dry weights of day t and day 0, respectively. Doubling time was calculated by $\ln 2 / \mu$.

The dry weight was measured through the gravimetric method [9]. Briefly, a 10-ml cell suspension was centrifuged at 5,000 g for 10 min. The pellet was rapidly washed in distilled water by centrifugation, dried overnight on a Petri dish at 60°C and subsequently weighted. The control was medium only.

To investigate the relationships among optical density (OD), cell density and dry weight, the suspension culture in exponential growth (at 2.10^5 cells. ml^{-1}) was diluted to different cell densities by serial dilutions. The dry weight was also calculated based on the correlation between the OD of cell suspension at 750 nm and dry weight [13, 18]. The obtained data were used to plot based on the correlation among OD values and the dry weights of different dilution folds. The doubling time in the logarithmic phase was also estimated by counting the cell number.

Influence of temperature

Temperature is a key factor that directly affects the

specific growth rate of *H. pluvialis*. In this study, *H. pluvialis* cells were cultured in a 500-ml flask placed in a water bath at different temperature points (18, 20, 23, 25, 28, 30 and 32°C) and monitored via a Hailea HC-150A chiller system and air conditioner. The cell suspension was illuminated with 40W fluorescent lamps at 35 $\mu\text{moles} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. The effect of temperature on the growth of *H. pluvialis* was characterized by a specific growth rate in the logarithmic phase.

Influence of inoculum size

The effect of inoculum size on the growth of *H. pluvialis* was investigated in 200 ml of different initial biomass densities from 0.5×10^4 to 4×10^4 cells. ml^{-1} . Stock culture was used to prepare cells for inoculum experiments. The stock culture was centrifuged at $2,500 \times g$ for 5 min, and the collected cells were resuspended in basal medium with initial cell densities that vary from 0.5×10^4 to 4×10^4 cells. ml^{-1} at 25°C. The effect of inoculum size on the growth of *H. pluvialis* was studied at a volume of 200 ml. The optimal seeding size was recorded based on the specific growth rate.

Influence of CO₂ supply

The influence of CO₂ on the growth of *H. pluvialis* was evaluated by measuring the growth rate and the change of pH at different concentrations: control (CO₂ in air 0.04%), 1.0, 2.5, 5.0, 7.5 and 10.0% (volume air/volume culture/min). CO₂ was introduced to the culture medium by using a stainless needle with a 0.6 mm diameter. The flow of CO₂ was monitored through a CO₂ meter. The parameters of specific growth rate and pH were measured.

Influence of light sources

Three light sources were used in this study, namely 40W fluorescent lamps, 25W red 630 nm LEDs and 25W blue 430 nm LEDs. The intensity of light sources was adjusted by maintaining the distance from the light source to the cultivation flasks and monitored through a light intensity meter (Teslo 545). Different combinations of these light sources, including red LEDs, blue LEDs and a combination of both red LEDs and blue LEDs and fluorescent lamps, were investigated.

Measurement of pigments

The chlorophyll concentration was determined spectrophotometrically using the extinction coefficients in 100% methanol as described by Wellburn [19] and Lichtenthaler and Wellburn [20]. Briefly, a 2ml cell suspension was centrifuged at $2,500 \times g$ for 10 min, and the supernatant was discarded. Two ml of methanol were subsequently added to the pellet and mixed thoroughly.

The samples were mixed and incubated in a water bath at 70°C for 10 min and then centrifuged at 2,500×g for 10 min. The liquid was read spectrophotometrically at different wavelengths (653, 665, 649 and 470) to determine chlorophyll a (1), chlorophyll b (2) and carotene (3).

$$\text{Chlorophyll a } (C_a) = 15.65 \times A_{666} - 7.34 \times A_{653} \quad (1)$$

$$\text{Chlorophyll b } (C_b) = 27.05 \times A_{649} - 5.32 \times A_{665} \quad (2)$$

$$\text{Carotene} = (1,000 \times A_{470} - 2.86 \times C_a - 129.2 \times C_b) \times 221 \quad (3)$$

where: A_x is the absorbance at x wavelength, and C_a and C_b represent the concentrations of chlorophyll a and chlorophyll b ($\mu\text{g} \cdot \text{ml}^{-1}$), respectively.

Removal of chlorophyll: the cell pellets of *H. pluvialis* in the cyst stage were treated with 5% KOH in 30% methanol and incubated at 70°C for 5 min to remove chlorophyll. After being washed with distilled water, cell pellets were used for astaxanthin extraction and quantitative analysis [1].

Astaxanthin extraction and analysis: Chlorophyll-removed cells were treated with 2N hydrochloric acid (HCl) and incubated at 70°C. The samples were centrifuged at 2,500×g for 10 min at 4°C, and the supernatants were removed and washed twice with distilled water. The cells were treated with methanol for 1 h and subsequently centrifuged as described. The supernatants were used for estimating the extractable astaxanthin (as carotene). In this study, astaxanthin content was calculated by using a calibration curve constructed via pure astaxanthin purchased from Sigma Aldrich (CAS Number 472-61-7). Pure astaxanthin was prepared in absolute methanol in different concentrations from 10 to 100 $\mu\text{g} \cdot \text{ml}^{-1}$ and measured at 470 nm. All of the steps in the astaxanthin extraction and sample preparation were carried out in dark [21]. Data on astaxanthin content calculated from the calibration curve were also compared with the data computed from equation (3) to adjust for rapid estimation in experiments.

Data analysis

In this study, data were analysed from three replicates and represented as mean values with standard deviations (SDs). Graphs were plotted using Microsoft Excel 2016. Analysis of variance was applied to assess the significant differences among the groups. Tukey's test was used to verify all of the pairwise differences among the means of the experiment groups.

Results and discussion

Influence of temperature

To determine the effect of temperature on the growth

of *H. pluvialis*, a serial of cell suspensions at 3.10^4 cells. ml^{-1} in the green vegetative state was inoculated at 18, 22, 25, 28, 30 and 32°C under fluorescent light at 50 $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ with a dark/light cycle of 8:16 h. The results indicated that the specific growth rates in the logarithmic phase increased when temperature increased from 18 to 27°C. However, a decrease in the specific growth rate was observed when the temperature was higher than 28°C. In this study, cells were unable to grow when the temperature was over 30°C (Fig. 1).

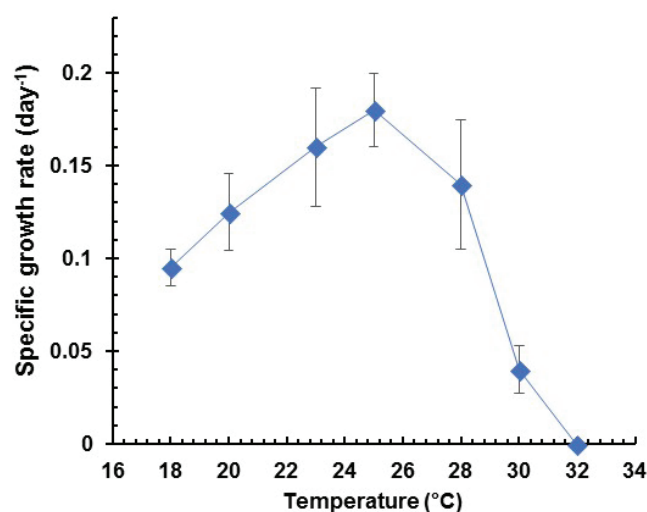


Fig. 1. Influence of temperature on the specific growth rate of *H. pluvialis*. Cell suspensions were cultured under fluorescent light at 50 $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ with a dark/light cycle of 8:16 h. The specific growth rate was measured in the logarithmic phase for 72h from days 10 to 14 after inoculation.

The optimal temperature for the growth of this *H. pluvialis* was 25°C, and the specific growth rate (μ) under this condition in the logarithmic phase was 0.18 ± 0.025 per day or 0.0075 h^{-1} , which was equivalent to the doubling time of 3.85 days or 84h. Studies revealed that the specific growth rate of *H. pluvialis* was affected by many cultivation factors and varied from 0.007 to 0.05 per h [1, 17, 22, 23]. Depending on strains, most studies reported that the optimal temperature for *H. pluvialis* strains was within the range of 25 to 28°C [1-3, 13].

Technically, the best method for cell density measurement is through a haemocytometer, but it is time consuming and inconvenient. Based on the findings of Chekanov about the correlation between OD_{750} , cell density and dry weight [13], a relationship among cell density, OD_{750} and dry weight was established (Fig. 2). A linear correlation emerged between OD_{750} and dry weight in the logarithmic phase (Fig. 2, inner graph).

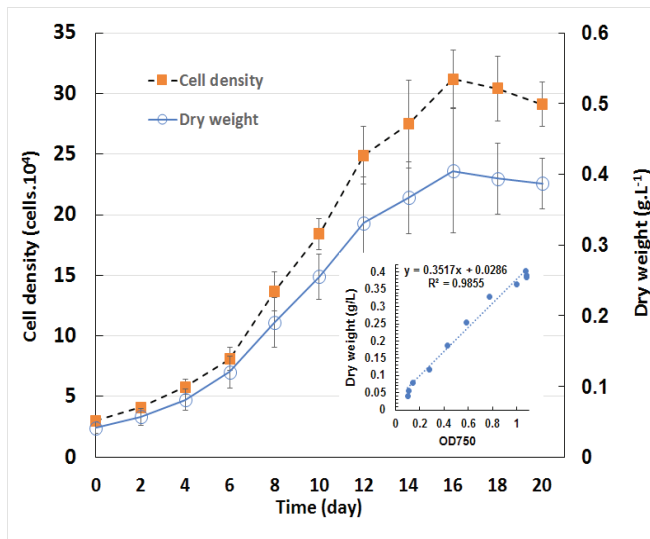


Fig. 2. Relationship between cell density and dry weight. Outer graph: relationship between cell density and dry weight over 20 days. Inner graph: correlation between dry weight and OD_{750} . *H. pluvialis* was inoculated at 3×10^4 cells.ml⁻¹ and cultured for 20 days at 25°C with light intensity of 50 μ E.m⁻².s⁻¹ with a dark/light cycle of 8:16 h and continuously bubbled with air at 10% (v/v).

Influence of inoculum size

All of the cell suspensions were cultured at 25°C in a 500-ml flask with a dark/light cycle of 8:16 h and bubbled with air (10% v/v). Data revealed that cell density increased proportionally with the initial biomass density. The highest cell density was observed at approximately 2.874×10^5 cells.ml⁻¹ (equivalent to 209.13 mg.ml⁻¹) when inoculum size was 3×10^4 cells.ml⁻¹. At a lower inoculum size, the maximum cell density was reduced significantly, and the duration from the time point of inoculation to the stationary phase was extended (data not shown). In the stationary phase, the morphology of the cells changed slowly from green active to inactive without flagella. No significant difference was observed between two initial biomass densities of 3×10^4 cells.ml⁻¹ and 4×10^4 cells.ml⁻¹ (Table 1). This result indicated the probability that a higher initial biomass density was not better at all times. The outcome might be due to the mutual shading of cells in the culture, the frequency of light/dark cycle and the total length of light period during which individual cells were exposed to incident light intensity [17, 22]. Several studies have reported that the higher initial biomass density, the higher frequency of light/dark cycle and the shorter the light exposure time individual cells receive from each light/dark cycle [17]. This result suggested that the initial biomass density of 3×10^4 cells.ml⁻¹ was suitable for biomass production in this study.

Table 1. Effect of inoculum size on the growth of *H. pluvialis*.

Inoculum size ($\times 10^4$ cells.ml ⁻¹)	0.5	1.0	2.0	3.0	4.0
Max cell density ($\times 10^4$ cells.ml ⁻¹)	16.12 $\pm$ 1.81 ^(a)	21.63 $\pm$ 2.51 ^(b)	25.16 $\pm$ 3.05 ^(c)	28.74 $\pm$ 3.11 ^(d)	24.69 $\pm$ 2.17 ^(c)
Max specific growth rate (day ⁻¹)	0.138 $\pm$ 0.019 ^(a)	0.153 $\pm$ 0.022 ^(b)	0.184 $\pm$ 0.024 ^(c)	0.197 $\pm$ 0.021 ^(d)	0.182 $\pm$ 0.023 ^(c)
Doubling time in log phase (day ⁻¹)	5.07 $\pm$ 0.41 ^(a)	4.57 $\pm$ 0.35 ^(b)	3.80 $\pm$ 0.26 ^(c)	3.55 $\pm$ 0.35 ^(d)	3.84 $\pm$ 0.43 ^(c)
Duration in vegetative stage (day)	16 $\pm$ 2 ^(a)	14 $\pm$ 2 ^(b)	12 $\pm$ 2 ^(c)	11 $\pm$ 2 ^(d)	12 $\pm$ 2 ^(c)
pH at stationary phase	9.12 $\pm$ 0.46 ^(a)	9.10 $\pm$ 0.64 ^(a)	9.06 $\pm$ 0.59 ^(a)	8.91 $\pm$ 0.47 ^(b)	8.87 $\pm$ 0.52 ^(b)

Note: means that share the same superscript are not significantly different at $p < 0.05$.

Effects of LED lights on the growth and astaxanthin production

Several studies have demonstrated the effect of red and blue LEDs on the growth and astaxanthin accumulation in *H. pluvialis* [14-16]. In this study, different light sources were used separately or in combination (Fig. 3).

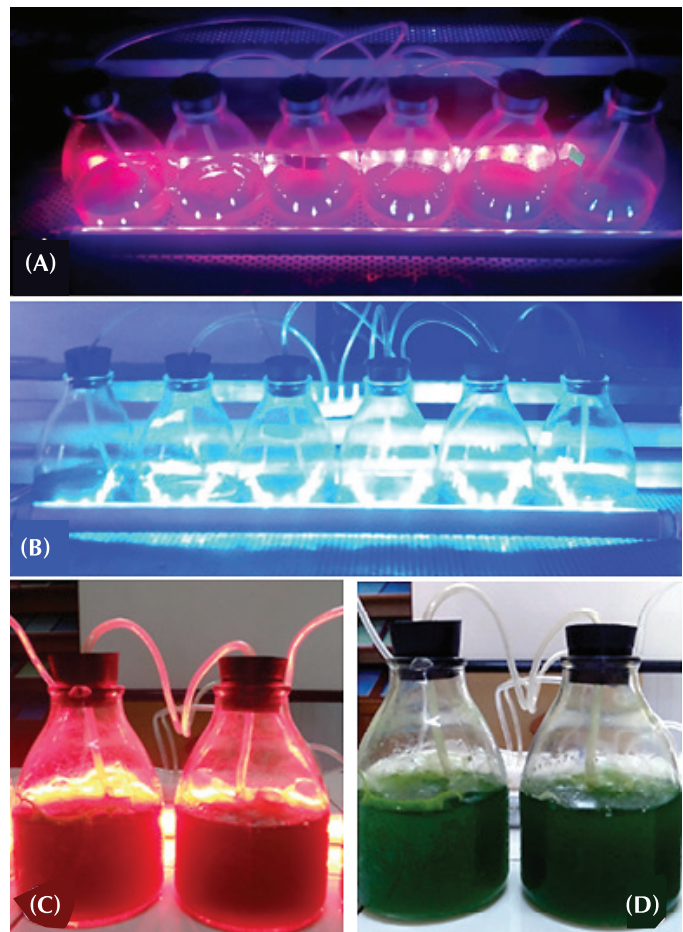


Fig. 3. Effect of LEDs on the growth and cell density of *H. pluvialis*: (A) Experiments with both red LEDs and blue LEDs at 50 μ mol.m⁻².s⁻¹; (B) Experiments with only blue LEDs at 50 μ mol.m⁻².s⁻¹; (C) Experiments with only red LEDs at 50 μ mol.m⁻².s⁻¹; (D) Cell suspension illuminated by fluorescent lamp at 50 μ mol.m⁻².s⁻¹.

In the first four days, no difference in cell density was observed among treatments. However, the density increased significantly from days 6 to 14 (Fig. 4). The red LEDs evidently exhibited a positive effect on the growth of *H. pluvialis*, whereas the blue LEDs did not promote the growth of these microalgae. The specific growth rate was affected by the intensity of light (Table 2). No significant difference emerged between a combination of red LEDs with fluorescent light and only red LEDs. Data analysis indicated that cell density increased significantly in the case of red LEDs.

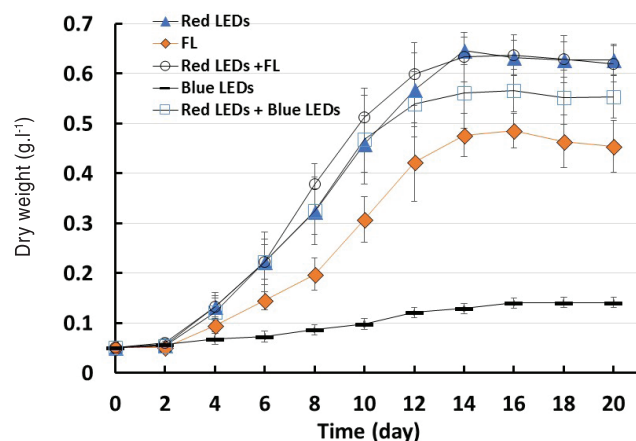


Fig. 4. Effect of light source on the growth of *H. pluvialis*. Inoculation density was 3.10^4 cell.mL⁻¹ in all experiments. Red LEDs: 25W red LEDs, 630 nm ($50 \mu\text{mol.m}^{-2}.\text{s}^{-1}$); Blue LEDs 25W, 430 nm ($50 \mu\text{mol.m}^{-2}.\text{s}^{-1}$); FL: 40W fluorescent lamp ($50 \mu\text{mol.m}^{-2}.\text{s}^{-1}$); Red LEDs + FL (using both red LEDs and fluorescent lamp); Red LEDs + Blue LEDs (using both red LEDs and blue LEDs).

Although illumination with blue LEDs did not significantly promote the growth of cells, the number of immotile cells increased rapidly, and cells changed into mature cysts within 12 days (Table 2). Harvested cells in the cyst state were extracted after removing the chlorophyll. In this study, astaxanthin was extracted from cells illuminated with red LEDs and blue LEDs after 14 and 16 days of illumination, respectively (Table 2). Morphological analysis revealed that red LEDs did not significantly stimulate astaxanthin accumulation, and the cells were in the resting (cyst) stage without cell division after 14 days of illumination (data not shown). In case of illumination with blue LEDs at $120 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ for 16 days, the carotene content was $2.36 \pm 0.46 \mu\text{g.mL}^{-1}$ or 0.5% dry weight (Table 2 and Fig. 5). Several attempts were undertaken to stimulate the accumulation of astaxanthin at different temperatures in combination with treatment with blue LEDs. The accumulation of astaxanthin by blue LED treatment was likely to be more efficient when the temperature was within the range of 25 to 27°C (data not

shown). This effect was significant only if the high intensity of blue LEDs was applied ($120 \mu\text{mol.m}^{-2}.\text{s}^{-1}$, Fig. 5). A higher temperature (above 28°C) indicated an adverse effect and a significant decrease in the number of cells. Several studies underscored the similar effect of LED light [10–13]. In addition, the accumulation of astaxanthin required a prolonged stimulation period of 16 days (Fig. 5). It suggested that illumination by using blue LEDs at 430 nm induced astaxanthin production. However, the application of blue LEDs only for the stimulation of astaxanthin production was inefficient. Additional investigations that use other factors such as chemical reagents are necessary to obtain better results.

Table 2. Effect of light intensity on the growth of *H. pluvialis*.

	Light sources and intensity					
	Red LEDs			Blue LEDs		
Light intensities ($\mu\text{E.m}^{-2}.\text{s}^{-1}$)	50	80	120	50	80	120
Max cell density (10^4 cells.mL ⁻¹) (*)	$31.84 \pm 4.12^{(a)}$	$35.42 \pm 4.42^{(b)}$	$35.67 \pm 4.67^{(b)}$	$6.31 \pm 0.71^{(c)}$	$4.45 \pm 0.50^{(d)}$	$2.26 \pm 0.315^{(e)}$
Max specific growth rate (day ⁻¹)	$0.188 \pm 0.02^{(a)}$	$0.196 \pm 0.023^{(b)}$	$0.187 \pm 0.021^{(ab)}$	$0.052 \pm 0.012^{(c)}$	$0.039 \pm 0.011^{(d)}$	$-0.013 \pm 0.001^{(e)}$
Doubling time (day ⁻¹)	$3.52 \pm 0.41^{(a)}$	$3.34 \pm 0.26^{(a)}$	$3.32 \pm 0.46^{(a)}$	$13.4 \pm 1.50^{(b)}$	$18 \pm 2.06^{(c)}$	-
Highest astaxanthin content ($\mu\text{g.mL}^{-1}$)	(**)	(**)	$0.12 \pm 0.02^{(a)}$	$0.25 \pm 0.03^{(b)}$	$1.05 \pm 0.02^{(c)}$	$2.36 \pm 0.46^{(d)}$
% motile cells	$75.13 \pm 6.71^{(a)}$	$65.26 \pm 6.12^{(ab)}$	$62.15 \pm 7.97^{(b)}$	$10.26 \pm 1.12^{(c)}$	$6.14 \pm 0.84^{(d)}$	0
% cysts	$4.53 \pm 0.31^{(a)}$	$5.71 \pm 0.41^{(ab)}$	$6.25 \pm 0.52^{(b)}$	$20.25 \pm 4.12^{(c)}$	$20.11 \pm 3.95^{(c)}$	$60.16 \pm 4.12^{(c)}$

Notes: means that share the same superscript are not significantly different at $p < 0.05$. The initial biomass density was 3.10^4 cell.mL⁻¹ in all experiments. A 200-ml cell suspension was cultured in a 500-ml flask with a dark/light cycle of 8:16 h and continuously bubbled with filtered air at 10% (v/v); (*) data were measured on day 14 with red LEDs or day 16 with blue LEDs (see also Fig. 4); (**) negligible.

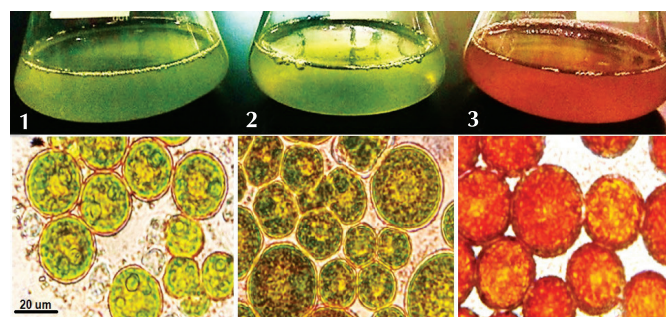


Fig. 5. Effect of LEDs on astaxanthin accumulation in *H. pluvialis*. Cells in the cyst stage and astaxanthin accumulation after treatment with blue LEDs at $50 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ (1), $80 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ (2) and $120 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ (3) for 16 days at 27°C.

Effects of CO₂ concentration

CO₂ is a critical factor that affects the growth of microalgae by providing a carbon source for photosynthesis. As a rule, high cell density and synchronization in the vegetative stage are the most important parameters in the two-stage cultivation of *H. pluvialis*. In this study, cell

suspensions were cultured at 25°C and illuminated by red LEDs at 80 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ in the presence of different CO_2 concentrations 0.04 (air/control), 1.0, 3.0, 5.0, 7.5 and 10% (v/v). The influence of CO_2 on the growth parameters of *H. pluvialis*, including pH, cell density, specific growth rate, doubling time and pigment contents, was investigated. The results are presented in Table 3.

Table 3. Effect of CO_2 on growth parameters.

CO_2 concentration (% v/v)	0.04 (*)	1	2.5	5	7.5	10
Initial pH	7.5	7.5	7.5	7.5	7.5	7.5
pH at log phase	8.33 $\pm$ 0.23 ^(a)	8.11 $\pm$ 0.22 ^(a)	7.61 $\pm$ 0.26 ^(b)	6.95 $\pm$ 0.21 ^(c)	6.63 $\pm$ 0.22 ^(cd)	6.43 $\pm$ 0.21 ^(d)
pH at stationary phase	9.50 $\pm$ 0.31 ^(a)	8.87 $\pm$ 0.38 ^(ab)	8.25 $\pm$ 0.33 ^(b)	6.68 $\pm$ 0.24 ^(c)	6.16 $\pm$ 0.21 ^(cd)	5.75 $\pm$ 0.28 ^(d)
Maximum cell density (10^4 cells. ml^{-1})	25.79 $\pm$ 3.45 ^(a)	31.46 $\pm$ 4.16 ^(ab)	36.46 $\pm$ 3.25 ^(bc)	42.79 $\pm$ 4.65 ^(c)	41.91 $\pm$ 6.37 ^(c)	33.52 $\pm$ 3.19 ^(b)
Maximum specific growth rate (day^{-1})	0.196 $\pm$ 0.015 ^(a)	0.213 $\pm$ 0.016 ^(ab)	0.231 $\pm$ 0.011 ^(b)	0.242 $\pm$ 0.022 ^(b)	0.236 $\pm$ 0.021 ^(b)	0.208 $\pm$ 0.026 ^(ab)
Doubling time in log phase (day^{-1})	4.89 $\pm$ 0.46 ^(a)	3.28 $\pm$ 0.43 ^(b)	3.03 $\pm$ 0.24 ^(bc)	2.89 $\pm$ 0.33 ^(c)	2.96 $\pm$ 0.26 ^(c)	3.36 $\pm$ 0.44 ^(c)
Maximum chlorophyll in log phase ($\mu\text{g}.\text{ml}^{-1}$)	8.77 $\pm$ 1.02 ^(a)	12.28 $\pm$ 1.56 ^(b)	16.38 $\pm$ 2.06 ^(c)	18.2 $\pm$ 1.92 ^(c)	18.5 $\pm$ 2.06 ^(c)	12.4 $\pm$ 1.54 ^(b)
Maximum carotene in stationary phase ($\mu\text{g}.\text{ml}^{-1}$)	1.57 $\pm$ 0.20 ^(a)	2.07 $\pm$ 0.17 ^(bc)	2.44 $\pm$ 0.26 ^(cd)	2.81 $\pm$ 0.26 ^(d)	2.93 $\pm$ 0.41 ^(cd)	2.87 $\pm$ 0.37 ^(cd)

(*) Notes: CO_2 from air (control). Cell density was measured by OD_{750} on the day after inoculum at 3×10^4 cell. ml^{-1} . Means that share the same superscript are not significantly different at $p < 0.05$.

Experiments with different CO_2 concentrations and red LEDs at 80 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ were conducted. The growth of *H. pluvialis* increased with the elevation of CO_2 concentration. An elevation of CO_2 concentration from 0.04% (air) to 5% promoted cell growth, and the highest density of approximately $4.279 \pm 5.65 \times 10^5$ cells. ml^{-1} was reached on day 14 at 5% CO_2 (Table 3 and Fig. 6). Under this condition, pH in the logarithmic phase was nearly neutral at 6.95 ± 0.21 and decreased slightly to 6.68 ± 0.24 at the stationary phase (Table 3). This outcome revealed that the supply of CO_2 at 5% in basal medium stabilized the pH value throughout the culture period. As previously stated, pH was strongly dependent on the buffer capacity of the media [3, 18, 22-24]. A lower concentration of CO_2 at 0.04% (air), 1% and 2.5% was insufficient, and the pH of the medium became more alkaline over time. A higher concentration of CO_2 (7 and 10%) resulted in a significant reduction of pH and a specific growth rate (Table 3 and Fig. 6). No significant difference emerged between two concentrations of CO_2 , 5.0 and 7.5% (v/v). A slight difference of all the investigated parameters between two concentrations of CO_2 , 2.5 and 5.0% (v/v) was observed. The concentration of 5% CO_2 was likely to be more suitable for the growth of this strain.

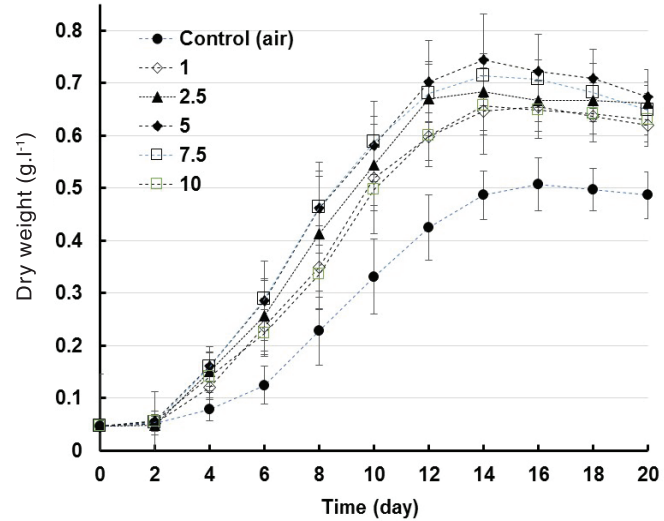


Fig. 6. Effect of CO_2 and red LEDs on the growth and cell density.

Numerous studies on both indoor and outdoor systems similarly emphasized that a higher concentration of CO_2 reduced pH at the stationary phase [18, 23, 24]. The reduction of pH value denoted a significant effect on the growth of *H. pluvialis* (Table 3). In addition, the doubling time was shortened significantly when 5% or 7.5% of CO_2 was applied. This result suggested that a concentration of 5% CO_2 was optimal for the culture of this strain. The concentration of 10% CO_2 did not exhibit a positive effect on the growth and chlorophyll content. However, data analysis indicated that the elevation of CO_2 concentration significantly increased carotene content (Table 3). An illumination by using blue LEDs at 120 $\mu\text{E.m}^{-2}.\text{s}^{-1}$ for 16 days stimulated astaxanthin accumulation in the study strain with maximum content (calculated based on β -carotene) of approximately 2.93 ± 0.41 $\mu\text{g}.\text{ml}^{-1}$ and 2.81 ± 0.26 $\mu\text{g}.\text{ml}^{-1}$ at concentrations of CO_2 7.5% and 5%, respectively. Data analysis revealed the lack of a significant difference between two concentrations of CO_2 5% and 7.5% CO_2 in a light/dark cycle of 16:8 h. Therefore, the concentration of CO_2 at 5% should be used for large-scale production due to economic issues. In comparison with published data, the results obtained in this study were also matched with the findings of several studies [18, 22-24].

Conclusions

In this study, the optimal condition for the growth of *H. pluvialis* Flotow was at a temperature of approximately 25°C and the inoculum size at 3×10^4 cells. ml^{-1} . Under the condition of red light LEDs (630 nm) at 80 $\mu\text{E.m}^{-2}.\text{s}^{-1}$, the highest specific growth rate and maximal density were $0.196.\text{day}^{-1}$ and 3.56×10^5 cells. ml^{-1} , respectively. A concentration of 5% CO_2 (v/v) was the optimal dose

for this strain. Under the conditions of both 5% CO₂ and illumination with red light LEDs at 80 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$, the maximal specific growth rate was 0.242 ± 0.022 , the doubling time was 2.89 days or 69.36 hours and the cell density reached approximately 4.28×10^5 cells.ml⁻¹. Illumination by using blue LEDs at 120 $\mu\text{E.m}^{-2}.\text{s}^{-1}$ for 16 days in a light/dark cycle of 16:8 h stimulated astaxanthin accumulation in the study strain with a maximum content of roughly 2.81 ± 0.26 $\mu\text{g.ml}^{-1}$.

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The authors declare that there is no conflict of interest regarding the publication of this article.

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Influence of microalgae retention time on biomass production in membrane photobioreactor using human urine as substrate

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Abstract:

Human urine is known as the excreta with a high concentration of nitrogen and phosphorus, causing eutrophication in water bodies. In this study, human urine was used to feed microalgae (*Chlorella vulgaris*) in a membrane photobioreactor (MPBR) at various microalgae retention times (MRTs) and hydraulic retention time (HRT) of 2 days to evaluate its biomass production. The results indicate that MPBR was operated under MRT of 2 to 5 days and HRT of 2 days, which performed the optimum condition with biomass productivity from 146.43 ± 8.52 to 151.93 ± 15.05 mg.l⁻¹.day. Moreover, the MPBR using the urine as a nutrient source demonstrated the high performance in biomass production and strong growth of microalgae.

Keywords: biomass production, human urine, membrane photobioreactor, microalgae, nutrient removal.

Classification number: 3.5

Introduction

Domestic wastewater has negatively affected the aquatic environment when human urine is discharged directly into the environment without sufficient treatment, thereby causing eutrophication. Urine contains a high concentration of nutrients (mostly nitrogen and phosphorus); it can therefore be used as a liquid fertilizer or even as a slowly soluble fertilizer (in the form of struvite - $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$) [1]. Additionally, it offers a high potential to cultivate microalgae for nutrient recovery. Microalgae biomass production is a potential source of feedstock for the bio-based production of biochemicals, biofuels, fertilizer, feed for cattle, food for health, and cosmetics for humans [2]. In addition, many types of wastewaters from agricultural, industrial, synthetic, and municipal activities which have been used for microalgae cultivation coupling with wastewater treatment is regarded as a more economical and sustainable option [3, 4]. Human urine contains about 80% of the nitrogen loading in wastewater; therefore, separating urine at the source to cultivate microalgae can help to improve effluent quality, save energy consumption, and recover the investment cost of the wastewater treatment plant [1].

The cultivation of microalgae using wastewater in photobioreactors is a novel, prospective, and sustainable method to remove contaminants (mostly nutrients) from wastewater and simultaneously produce useful microalgae biomass. Significant effort has been dedicated to developing the performance and cost-effectiveness of microalgae cultivation systems. The pilot scale or commercial cultivation system are often based on open ponds technology. However, this pond technology presents many disadvantages, such as water evaporation, extensive space requirements, contamination of algal cultures, and lack of control over operating parameters [5, 6]. To overcome these issues with open pond technology, the photobioreactor (PBR) has been designed to tackle these drawbacks [4]. However, PBRs present additional challenges, such as poor settling ability, biomass washout, and harvesting limitations [7]. Therefore,

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the microalgae cultivation system has been improved by combining it with membrane separation in PBR, rendering it the membrane photobioreactor (MPBR). The advantages of MPBR relative to PBR included decoupling the hydraulic retention time (HRT) and microalgae retention time (MRT), preventing biomass washout, higher biomass production, enhanced nutrient removal efficiency, and reduced land requirement, which contributed to a decrease in construction and operation costs.

There was minimal available knowledge regarding microalgae cultivation by using human urine as a substrate incorporated with a membrane photobioreactor [2]. In several previous studies, synthetic or real urine was applied as a nutrient medium for microalgae growth [2, 8, 9]. However, ammonia production, high pH, and key-element precipitation that occurred during urea hydrolysis in concentrated urine would produce microalgae growth difficulties and render nutrient recovery ineffective [9]. In fact, Jaatinen, et al. (2016) reported that 1:25-diluted urine could be used for microalgae biomass production [8]. In addition, *Chlorella vulgaris* was known to be easy to cultivate in an inexpensive nutrient medium and exhibited a fast growth rate and a high biomass productivity [10]. At HRT of 2 days, microalgae concentration and biomass productivity of MPBR achieved 3.5-fold and 2-fold higher compared to those of PBR respectively [11]. Therefore, the first time that *Chlorella vulgaris* was grown in the MPBR system with diluted human urine as nutrients source in this study, the reactor was operated under conditions in which HRT was fixed at 2 days, and the MRT was variable. This study aims to investigate the effect of various microalgae retention times (MRTs) on algae biomass production.

Materials and methods

Membrane photobioreactor structure

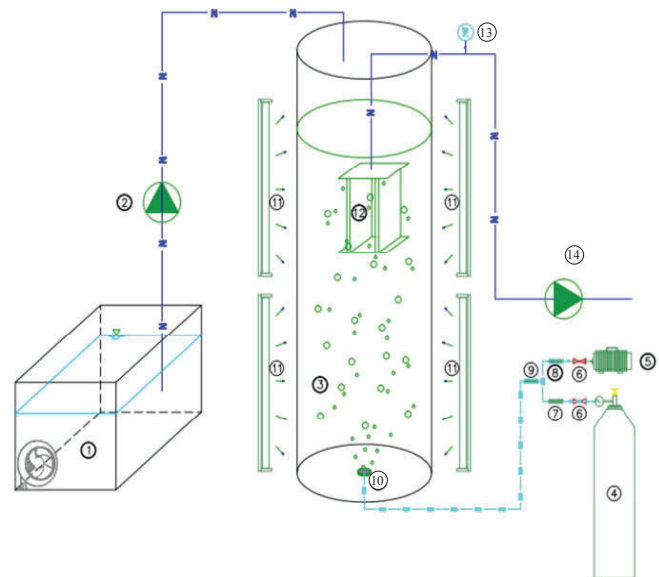
The MPBR system was installed in a wooden box with a thickness of 10 mm to prevent temperature change. It was then continuously illuminated with four 18 W white fluorescent lamps (11), and the intensity of the lighting was 4.4 kLux. MPBR (3) was made from transparent acrylic and designed with an internal diameter of 100 mm and 1200 mm in height; the working volume was 8 l. A hollow fiber membrane module (12), which was made from polyvinylidene fluoride (PVDF) (Mitsubishi, Japan) and had a pore size of 0.4 μm with a membrane area of 0.035 m^2 ; it was submerged in the reactor.

Operating conditions of the MPBR system

The flow rates of CO_2 (4) and air (5) mixture, which were 0.1 l/min and 4.0 l/min respectively, were injected into the MPBR via a 20 mm-diameter air diffuser installed at the bottom of the reactor.

The diluted human urine (30 times) was pumped from the feed tank (1) into the MPBR by an automatic feed pump

(2). The permeate was intermittently withdrawn in a cycle (8 min of operation and 2 min idle) by a suction pump. A digital pressure gauge (13) was installed on a pipe connected with a permeate pump (Fig. 1).



1: feed tank; 2: feed pump; 3: photobioreactor; 4: compressed CO_2 cylinder; 5: air blower; 6: valve; 7-9: rotameters; 10: air distributor; 11: fluorescent lamp; 12: membrane module; 13: digital pressure gauge; 14: permeate pump.

Fig. 1. Schematic diagram of lab-scale membrane photobioreactor.

Microalgae retention time (MRT, day) was calculated by the following expression [11]:

$$MRT = \frac{V}{F_{\text{retentate}}}$$

where V was volume of reactor (l), and $F_{\text{retentate}}$ was daily volume of wasted retentate (l/day).

To determine the optimum MRT, MPBR was operated in four phases at MRTs changing from 5 days (during operation period from day 18 to day 113), to 3 days (between day 114 and day 175), to 2 days (between day 176 and day 190) and 1.5 days (from day 191 to day 218) and the discharged biomass amounts were 1.6, 2.67, 4.0, and 5.3 l/day, respectively. However, the reactor was operated in during the start-up time (from day 0 to day 17) to achieve a sufficiently high initial microalgae concentration. While MRT was changed in turn, HRT was controlled at 2 days for all operated MRTs. HRT (day) was defined by the following expression [11]:

$$HRT = \frac{V}{F_{\text{in}}}$$

where F_{in} was influent flowrate (l/day).

Feed wastewater characteristics and microalgae strain

Chlorella vulgaris was used in this study provided by The Research Institute for Aquaculture No. 2, Ho Chi Minh city, Vietnam with initial dry weight of 36 mg/l.

Fresh human urine was collected from male toilet in Ho Chi Minh city University of Technology and stored at 4°C in a refrigerator to reduce the effect of urea hydrolysis before use. Then urine was diluted 30 times with tap water and contained in feed tank. The diluted urine contained PO_4^{3-}P of 4-8 mg/l, total phosphorus (TP) of 8-15 mg/l, $\text{NH}_4^+\text{-N}$ of 6-12 mg/l and total Kjeldahl nitrogen (TKN) of 180-350 mg/l.

Analysis

Daily, 200-ml samples were taken from influent and permeate for analysis. In addition, 50-ml samples of mixed liquor suspended solids (MLSS) were taken from middle of MPBR to measure biomass concentration [10]. MLSS was measured using a Whatman glass fiber filter membrane and then drying biomass after filtering until a constant weight was reached at 105°C [12]. The water quality parameters including TKN, TP, nitrite, nitrogen ($\text{NO}_2^-\text{-N}$), nitrate nitrogen ($\text{NO}_3^-\text{-N}$), and biomass concentration were analysed, following the Standard Method for The Examination of Wastewater [12]. pH was measured using a pH meter (HANA, USA).

Biomass productivity (P, $\text{mg.l}^{-1}.\text{day}$) was calculated based on the following expression [11]:

$$P = X_{MPBR} \times \frac{D}{v} = X_{MPBR} \times \frac{1}{HRT} \times \frac{HRT}{MRT} = \frac{X_{MPBR}}{MRT}$$

where, X_{MPBR} was biomass concentration in MPBR (mg/l), D was dilution rate (day^{-1}), and v was dilution factor.

The nutrients loading ($\text{mg.l}^{-1}.\text{day}$) and food/microorganism (F/M) ratio of MPBR were calculated using the following equation [13]:

$$\text{Nutrients loading} = \frac{C_{inf} \times Q}{V}$$

$$\frac{F}{M} = \frac{Q \times C_{inf}}{V \times X_{MPBR}}$$

where, C_{inf} was the concentration (mg/l) of TN (or TP) in the influent.

Microalgae cell density was determined every day by counting method following Fuchs-Rosenthal and Burkner method with hemocytometer (Germany). After counting the microalgae cell via light microscope, cell density is calculated by the following formula:

$$\text{Cell density} = \frac{\text{number of cell}}{\text{ml}} = \frac{\text{number of cell on a large square}}{\text{volume of a large square} \times \text{dilution rate}}$$

Results and discussion

Figure 2 demonstrates that the variation of *Chlorella vulgaris* biomass concentration in MPBR operated at different MRTs during the entire cultivation period of 218 days. At the start-up period, biomass concentration achieved 615 mg/l at day 9. Based on the observed results, there was no lag phase in the first 18 days (start-up period), which reflected the results of Gao, et al. [13]. This proved that *Chlorella vulgaris* adapted effectively to human urine as a feeding substrate.

At MRT of 5 days, biomass concentration was maintained in the range of 540-860 mg/l. This high concentration of microalgae was achieved through the effect of the submerged membrane in MPBR, which allowed the reactor to operate under a longer MRT but a shorter HRT [4]. However, at the initial time

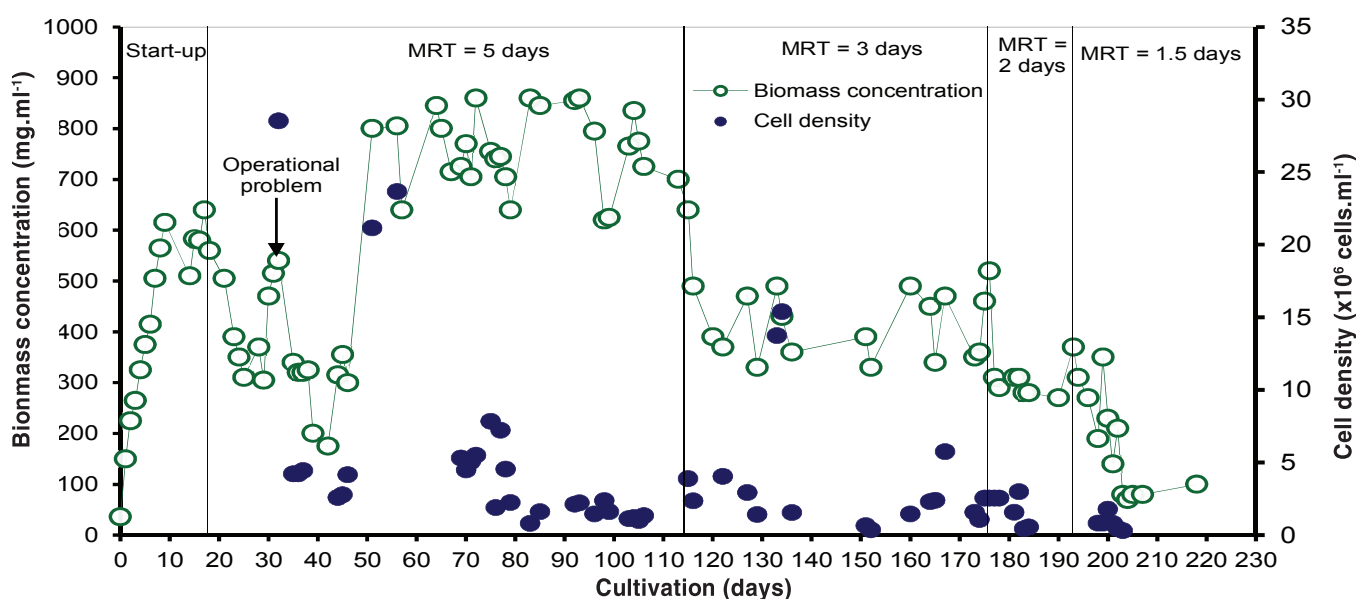


Fig. 2. Microalgal growth curve and cell density of *Chlorella vulgaris* at different MRTs.

of this MRT, biomass concentration was reduced from 560 mg/l on day 18 to 305 mg/l on day 29 due to the operational problem (clogging of the electrical floater) of the system. Biomass concentration was then continuously increased to 540 mg/l on day 32. Similarly, on day 32, a biomass washout incident again occurred due to the previously described operational problem. Therefore, biomass concentration was again gradually reduced to 175 mg/l on day 42. From day 46, biomass concentration was restored and achieved a steady state (800 mg/l) from day 51 onwards. At the steady state of 5-day MRT, the average biomass productivity was $151.93 \pm 15.05 \text{ mg.l}^{-1}.\text{day}$ (Fig. 3).

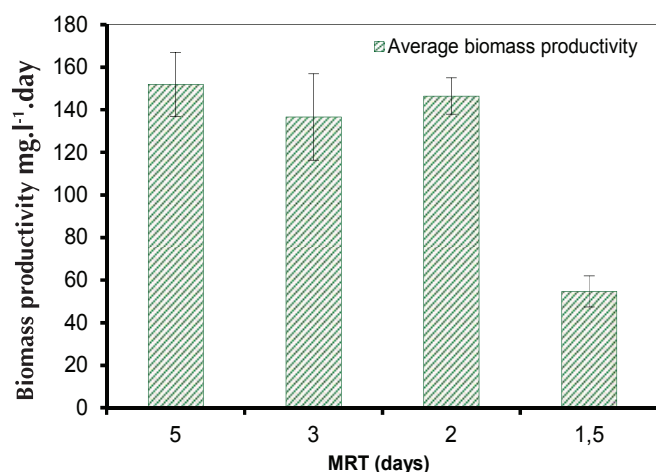


Fig. 3. Biomass productivity of *Chlorella vulgaris* at different MRTs.

At MRT of 3 days, average biomass concentration and biomass productivity reached 410 mg/l and $136.67 \pm 20.34 \text{ mg.l}^{-1}.\text{day}$, respectively. The system was stable after several days and operated for 50 days at 3-day MRT.

At MRT of 2 days, microalgae biomass concentration achieved a steady state quickly for several days. During 15 days of operation, average biomass concentration and biomass productivity were 292.86 mg/l and $146.43 \pm 8.52 \text{ mg.l}^{-1}.\text{day}$, respectively.

When MRT was controlled at MRT of 1.5 days, the biomass concentration began to decrease significantly from 310 mg/l (day 194) to 80 mg/l (day 203); it then became steady at this value. At this stage, average biomass concentration and biomass productivity achieved 82 mg/l and $54.67 \pm 7.30 \text{ mg.l}^{-1}.\text{day}$, respectively.

The biomass growth in MPBR was measured as MLSS. This value included living, dead algae, protozoa and bacteria. However, based on cell counts and microscopic observation, living algae was observed to be dominant in the biomass mixture during the cultivation period, which ranged from 0.3×10^6 to 28.5×10^6 cells/ml (Fig. 2). Flocs formation of microalgae occurred in MPBR at the beginning of the stationary phase; therefore, the counting number of algae was hardly estimated because flocs formation was occurred in the reactor. The appearance of flocs in MPBR could be due to

the competition of bacteria and their extracellular polymeric substance [14] and the intracellular substances was released by dead algae [8]. Bacteria growth could not cause a 'shut down' of the photobioreactor and the microalgae dominant, although bacteria, protozoa, and flocs formation occurred in the MPBR at almost MRTs. Moreover, the influence of bacteria was effectively prevented by withdrawal of biomass and a microfiltration membrane module in the photobioreactor.

The longer MRT corresponded with high biomass concentration (Table 1), which may lead to the rapid removal of nitrogen [15, 16]. However, the high concentration indicates low nutrient loading rates or low F/M ratios. In this study, these ratios were 0.13, 0.22, 0.3, and 1.21 for nitrogen and 0.01, 0.01, 0.02, and 0.04 for phosphorus corresponding with MRT of 5, 3, 2, and 1.5 days, respectively. Therefore, at MRT of 5 days, MPBR performed the optimum biomass productivity; the productivity at 2 days was then $136.67 \pm 20.34 \text{ mg.l}^{-1}.\text{day}$. Relative to MRT of 2 days, the lower biomass productivity was achieved at MRT of 3 days due to lower F/M ratio. In contrast to MRT of 3 days, the lowest microalgae productivity occurred at 1.5 days because of the overly high F/M ratios. In addition, light may limit the microalgal growth due to self-shading at high biomass concentration; therefore, dark respiration of algae occurs in MPBR [17]. This was not proved in this study.

Based on the observed results, it is clear that the MRT as short as 1.5 days could cause the biomass productivity to decrease significantly due to low algal biomass concentration retained in the reactor. MRT of lower than 2 days strongly affects the biomass concentration and biomass productivity of the MPBR. In addition, the suitable MRTs for MPBR in this study ranged between 2 and 5 days. The average biomass productivity ranged between 146.43 ± 8.52 and $151.93 \pm 15.05 \text{ mg.l}^{-1}.\text{day}$ for MRT of 2 to 5 days (Table 1).

Table 1. Comparison of performance of MPBRs.

References	MPBR	Influent concentrations		Nutrients loading		Growth of microalgae	
	SVR (m ²)	TN (mg N/l)	TP (mg P/l)	TN (mgN.l ⁻¹ .day)	TP (mgP.l ⁻¹ .day)	MLSS (mg/l)	Microalgae productivity (mg.l ⁻¹ .day)
5-day MRT (this study)	39.2	200.1	10.2	86.30	5.13	759	151.93±15.05
3-day MRT (this study)		184.0	9.4	92.01	4.70	410	136.67±20.34
2-day MRT (this study)		176.5	12.5	88.28	6.29	292	146.43±8.52
1.5-day MRT (this study)		198.8	9.3	99.44	4.66	82	54.67±7.30
Marbelia, et al. (2014) [11]	20.0	7.4	1.6	3.74	0.84	590	27.00
Gao, et al. (2014) [3]	32.3	19.1	1.24	8.39	0.56	-	39.93
Gao, et al. (2016) [13]	57.5	13.3	0.72	6.66	0.36	1724	50.72
Gao, et al. (2016) [18]	56.2	6.8	0.42	6.81	0.42	1100	42.60

Remarks: SVR = surface volume ratio; TN = total nitrogen; TP = total phosphorus; MLSS = mixed liquor suspended solids.

Because of the high nutrient media in this study, which were 10- to 28-fold and 6- to 24-fold higher than these wastewaters respectively, the microalgae productivity in this study was higher than in previous studies [3, 11, 13, 18]. Relative to other studies, the nutrient loading in this study was higher. This

proved that the 1:30-diluted human urine provided sufficient nutrients for microalgae production, while Jaatinen, et al. (2016) reported that the 1:25-diluted urine was the optimal medium for *Chlorella vulgaris* cultivation [8]. The submerged membrane demonstrated the effectiveness in preventing wash-out of biomass and improvement of nutrient loading. The highest biomass concentration of 759 mg/l at MRT of 5 days was achieved.

In this study, the MPBR exposed an illumination area of 0.32 m² and yielded the surface to volume (S/V) ratio of m²/m³, which was lower than the optimum S/V ratios of 80-100 m²/m³ in PBR [11]. However, the reactor's biomass and biomass productivity were respectively 759 mg/l and 151.93±15.05 mg.l⁻¹.day. This value was higher than that yielded by other MPBRs [3, 11]. Therefore, the performance of MPBR could be minimised by effective mixing of air bubbles. Moreover, the S/V ratio was smaller than the ratio in previous studies by Gao, et al. [13, 18]; nevertheless, the higher production was achieved in this study due to the lower biomass concentration (Table 1). The high concentration of algae could cause the respiration in the dark [17] and the smaller production in these studies.

The N/P ratio of diluted human urine in this study was 20:1, which was higher than the ratio of microalgal biomass (CO_{0.48}H_{1.83}N_{0.11}P_{0.01}) [5] and Redfield ratio (16:1) [18]; therefore, P was the limiting factor for microalgal growth. In addition, the N/P ratio of 15:1 was regarded as the optimum ratio for microalgal growth with maximum biomass concentration of 3568 mg/l [19]. Additionally, other types of wastewater containing the lower N/P ratio can be mixed with human urine for microalgal cultivation. For example, the shrimp farming wastewater containing TN and TP was 159 and 19.6 kg/ha.crop (the N/P ratio was 8:1), which is one of the potential sources for eutrophication in the Mekong Delta [20].

Conclusions

This study illustrates the potential of applying human urine for biomass production. Urine can be an ideal nutrient to cultivate microalgal biomass. The average biomass productivity was as high as 146.43 to 151.93 mg.l⁻¹.day at the operated MRT of 2 to 5 days. The MRT shorter than 1.5 day caused a significant reduction of biomass productivity.

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The authors declare that there is no conflict of interest regarding the publication of this article.

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Water quality assessment using the Pollution Index (PI) and statistical tools: a case study of Thi Vai river, Dong Nai, Vietnam

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Abstract:

The quality of river water is influenced by two factors: nature and man. The aims of this paper are to determine the influence of anthropogenic sources of pollution on water quality, by assessing integrated measurements from use of the Pollution Index (PI) of Indonesia and diverse statistical techniques, including one-way analysis of variance (ANOVA) and ArcGIS. In this study, 10 physicochemical parameters for the determination of water quality, from surface water taken the Thi Vai river, are examined: dissolved oxygen (DO), biochemical oxygen demand (BOD_5), chemical oxygen demand (COD), ammonium (NH_4^+-N), phosphate ($PO_4^{3-}-P$); total dissolved solids (TSS), pH, nitrite (NO_2^-), nitrate (NO_3^-), total coliforms, and fecal E. coli. The samples were collected from seven monitoring sites, for assessing spatial and temporal water quality, in the three years 2015 to 2017.

The findings revealed that water quality index values within the study area showed a significant pollution level for nitrite, and fecal E. coli. Water quality was detrimental at the sites TV2, TV3, and TV4. A further finding was that there was significant variation recorded between the two methods of measuring PI - that of the Ministry of the Environment, Indonesia, and that of Vietnam. Finally, this integrated technique could, it is suggested, be an effective approach for communicating information on water quality for sustainable waste management in Thi Vai river.

Keywords: affect, assessment, Dong Nai, environment, Pollution Index, surface water, Thi Vai, Water Quality Index.

Classification number: 5.1

Introduction

Human beings in modern society adversely affect the quality of surface water through various areas of activity, such as agriculture and industry. Natural forces such as stormwater run-off events, can also cause problems, such as the seasonal phenomenon of soil erosion, which is largely affected by factors such as climate, land cover, land slope, and soil resilience [1].

Without doubt, a variety of physical, chemical, and biological factors can be harmful to human health, if they occur over and above permissible limits [1-3]. The PI is one of the most effective methods for assessing the status of water. The values of the Water Quality Index (WQI) or PI (PI) can be used to modify policies and to forward feasible measures for management and use of surface water resources, as formulated by various environmental monitoring agencies [4-6].

River water quality is readily affected by both anthropogenic impacts and natural processes, leading to degradation of surface water, which in turn fails to meet various purposes [1, 2]. Furthermore, the WQI has been considered to pose sorting water quality [6, 7].

Statistical techniques are useful for verifying changes over time and space that are caused by natural and anthropogenic processes [1, 2]. Of these, Analysis of Variance (ANOVA) was applied to evaluate the significant disparity between groups of monitoring stations and across seasons. Assessing the relationships between dependent and independent variables by use of Spearman's Correlation Analysis (SCA) has been popular in scientific research [2-4, 8].

QCVN 08-MT:2015/BTNMT is presently being harnessed as the national technical regulation on evaluation of surface water in Vietnam. This monitoring programme

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requires numerous parameters to be measured, analyzed, and explained, through the multivariate approach. The Water Quality Index (WQI), which involves a single number expressing water quality by integrating measurement values across many physicochemical parameters, is used to indicate the overall status of surface water quality [4-7].

The Ministry of Natural Resources and Environment of Vietnam (MONRE) has developed the WQI approach, as explained in Decision No. 879/QĐ-TCMT, to create guidelines on surface water quality for the protection and management of water resources. The WQI, which suits conditions in Vietnam well, evolved from the combination of weighted arithmetic WQI values with River Status Index (RSI) [4]. Accordingly, nine water quality parameters, those of temperature, DO, BOD₅, COD, NH₄⁺-N, PO₄³⁻-P; TSS, pH, and total coliforms, were chosen to calculate WQI. The WQI number ranges, which are colour-banded, are from 0 to 100; the higher the number, the better the water quality. This study also monitors additional indicators such as NO₂⁻, NO₃⁻ and fecal E. coli. Hence, another method, that of the PI (PI) of Indonesia, is also used to evaluate water quality. This method is based on the Indonesian Ministry of the Environment's Decree No115/2003 regarding the "Guidelines for determination of water quality status".

This topic will offer support for scientists as well as managers in the field, such as those working in ecology, resources, and environmental protection, who can use this study for monitoring the ecological health of Thi Vai river, Dong Nai province [9].

Materials and methods

Study area

The Thi Vai river starts in the Nhon Tho town of Dong Nai's Long Thanh province, running through the Tan Thanh district of Ba Ria - Vung Tau province and the Can Gio district of HCMC before flowing into the Eastern Sea. Its total length is approximately 76 kilometers and its total basin area around 300 square kilometers. The river receives around 34,000 cubic meters of discharged untreated wastewater daily, from nearly 200 operating enterprises situated along the basin; it also receives untreated wastewater from populated areas, aquaculture, fish farming, and cattle-raising farms.

The rainy season in the Thi Vai river area begins at the end of May and ends in the last week of October. This accounts for 90% of the whole year's rainfall; the remainder of the year is the dry season.

The total discharged volume into Thi Vai river directly from industrial activities in Dong Nai and Ba Ria - Vung Tau provinces, is 36,357 m³/day. Besides the direct sources mentioned above, the river also receives indirect wastewater from production facilities and industrial zones in Long

Thanh and Nhon Trach districts, through canals which flow into the river. One of the most common sources of pollution is that of water pollution, which is characterized by the presence of organic pollutants (BOD₅, COD), TSS, nutrients, oils, and microorganisms in the water. At present, urban centres in Dong Nai province do not have concentrated wastewater treatment systems, and wastewater is drained into the common drainage system. The results of a survey of 50 households situated along the Thi Vai river indicated that these households use groundwater, which is then discharged untreated directly into the canal, draining into Thi Vai river.

Most canals in the upstream area of the Thi Vai river have poor water quality. The parameters for COD, BOD₅, ammonium, nitrite, coliform, and E. coli exceed those specified in QCVN 08-MT:2015/BTNMT, column B₁, on multiple occasions.

Sampling, measuring, and analysis

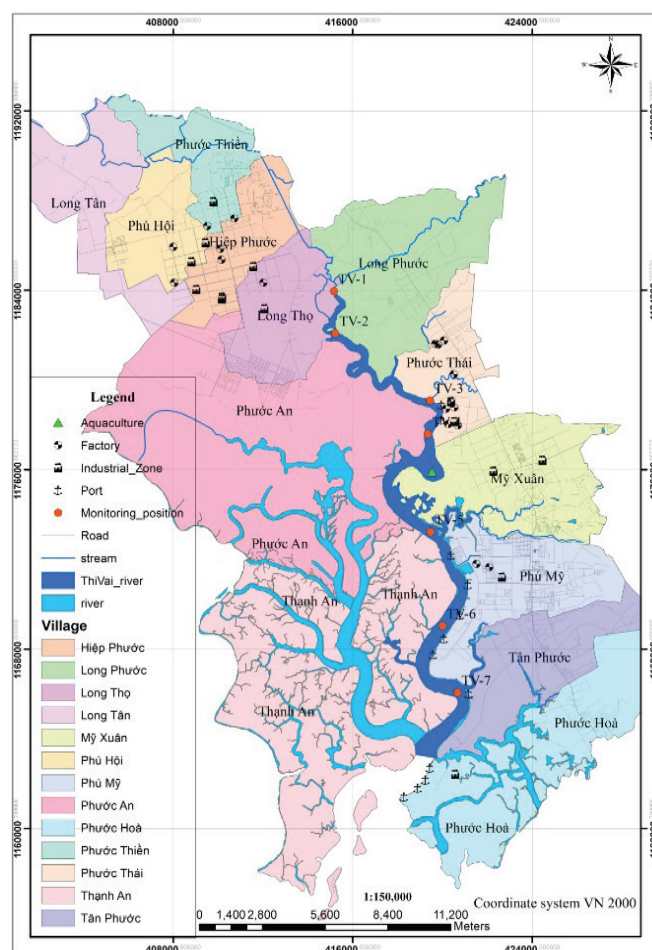


Fig. 1. Map of Monitoring Positions on Thi Vai river.

Where: TV1: confluence of Ba Ky canal on Thi Vai river; TV2: Long Tho Ward; TV3: Vedan large water ditch; TV4: Go Dau port; TV5: float number 23; TV6: Phu My Thermal Power Plant; TV7: float number 7.

Technical guidance for sampling water and sample treatments is specified in TCVN 6663-6:2008 (ISO 5667-6:2005), TCVN 6663-3:2003 (ISO 5667-3:1985), and TCVN 6663-6:2008 (ISO 5667-6:2005) respectively. The measurements for pH and DO were analyzed in the field; others samples were brought to the laboratory for analysis.

The data on 11 physical and chemical parameters for surface water quality were collected at seven sampling locations in the Thi Vai river basin, during the period 2015-2017, by the Centre for Monitoring Natural Resources and Environment (DONRE) of Dong Nai province (Fig. 1.) After collection, physicochemical parameters including DO, BOD₅, COD, NH₄⁺-N, PO₄³⁻-P, TSS, pH, NO₂⁻, NO₃⁻, total coliforms, and fecal coli were analyzed according to the procedures laid down in APHA, 1999 [10]. The data were then structured through use of the SPSS software program for statistical analysis.

Statistical analysis and ArcGIS

The box-and-whisker plot statistical technique was used to evaluate seasonal variance in the pollution status of the Thi Vai river basin. One-way Analysis of Variance ($0.01 \leq \alpha \leq 0.05$) was used to investigate the substantial disparity in the mean of the PI across locations and seasons [2, 4]. Furthermore, Spearman's Correlation Analysis (SCA) was used to evaluate the relationships between the WQI (or PI) and physicochemical parameters. This study also monitored additional indicators such as NO₂⁻, NO₃⁻ and fecal E. coli. The PI of Indonesia was used to evaluate water quality, and was essential for this research. In this study, all statistical procedures were executed by using the SPSS 22.0 tool. In addition, ArcGIS 10.0 was also applied, to distinguish water quality for specific purposes [3, 4, 6, 7].

Water quality index (WQI)

The Vietnamese WQI Decision No. 879/QD-TCMT is used to determine water quality based on nine parameters: DO, BOD₅, COD, NH₄⁺-N, PO₄³⁻-P; TSS, pH, temperature, and total coliforms [8].

$$WQI = \frac{WQI_{pH}}{100} \left[\frac{1}{5} \sum_{a=1}^5 WQI_a \times \frac{1}{2} \sum_{b=1}^2 WQI_b \times WQI_c \right]^{\frac{1}{3}}$$

where: WQI_a is determined through five parameters: DO, BOD₅, COD, NH₄⁺-N, and PO₄³⁻-P; WQI_b is calculated by TSS and turbidity; WQI_c was calculated with total coliform parameter, and WQI_{pH} is determined by pH parameter.

WQI is distinguished according to a range from 0 to 100,

the values corresponding to specific colours, and higher numbers signifying better water quality. The WQI process of water quality ranking was performed as in Table 1.

Table 1. Surface water quality classification based on WQI.

WQI range/colour	Water quality rating
91-100 (Blue)	Excellent water quality
71-90 (Green)	Good water quality
51-70 (Yellow)	Medium water quality
26-50 (Orange)	Poor water quality
0-25 (Red)	Very bad water quality

The analysis of river water quality according to the PI followed the guidelines designated by the Ministry of Natural Resources and Environment's Decree No 115/2003, which uses the equation below:

$$PI_j = \sqrt{\frac{(C_i/L_{ij})_M^2 + (C_i/L_{ij})_R^2}{2}}$$

where: L_{ij} is the concentration of water quality parameters; C_i: concentration of water quality parameters; PI_j: PI of water; R: average; M: maximum.

Assessment of the PI estimate is: $0 \leq PI_j \leq 1.0$: meets standards of excellent quality; $1.0 < PI_j \leq 5.0$: slightly polluted; $5.0 < PI_j \leq 10$: steadily polluted; $PI_j > 10$: drastically polluted.

Results and discussion

Water quality assessment based on physicochemical parameters

Table 2 and Figure 2 below compare the disparity in the percentage of samples that failed to meet Vietnamese technical requirements regarding surface water quality QCVN 08-MT:2015:BTNMT (B1) during the period 2015 to 2017.

Overall, what is striking from looking at the table and graphs is that surface water is polluted significantly by concentrations of COD, NO₂⁻, and E. coli. In fact, COD concentrations in both the dry and the rainy season failed to meet admissible standards and varied considerably, at 33.33% and 46.33% respectively, with great variance between the seasons. Likewise, there was an upward trend in E. coli, the samples of which exceeded the permissible levels in the dry season and the wet season, at 13.10 and 24.14, respectively, again with a great difference between the two seasons. With regards to NO₂⁻, almost 100% of the sampling sites failed to meet acceptable standards, with an

Table 2. The proportion of samples that failed to meet the necessary standards.

Parameter	QCVN*	TV-1		TV-2		TV-3		TV-4		TV-5		TV-6		TV-7		Mean	
		Dry (%)	Wet (%)	Dry (%)	Wet (%)	Dry (%)	Wet (%)	Dry (%)	Wet (%)	Dry (%)	Wet (%)	Dry (%)	Wet (%)	Dry (%)	Wet (%)	Dry (%)	Wet (%)
pH	5,5-9	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0.00
DO	≥4 mg/l	0.00	0	12.50	12.5	0.00	12.5	12.50	25	0.00	0	0.00	0	0.00	0	1.79	3.57
BOD ₅	<15 mg/l	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0.00
COD	<30 mg/l	50.00	50	50.00	50	37.50	62.5	37.50	62.5	62.50	62.5	16.67	60	16.67	40	33.33	46.43
NH ₄ ⁺ -N	<0.9 mg/l	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0.00
TSS	<50 mg/l	12.50	0	12.50	12.5	12.50	0	0.00	0	0.00	0	16.67	0	0.00	0	7.74	1.79
NO ₂ ⁻ -N	<0.05 mg/l	100.00	100	100.00	100	100.00	100	100.00	100	100.00	100	83.33	80	50.00	60	76.19	77.14
NO ₃ ⁻ -N	<10 mg/l	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0.00
PO ₄ ³⁻ -P	<0.3 mg/l	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0.00
E. Coli	<100 mg/l	37.50	50	25.00	50	0.00	37.5	12.50	37.5	12.50	12.5	16.67	20	0.00	20	13.10	27.14
Coliform	<7,500 mg/l	0.00	0	0.00	0	0.00	0.00	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0.00

Note: QCVN*: national technical regulations on surface water quality, QCVN 08-MT:2015/BTNMT (B1). B1 is the surface water source for irrigation or other purposes.
Dry: dry season; Wet: wet season.

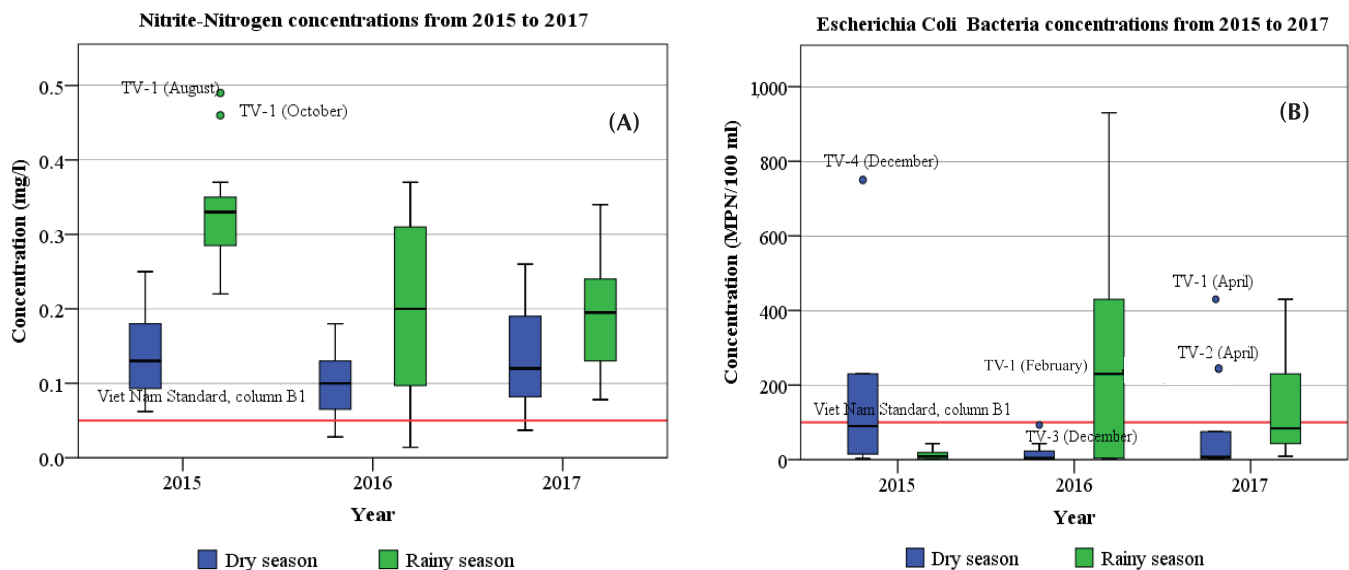


Fig. 2. Nitrite (A) and E. Coli (B) concentrations across the seasons, in the observed period of 2015-2017.

average range of 91.56 ± 9.01 during the observed period, and significant seasonal variation. The causes of this issue could include the discharge of untreated wastewater from domestic use, seafood processing, aquaculture, fish farming, industrial activities, and agricultural run-off.

Water quality index assessment

The Vietnamese WQI is calculated on nine parameters. These do not include NO_2^- or NO_3^- . However, the water quality of the Thi Vai river was polluted by these indicators. Therefore, the results of the statistical analysis illustrate the

great disparity between the WQI method of Vietnam and the PI of Indonesia. Hence, this study uses the Indonesian PI method for assessing water quality.

PI analysis

The striking observation from Fig. 3 is that the period in question witnessed a considerable downward trend in PI from upstream to downstream, corresponding to improved water quality (Fig. 3, Table 3). Furthermore, PI value was the highest in 2015, estimated at 2.09 ± 0.68 ; this was followed by the PI value of 2017 (1.69 ± 0.54); with the smallest estimation being 1.59 ± 0.63 in 2016 (Table 3, Figs.

4, 5). Thus, the average difference between 2015, 2016, and 2017 was negligible (Fig. 4). Furthermore, the statistical analysis by ANOVA showed no dramatic difference in PI value between the three years.

The Box-and-whisker plots (Fig. 3) show that the PI values at the various sampling locations witnessed remarkable differences during the period 2015 to 2017, with PI values in the wet season being much greater than in the dry season. This seasonal change is most marked in 2016 (Fig. 3A). Moreover, in the wet season of 2015, the values for the two observations were outside the graph range. It is therefore important to determine the exact causes of this phenomenon, in order to bring in effective measures for the Thi Vai river. Furthermore, the PI value demonstrated a surge from TV1 to TV2, after which it declined slightly, from TV2 to TV4, then continued to drop rapidly to TV7. In general, pollution is seen to be mainly concentrated in the upstream areas of the river, such as the Ba Ky - Thi Vai canal, Long Tho, Vedan, and Go Dau areas. It is noteworthy that sites TV2 and TV3 receive a large quantity of untreated waste from industrial zones, fish farming, and aquaculture, which would have a detrimental effect on water quality.

The summary in Table 2 shows that at locations TV1, TV2, TV3, TV4, TV5, and TV6, water quality failed to meet permissible standards for the supply of residential domestic water, and was lightly polluted. The exception to this was site TV7, where water quality met the necessary standards (Table 3, Figs. 5, 6).

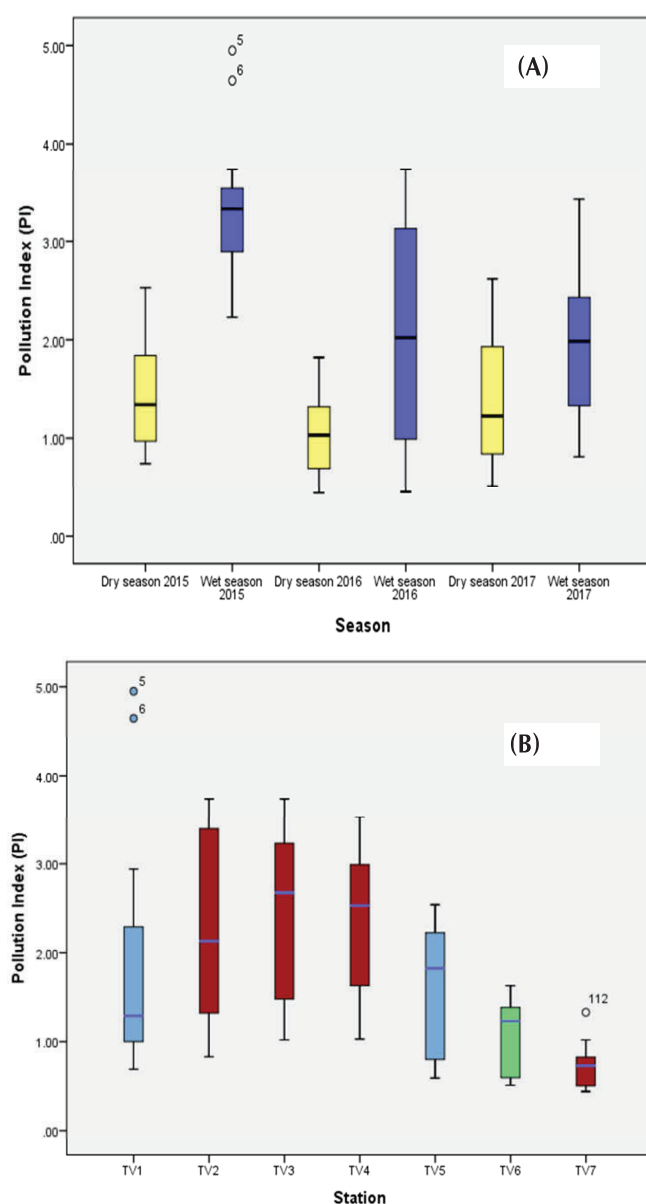


Fig. 3. (A) was about seasonal changes; (B) was about spatial changes.

Table 3. Average annual PI and rankings.

Code	Average annual PI			Ranking
	2015	2016	2017	
TV-1	2.68	1.45	1.36	Lightly polluted (LP)
TV-2	2.55	2.25	1.88	Lightly polluted
TV-3	2.56	2.32	2.49	Lightly polluted
TV-4	2.48	2.30	2.21	Lightly polluted
TV-5	1.89	1.14	1.87	Lightly polluted
TV-6	1.34	0.94	1.20	Lightly polluted
TV-7	0.77	0.75	0.85	Met quality standards
Mean	2.09 ± 0.68	1.59 ± 0.63	1.69 ± 0.54	

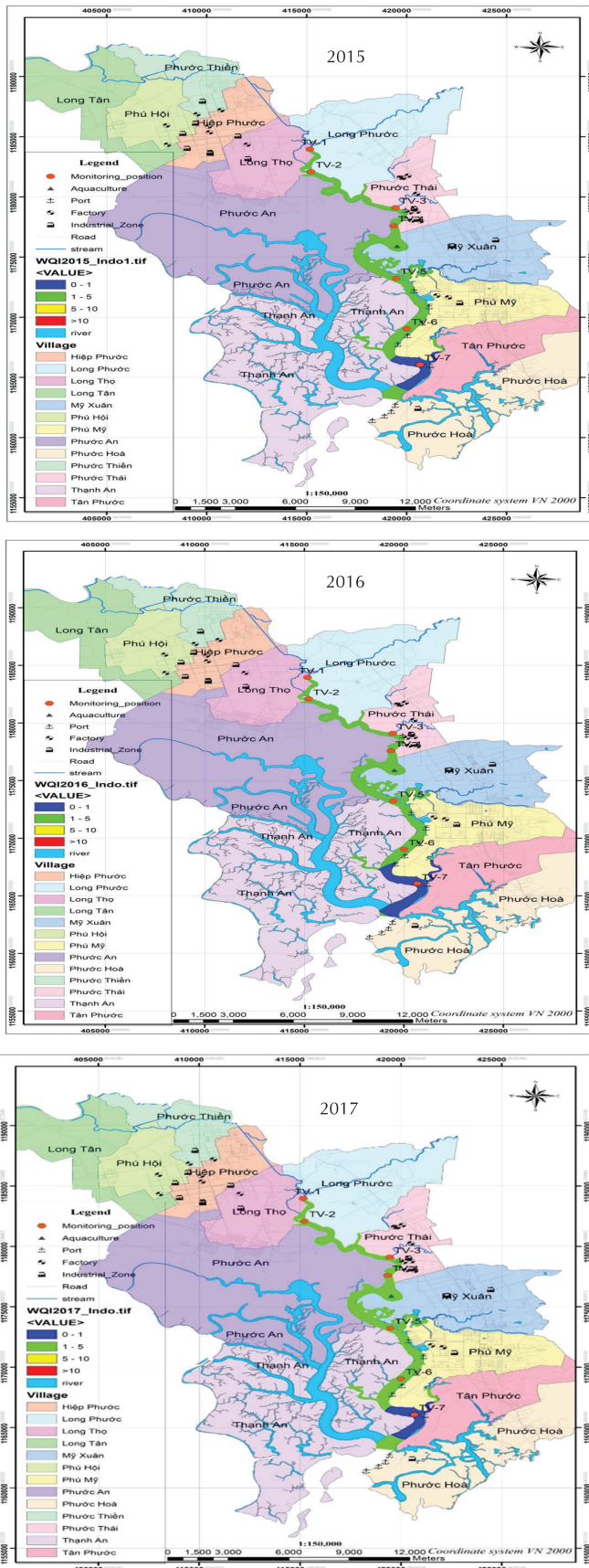


Fig. 4. PI of the Thi Vai river from 2015 to 2017.

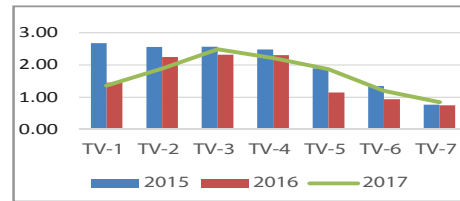


Fig. 5. Average annual PI.

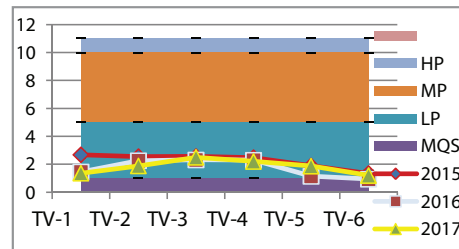


Fig. 6. Partition water quality according to PI.

Spatial and temporal variations at the monitoring sites (ANOVA)

The results of the one-way ANOVA analysis illustrate that the mean PI value in the dry season is dramatically different to that in the wet season (Table 4). Indeed, the statistical results indicate the seasonal changes were very distinct in 2015 and 2016, but not for 2017. The results also indicate the efficacy of the ANOVA tool in reference to this study. Seasonal factors strongly impact water quality in each of the studied areas (Sig.=0.000, $\alpha=0.05$), which is a beneficial finding for the management of the water environment. A wide range of parameter concentrations and indications of pollution at the monitoring stations were recorded during the period in question. This indicates that the sources of pollution at each sampling site may be different (Table 5).

Table 4. Differences in WQI value between the two seasons over the observed period.

(I) Season		Mean difference (I-J)	95% Confidence interval	
			Lower bound	Upper bound
Dry 2015	Wet 2015	-1.80467*	-2.61	-1.00
	Dry 2016	.44	-0.30	1.18
	Wet 2016	-.56	-1.30	0.18
	Dry 2017	.13	-0.69	0.95
Wet 2015	Wet 2016	2.24848*	1.48	3.01
	Dry 2016	1.24229*	0.48	2.01
	Wet 2017	1.93252*	1.09	2.77
	Dry 2017	1.29110*	0.45	2.13
Dry 2016	Wet 2016	-1.00619*	-1.71	-0.31
	Dry 2017	-.32	-1.10	0.47
	Wet 2017	-.95738*	-1.74	-0.18
	Dry 2017	.69	-.09	1.47
Wet 2016	Wet 2017	.05	-.73	.83
	Dry 2017	-.64	-1.50	.21

*The mean disparity is significant at 0.05.

Table 5. The difference in WQI values between monitoring sites during the observed period.

(I) Station		Mean difference (I-J)	95% Confidence interval	
			Lower bound	Upper bound
TV1	TV2	-.38	-1.33	0.57
	TV3	-.55	-1.51	0.40
	TV4	-.45	-1.40	0.51
	TV5	.28	-0.67	1.24
	TV6	.82	-0.24	1.87
	TV7	1.15432*	0.10	2.21
TV2	TV3	-.17	-1.13	0.78
	TV4	-.07	-1.02	0.89
	TV5	.66	-0.29	1.62
	TV6	1.19614*	0.14	2.25
	TV7	1.53432*	0.48	2.59
TV3	TV4	.107	-.847	1.062
	TV5	.836	-.118	1.791
	TV6	1.37114*	0.31	2.43
	TV7	1.70932*	0.65	2.77
TV4	TV5	.73	-0.23	1.68
	TV6	1.26364*	0.21	2.32
	TV7	1.60182*	0.54	2.66
TV5	TV6	.53	-0.52	1.59
	TV7	.87	-0.18	1.93
TV6	TV7	.34	-0.81	1.49

*The mean disparity is significant at 0.05.

Table 6. Summarized correlation between PI and seven physicochemical parameters.

	DO	TSS	COD	BOD ₅	NH ₄ ⁺ -N	NO ₂ ⁻ -N	NO ₃ ⁻ -N	PO ₄ ³⁻ -P	E.Coli	Coliform	PI
DO	1.000	.017	-.219*	-.287**	.112	-.527**	-.115	-.111	-.279**	-.292**	-.529**
COD	-.219*	.315**	1.000	.912**	-.256**	.227*	-.321**	-.216*	-.120	-.121	.244*
BOD ₅	-.287**	.263**	.912**	1.000	-.200*	.309**	-.346**	-.131	-.125	-.121	.326**
NO ₂ ⁻ -N	-.527**	-.111	.227*	.309**	-.007	1.000	.220*	.034	.268**	.301**	.999**
NO ₃ ⁻ -N	-.115	-.153	-.321**	-.346**	.161	.220*	1.000	.228*	.377**	.439**	.218*
E.Coli	-.279**	.037	-.120	-.125	.418**	.268**	.377**	.333**	1.000	.932**	.272**
Coliform	-.292**	-.043	-.121	-.121	.412**	.301**	.439**	.270**	.932**	1.000	.305**

*Correlation is significant at the 0.05 level (2-tailed).

**Correlation is significant at the 0.01 level (2-tailed).

Statistical correlations between physicochemical parameters and the PI

The correlation between PI and specific parameters is indicated in Table 6. The main observation from this is the considerable negative correlation between PI value and DO, at $r=-0.53$. Considerable positive correlations can be observed between PI values and COD, BOD, NO₃⁻, E. coli, and coliform concentrations, at $r=0.24, 0.33, 0.22, 0.27, 0.31$, respectively. In particular, there was a strong positive correlation between PI and NO₂⁻ concentration, at $r=0.99$, which indicates that NO₂⁻ concentrations substantially impact PI value, followed by DO. Other parameters had a low correlation with PI, because these showed good quality and mostly met the required standards. Thus, it can be stated that

declining NO₂⁻ concentrations and increasing DO are the urgent issues.

Conclusions

The PI and statistical tools are effective and useful methods for communicating information on, and managing, water quality, both with regard to citizens and policymakers. The surface water quality of the Thi Vai river can be clarified in the following order: TV7>TV6>TV5>TV1>TV4>TV3>TV2. Furthermore, in 2015 and 2016, a strong difference between parameter in the dry season and those in the wet season can be observed; in addition, concentrations in the wet season were consistently higher than in the dry season. This study also indicates the anthropogenic effects of activities such as aquaculture industries and fish farming, which are seen to be principal sources of pollution. Furthermore, the efficacy of the Indonesian PI method is clear when assessing water quality in the Thi Vai river. Based on investigation of PI results, it can be concluded that effective treatment solutions and appropriate management processes are urgently required to enhance the water quality of the Thi Vai river.

The authors declare that there is no conflict of interest regarding the publication of this article.

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Evaluation of iron-rich adsorbent to remove arsenic from groundwater in decentralised water supply treatment

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Abstract:

This paper evaluates the adsorption capacity of iron-rich adsorbent to remove arsenic from groundwater in decentralised water supply treatment; a pilot-scale with a capacity of 5 m³/day has been investigated at Cu Da Nursery School in Hanoi city, in which the concentration of arsenic in groundwater is abnormally high (175–400 µg/l). The pilot system was designed to include a series of processes, such as aeration, sedimentation, sand filtration, and adsorption, using the iron-rich adsorbent and activated carbon from coconut shells. The pilot system was tested with three operated modes. The results demonstrate that the treatment efficiency of the overall treatment processes was significantly higher than that of the traditional treatment system used by local people. After aeration, sedimentation, and sand filtration, the water quality was discovered to respond substantially in reducing iron by over 96% (from 8–12 mg/l to less than 0.3 mg/l) arsenic by 55 to 60%, but after iron-rich adsorbent, the concentration of arsenic decreased to less than 10 µg/l, which fulfills Vietnam National technical regulations concerning drinking water quality.

Keywords: adsorbent, arsenic, decentralised water supply groundwater.

Classification number: 5.1

Introduction

Groundwater is a water source used for living and drinking purposes. In Vietnam, arsenic concentrations in groundwater are exceptionally high in many areas. A broad survey of arsenic pollution in the 12 provinces of the Red River Delta, Central area, and Mekong Delta was conducted by the Vietnamese government with UNICEF funding in 2008. A total of 12,439 water samples were analysed with arsenic in 419 communes of 33 districts in these 12 provinces. Analytical results indicate that some provinces of the Red River Delta and Mekong Delta had tube well water in which arsenic concentrations significantly exceeded Vietnamese technical regulations (≤ 10 µg/l for drinking water and ≤ 50 µg/l for domestic water); 34.9% and 21.1% of tube wells had arsenic concentrations above 10 µg/l and 50 µg/l, respectively [1]. The concentration of arsenic in groundwater in the Red River Delta was between 1 and 3,050 µg/l (average 159 µg/l) [2]. An estimated 17 million Vietnamese may face the potential risk of arsenic poisoning from using groundwater [3].

Decentralised water treatment is an effective and appropriate solution for households or groups of households in rural and suburban areas in Vietnam. This may also be effective in areas where the central water supply treatment system is lacking, when re-pollution occurs in the pipeline system before reaching end users, or in emergency cases, such as flooding, epidemic disease, etc. [4]. A decentralised water supply system may be more cost-effective than a centralised system because the central conveyance, treatment capacity, and potable water transmission reduce costs [5]. In rural areas of Vietnam, groundwater has been pumped from drilled wells and treated through a simple filtering system that involves rock, sand, and activated carbon or used directly; this was unable to remove arsenic in water.

To provide safe drinking water, effective treatment technologies for removing arsenic from groundwater must be applied. However, the implementation of efficient water treatment technologies may require significant capital investment and high operating costs for households. In Vietnam, some low-cost technologies have been investigated in relation to their

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ability to adsorb arsenic through natural iron-rich or manganese-rich materials such as red mud, laterite, etc. This research has yielded positive results at the laboratory scale [6]. The results have demonstrated that the highly-efficient adsorbents were made from iron-rich materials. This paper aims to evaluate the potential to remove arsenic from groundwater using iron-rich adsorbents on a pilot scale, with a capacity of 5 m³/day at Cu Da Nursery School in Hanoi, where arsenic concentrations in groundwater are abnormally high (175-400 µg/l).

Materials and methods

Iron-rich adsorbent preparation

The iron-rich material iron (III) hydroxide was prepared in the laboratory based on the reaction of FeCl₃ with NaOH. These materials were weighed and mixed based on the mixing ratio (% by weight) of 40% ferric hydroxide, 10% aluminum hydroxide, 7.5% additive 1, 18.5% additive 2, and 24% kaolin. Two types of mineral soils (K soil and yellow clay soil), which are common and easy to find in Vietnam and are regarded as additives, were added to create porosity and adhesive. The mixed materials were molded into cylinders which were 10 mm in diameter and 10 to 15 mm in height. Cylinders were then dried at 50°C for 24 hours and burned at 500°C for 10 hours. Finally, iron-rich adsorbents were cooled naturally, and broken pellets were removed by sieves.

Pilot operation

The pilot-scale with a capacity of 5 m³/day was developed at Cu Da Nursery School in Hanoi city, where the concentration of arsenic in groundwater is abnormally high (175-400 µg/l). The adsorbent columns with dimensions of 760x1,500 mm (diameter x height) were created, fixed with 300 mm-high sand; the height of iron-rich adsorbents varies with each mode (Fig. 1). The pilot system was tested with three operated modes: (i) without aeration and 120 mm of iron-rich adsorbents, (ii) with aeration and 120 mm of iron-rich adsorbents, and (iii) with aeration and 250 mm of iron-rich adsorbents.

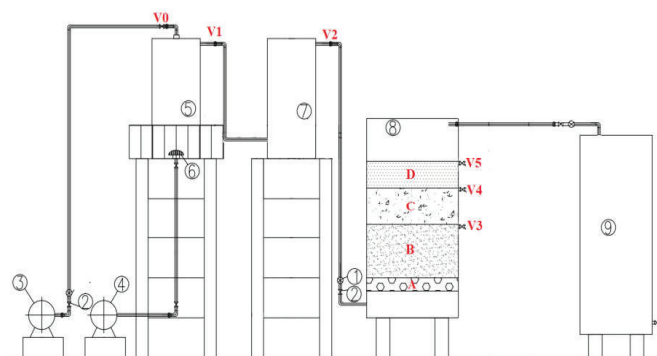


Fig. 1. Pilot system model in second and third modes

Note: 1 - flow meter, 2 - valve, 3 - pump, 4 - air blower, 5 - aeration tank, 6 - aeration tube, 7 - sedimentation tank, 8 - main treatment tank, 9 - bunker tank.

A - gravel layer, B - sand layer, C - iron-rich adsorbents, D - activated carbon layer.

pH was measured at the sampling site. The concentrations of iron and arsenic in water were analysed by AAS in the laboratory (AAnalyst 400, Perkin Elmer Inc).

Water samples were captured at V0 - groundwater, V1 - after aeration, V2 - after sedimentation, V3 - after sand filtration, V4 - after iron-rich adsorbents, and V5 - treated water (after activated carbon layer). All sampling locations have a suitable sampling valve.

Results and discussion

Effect of pH on changing operation mode

Figure 2 indicates the pH change in water after passing the pilot system. The results from Fig. 2 illustrate that, at the second and third modes, the pH of the treated water samples were slightly increased by the removal of iron of the pilot system, from 6.9 to 7.8.

In the second and third modes, after forced aeration, pH of the water increased slightly (from 6.5 to above 7) due to oxidation and precipitation of iron. pH of the water remained stable after sand filtration (V3) (between 7 and 7.5) and slightly increased when it passed the iron-rich adsorbents because of the alkalinity of kaolin in the iron-rich adsorbents. The increase pH values in the aeration and precipitation processes displayed the tendency of the oxidation of Fe (II) and As (III) to precipitate Fe (III) and soluble As (V). The ability to remove arsenic from water was facilitated significantly by this conversion since As (V) is adsorbed more efficiently by Fe(OH)₃ colloids than As (III).

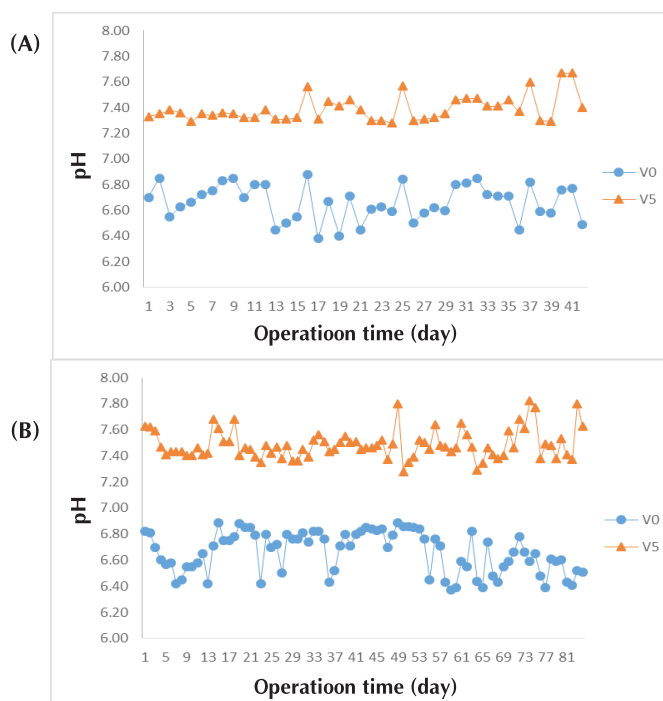


Fig. 2. pH of water samples in each mode. (A) The second mode and (B) the third mode.

Note: V0 - groundwater, V5 - treated water.

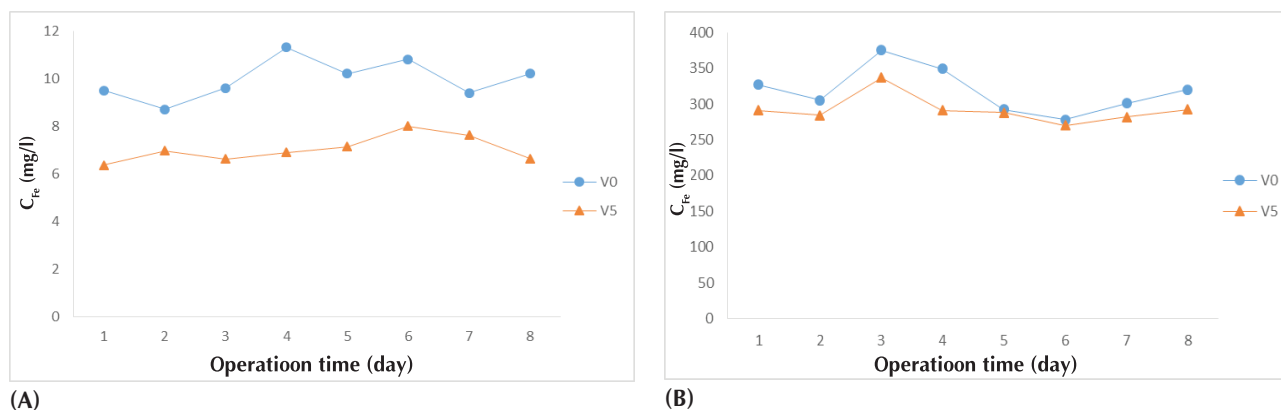


Fig. 3. Removal of iron and arsenic the first mode. (A) Removal of iron and (B) removal of arsenic.
Note: V0 - groundwater, V5 - treated water.

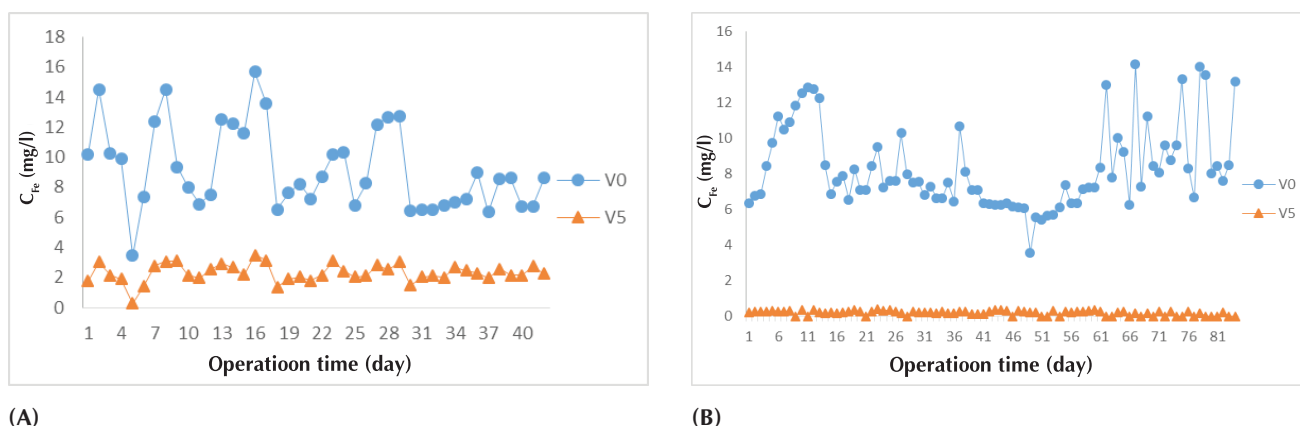


Fig. 4. Removal of iron in the second and third operated mode. (A) The second operated mode; (B) the third operated mode.
Note: V0 - groundwater; V5 - treated water.

Effect of aeration process on the efficiency of the pilot system

Figure 3 illustrates the first mode with a capacity of 5 m³/day, 120 mm-high iron-rich adsorbents, and the absence of aeration. The efficiency of removal of iron from groundwater was between 25 and 35%, whereas the treatment system was incapable of removing arsenic. The concentration of arsenic in V5 was insignificantly lower than that in V0.

In the second mode, the pilot system was installed with aeration. The adsorption column in the pilot scale achieved 76% removal of arsenic (ranged from 12 to 50 µg/l after overall treatment processes) and about 85 to 90% removal of iron from groundwater.

In the second mode, after sedimentation (V2), the concentration of arsenic decreased remarkably to 150 µg/l (approximately 50% relative to concentration of arsenic in groundwater V0) due to As (V) adsorbed into FeAsO₄ surfaces in the sedimentation tank. Precipitated Fe(OH)₃ particles which escaped from the sedimentation tank would be retained on the sand layer. When flow passed through the sand layer, As (V) continued to be adsorbed by Fe(OH)₃ precipitates in the sand layer, which decreased As (V) in water. The addition of a sprinkler in the third mode promoted the conversion of Fe (II) to precipitated Fe (III) relative to predecessor mode, meaning that arsenic in water was adsorbed more than in

the second mode.

This explains why the efficiency of the removal of iron and arsenic in the third mode is higher than in the second mode after sedimentation (V2) and after sand filtration (V3) because the efficiency of arsenic removal through iron hydroxide depends upon the Fe/As ratio in water (the higher the ratio, the higher the efficiency) [7]. In the third mode, a sprinkler was added at the position and pumped into the aeration tank.

The results of the analysis of iron concentrations in water samples indicated that after sand filtration (V3) in the third mode, iron concentration decreased to less than 0.3 mg/l and reached over 96% iron treatment efficiency in the third mode. After iron-rich adsorbents (V4) and activated carbon layer (V5), the iron concentration in the adsorbent column in pilot scale was nearly unchanged relative to the water sample in sand filtration (V3), which indicates that two types of adsorbents (iron-rich adsorbents and activated carbon) were almost not involved in iron treatment and that iron-rich adsorbents were not corroded; iron from iron-rich adsorbent was not to be released into water (Fig. 4).

Effect of height of iron-rich adsorbents on the removal of arsenic in the pilot system

The efficiency of removing arsenic from groundwater in the

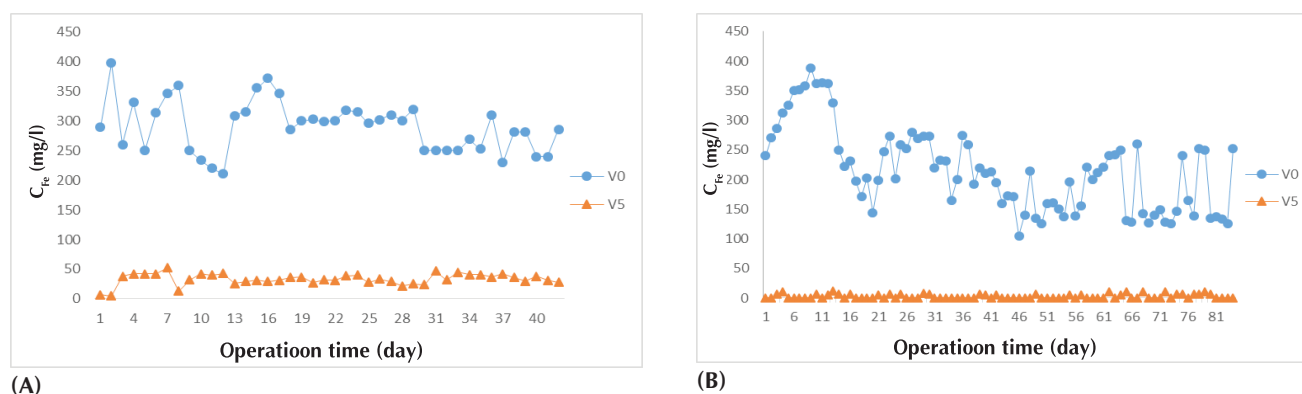


Fig. 5. Removal of arsenic in the second and third modes. (A) The second mode and **(B)** the third mode. Note: V0 - groundwater; V5 - treated water.

second mode was about 76%. The concentration of arsenic after overall treatment processes was between 12 and 50 $\mu\text{g/l}$, which does not fulfil Vietnam's technical regulations concerning drinking water quality. Therefore, the pilot system was developed in the third mode with twice the height of the iron-rich adsorbents relative to the predecessor system (250 mm).

The results of groundwater analysis at Cu Da Nursery School suggest that the concentration of arsenic in groundwater (V0) was between 250 and 400 $\mu\text{g/l}$. In the second mode, after the sand layer, the concentrations of arsenic in water were between 55 and 90 $\mu\text{g/l}$; after overall treatment processes (V5), concentrations of arsenic were between 12 and 50 $\mu\text{g/l}$. After the activated carbon layer (V5), the arsenic concentration in water was nearly unchanged relative to the water sample after iron-rich adsorbents (V4). Figure 5 illustrates the efficiency of arsenic removal in the second and third modes. The results indicate that the efficiency of arsenic removal increased with increasing adsorbent packed media. The concentration of arsenic in treated water (V5) decreased to less than 10 $\mu\text{g/l}$, which fulfils Vietnam's national technical regulations concerning drinking water quality (Fig. 5).

Of the changes in three different operated modes, the third mode corresponds with the most effective removal of iron and arsenic. The results of the analysis of the water samples demonstrated that after the addition of the aeration system, the sprinkler increased the efficiency of the removal of iron and arsenic in groundwater. Fe (II) and As (III) were converted to Fe (III) and As (V) (As (V) is adsorbed by $\text{Fe}(\text{OH})_3$ more easily than As (III)). In addition, changing the height of iron-rich adsorbents layer also affected the removal of arsenic from groundwater. With the processes and the 120 mm height of iron-rich adsorbents, the adsorption column in the pilot scale removed 76% of arsenic, while with a 250 mm height of iron-rich adsorbents, the water quality was demonstrated to respond significantly in reducing arsenic by over 94% (to less than 10 $\mu\text{g/l}$).

Conclusions

In the pilot scale, the efficiency of arsenic removal from groundwater was affected directly by the height of the iron-rich adsorbents layer and the aeration process. After passing through the pilot system, the concentration of arsenic and iron in groundwater

decreased to less than 10 $\mu\text{g/l}$ and less than 0.3 mg/l, respectively, and the pH of the treated water samples was slightly increased by the removal of iron of the pilot system and the alkalinity of kaolin in the iron-rich adsorbents (between 6.9 and 7.8). The parameters of pH, As, and Fe in the ground water after treatment fulfilled Vietnam technical regulations concerning drinking water quality. These confirmed that using iron-rich adsorbents to remove arsenic from groundwater in decentralised water supply treatment is an effective method both economically and in terms of ameliorating water quality.

The authors declare that there is no conflict of interest regarding the publication of this article.

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Projection of saline intrusion into groundwater in the context of climate change in the coastal zone of Ha Tinh province

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Abstract:

In addition to surface water, groundwater is an essential source of water for agriculture, industry, and living in Ha Tinh province (central Vietnam). However, overexploitation and unreasonable use of groundwater has put this resource at risk of endangerment and pollution. In the coastal areas especially, the impact of climate change and the rise in sea-level has increased the risk of salt-water intrusion into groundwater. In this study, the groundwater system model (GSM) is applied to simulate the intrusion of saline water in different climate change scenarios in the coastal area of Ha Tinh province. The result reveals that saline intrusion into groundwater is becoming more complex and is a rising trend in climate change scenarios RCP4.5 and RCP8.5.

Keywords: climate change, coastal, groundwater, Ha Tinh, saline intrusion.

Classification number: 5.2

Introduction

Salt-water intrusion into surface water and groundwater is a frequent problem in the coastal areas of Ha Tinh province, as well as in other provinces and cities. With the current socio-economic growth rate, water demand from various sectors is increasing dramatically; on the other hand, with the impact of climate change, surface water as a resource is diminishing and pollution levels are rising. This, in turn, depletes the available store of surface water that sectors depend on. In this context, groundwater would be an effective solution to provide for the needs of socio-economic development, especially where exploitation of surface water is no longer possible. However, as with surface water, groundwater also faces the risk of seawater intrusion; hence, if there are no solutions to reducing saltwater infiltration, or rationally using and supplementing fresh water for groundwater, coastal resources will diminish and fail to supply the needs of socio-economic development. In this study, the GSM is applied to simulate groundwater level and assess saline intrusion in climate change scenarios over extended periods of time in the coastal areas of Ha Tinh (including seven coastal districts, two towns, and one city: Nghi Xuan, Duc Tho, Can Loc, Loc Ha, Thach Ha, Cam Xuyen, and Ky Anh districts; the city of Ha Tinh; and the towns of Ky Anh, Hong Linh). The primary objective of this study is to assess the impact of climate change on coastal groundwater resources.

Method and data

Method

The GSM model was applied to simulate the groundwater resource for the coastal area of Ha Tinh province:

The GSM is a model that integrates the MODFLOW [1] groundwater flow model and the MT3DMS [2] water-quality model to simulate groundwater flow and quality.

In the MODFLOW model, the three-dimensional

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movement of a groundwater level of a constant density through porous earth material may be described by the following partial differential equation:

$$\frac{\partial}{\partial x} \left(K_{xx} \frac{\partial h}{\partial x} \right) + \frac{\partial}{\partial y} \left(K_{yy} \frac{\partial h}{\partial y} \right) + \frac{\partial}{\partial z} \left(K_{zz} \frac{\partial h}{\partial z} \right) + W = S_s \frac{\partial h}{\partial t} \quad (1)$$

where:

- K_{xx} , K_{yy} , K_{zz} : are values of hydraulic conductivity along the x, y, and z coordinate axes, which are assumed to be parallel to the major axes of hydraulic conductivity (L/T).

- h: is the potentiometric head (L).

- W: is a volumetric flux per unit volume representing sources and/or sinks of water, with $W < 0.0$ for flow out of the groundwater system, and $W > 0.0$ for flow into the system (T^{-1}).

- S_s : is the specific storage capacity of the porous material (L^{-1}).

- t: is time (T).

This equation describes water-level dynamics in heterogeneous and anisotropic environments.

With the MT3DMS water quality model, transporting solutions in a porous environment is a complex chemical and physical process. Two basic components of the process are (i) the transporting of hydrodynamics and (ii) diffusion of ions and particles are dissolved in water from the high concentration to the low concentration. When contaminated water flows through the porous environment, it mixes with uninfected water by means of mechanical dispersion that dilutes it and reduces its concentration. Molecular diffusion and mechanical dispersion cannot be separated in an underground stream and both processes are referred to as hydrodynamic dispersion.

The partial differential equation describing the fate and transporting of contaminants of species k in 3D, transient groundwater flow systems can be written as follows:

$$\frac{\partial(\theta C^k)}{\partial t} = \frac{\partial}{\partial x_i} \left(\theta D_{ij} \frac{\partial(C^k)}{\partial x_j} \right) - \frac{\partial}{\partial x_i} (\theta v_i C^k) + q_s C_s^k + \sum R_n \quad (2)$$

where:

θ : the porosity of the sub-surface medium, considered to be dimensionless.

C^k : dissolved concentration of species k, ML^{-3} .

t: time, T.

x_{ij} : distance along the relevant Cartesian coordinate axis, L.

D_{ij} : hydrodynamic dispersion coefficient tensor, L^2T^{-1} .

v_i : seepage or linear pore water velocity, LT^{-1} ; this is related to the specific discharge or Darcy flux by means of

the relationship $v_i = q_i / \theta$.

q_s^k : volumetric flow rate per unit of the volume of the aquifer, representing fluid sources (positive) and sinks (negative), T^{-1} .

C_s^k : concentration of the source or sink flux for species k, ML^{-3} .

$\sum R_n$: chemical reaction term, $ML^{-3}T^{-1}$.

The left-hand side of Equation 2 can be expanded into two terms:

$$\frac{\partial(\theta C^k)}{\partial t} = \theta \frac{\partial C^k}{\partial t} + C^k \frac{\partial \theta}{\partial t} = \theta \frac{\partial C^k}{\partial t} + q'_s C^k \quad (3)$$

where: $q'_s = \frac{\partial \theta}{\partial t}$ is the rate of change in transient groundwater storage (unit, T^{-1}).

The chemical reaction term in equation 2 can be used to include the effect of general biochemical and geochemical reactions on the fate and transport of contaminants. Considering only two basic types of chemical reactions, that is, aqueous-solid surface reactions (sorption) and first-order rate reactions, the chemical reaction term can be expressed as follows:

$$\sum R_n = -\rho_b \frac{\partial \bar{C}^k}{\partial t} - \lambda_1 \theta C^k - \lambda_2 \rho_b \bar{C}^k \quad (4)$$

where:

ρ_b : bulk density of the sub-surface medium, ML^{-1} .

$\bar{C}^k$: concentration of species k sorbed on the subsurface solids, MM^{-1} .

λ_1 : first-order reaction rate for the dissolved phase, T^{-1} .

λ_2 : first-order reaction rate for the sorbed (solid) phase, T^{-1} .

Substituting equations 3 and 4 into equation 2 and omitting the species index in order to simplify the presentation, Equation 2 can be rearranged and rewritten as:

$$\theta \frac{\partial C}{\partial t} + \rho_b \frac{\partial \bar{C}}{\partial t} = \frac{\partial}{\partial x_i} \left(\theta D_{ij} \frac{\partial C}{\partial x_j} \right) - \frac{\partial}{\partial x_i} (\theta v_i C) + q_s C_s - q'_s C - \lambda_1 \theta C - \lambda_2 \rho_b \bar{C} \quad (5)$$

Equation 5 is a mass balance statement, that is, the change in the mass storage (both dissolved and sorbed phases) at any given time is equal to the difference between the mass inflow and outflow due to dispersion, advection, sink/source, and chemical reactions.

Local equilibrium is often assumed for the various sorption processes (i.e., sorption is sufficiently fast relative to the transport time scale). When the local equilibrium

assumption is invoked, it is customary to express equation 5 in the following form:

$$R\theta \frac{\partial C}{\partial t} = \frac{\partial}{\partial x_i} \left(\theta D_{ij} \frac{\partial C}{\partial x_j} \right) - \frac{\partial}{\partial x_i} (\theta_i C) + q_s C_s - q'_s C - \lambda_i \theta C - \lambda_2 \rho_b \bar{C} \quad (6)$$

where: R is referred to as the retardation factor, which is a dimensionless factor defined as:

$$R = 1 + \frac{\rho_b}{\theta} \frac{\partial \bar{C}}{\partial C} \quad (7)$$

when the local equilibrium assumption is not appropriate, sorption processes are typically represented through a first-order kinetic mass transfer equation, as discussed in the section on chemical reactions.

Data

Input data for the GSM include:

- Hydrometeorological data: meteorological and hydrographic data up to 2014 from the project “Technical consultancy on the hydrological/hydraulic model of the Rao Cai river basin and the drainage model in the city of Ha Tinh, Ha Tinh province” were also part of the project “Integrated water resource management and urban development in Ha Tinh province”, conducted by the Vietnam Academy for Water Resources [3]. Additional data up to 2016 were collected from the Hydrometeorological Data Centre of the National Center of Meteorology and Hydrology (now the Meteorological and Hydrological Administration).

- Land-use data: land-use status data for Ha Tinh from 2015 were collected from Center for Land Assessment under Center for Land Survey and Planning under General Department of Land Administration.

- Stratigraphic data: the 2014 1:200,000-scale hydro-geological map of Ha Tinh province was sourced from the National Center for Water Resource Planning and Investigation.

- The stratigraphic data on hydro-geological boreholes were inherited from the project “Planning, exploitation, utilization, and protection of water resources in Ha Tinh province up to 2020”, conducted by the 2F Division for Water Resources Planning and Investigation of the Ministry of Natural Resources and Environment in 2011 [4].

- Survey data: survey data were collected by means of interviews with local people using pre-designed table templates, and by means of direct water sampling. The scope and subjects of the survey were the current status of water use in 330 households and 20 organisations in 10 coastal districts/cities/towns of Ha Tinh province.

- Climate-change scenarios: climate-change in Ha Tinh

province was examined in terms of two scenarios, RCP4.5 and RCP8.5, for temperature (Table 1), precipitation (Table 2) and sea level rise (Table 3) extraction from climate change and sea-level rise scenarios for Vietnam, which were updated by Ministry of Natural Resources and Environment in 2016 [5].

Table 1. Changes in temperature (°C) compared to the period 1986-2005 in terms of different climate change scenarios in Ha Tinh province.

Temperature	RCP4.5			RCP8.5		
	2016-2035	2046-2065	2080-2099	2016-2035	2046-2065	2080-2099
Annual	0.6 (0.3÷1.0)	1.5 (1.0÷2.1)	2.0 (1.4÷2.9)	0.9 (0.6÷1.3)	1.9 (1.3÷2.8)	3.5 (2.8÷4.8)
Winter	0.6 (0.3÷1.0)	1.3 (0.7÷1.8)	1.6 (1.0÷2.1)	0.9 (0.6÷1.2)	1.7 (1.2÷2.4)	2.8 (2.0÷3.7)
Spring	0.6 (0.1÷1.2)	1.3 (0.7÷1.9)	2.0 (1.2÷2.9)	0.9 (0.5÷1.3)	1.8 (0.9÷2.8)	3.2 (2.0÷4.5)
Summer	0.8 (0.4÷1.3)	1.9 (1.2÷3.0)	2.6 (1.8÷3.6)	1.0 (0.5÷1.5)	2.3 (1.4÷3.6)	4.1 (3.2÷5.7)
Autumn	0.6 (0.3÷1.1)	1.5 (1.0÷2.2)	2.0 (1.2÷2.9)	0.8 (0.4÷1.4)	2.0 (1.3÷3.0)	3.6 (2.7÷5.0)

Source: Vietnam Institute of Meteorology, Hydrology and Climate Change (IMHEN).

Table 2. Changes in rainfall (%) relative to the period 1986-2005 in terms of climate change scenarios in Ha Tinh province.

Rainfall	RCP4.5			RCP8.5		
	2016-2035	2046-2065	2080-2099	2016-2035	2046-2065	2080-2099
Annual	11.3 (6.0÷16.6)	16.3 (8.5÷24.4)	13.0 (3.4÷22.6)	12.9 (6.8÷18.9)	14.1 (8.9÷19.0)	17.4 (10.6÷24.4)
Winter	12.0 (4.1÷19.5)	21.0 (11.4÷30.4)	12.8 (5.4÷20.0)	3.5 (-2.1÷9.2)	13.0 (1.6÷24.4)	19.8 (6.5÷33.2)
Spring	2.8 (-3.7÷9.2)	14.5 (4.3÷23.9)	9.4 (-1.8÷20.5)	-4.2 (-14.4÷5.8)	5.0 (-3.5÷13.0)	16.1 (2.1÷30.5)
Summer	21.1 (-3.7÷44.7)	9.1 (-2.1÷20.3)	4.8 (-5.7÷16.1)	40.6 (5.0÷70.7)	18.6 (-6.5÷43.4)	22.2 (3.0÷41.8)
Autumn	9.9 (3.8÷16.1)	19.0 (5.2÷31.6)	17.6 (3.8÷30.3)	8.2 (-0.1÷15.8)	15.1 (6.6÷23.4)	17.6 (8.2÷27.0)

Source: IMHEN.

Table 3. Sea-level rise scenarios for the coastal areas of Ha Tinh province (cm).

Scenarios	Timeline of the 21 st century							
	2030	2040	2050	2060	2070	2080	2090	2100
RCP4.5	13 (8÷18)	17 (10÷24)	22 (13÷31)	27 (16÷39)	33 (20÷47)	39 (24÷56)	46 (28÷65)	53 (32÷75)
RCP8.5	13 (9÷18)	18 (12÷26)	25 (17÷35)	32 (22÷45)	40 (28÷57)	50 (34÷71)	60 (41÷85)	72 (49÷102)

Source: IMHEN.

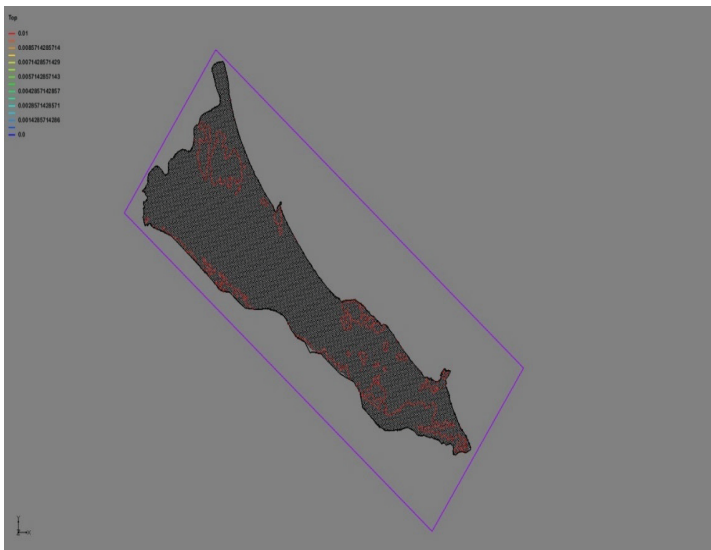


Fig. 1. Computational domain and computational grid of the research area.



Fig. 2. Sea and river boundaries in the research area.

Results and discussion

The GSM model constructed for the coastal area of Ha Tinh province

- The computational domain includes the coastal districts of Nghi Xuan, Duc Tho, Can Loc, Loc Ha, Thach Ha, Cam Xuyen, and Ky Anh; the city of Ha Tinh; and the towns of Ky Anh, Hong Linh (Fig. 1).

- The computational grid includes 563,060 grid points, including 294,865 computational points. Grid cell size is 200 m x 200 m.

- Boundary conditions:

+ The sea boundary is approximately 143 km, from the Lam river mouth to the end of Ky Anh town, next to Quang Binh province.

+ The river boundary comprises four major rivers, the Lam, Ha, Lui, Quyen, and their major branches (Fig. 2).

+ The groundwater restoration boundary was calculated by subtracting the evaporation boundary from the precipitation boundary in the corresponding exposure area of geological layers in the research area (Fig. 3 and Table 4).

Table 4. Classification of the groundwater restoration area in the coastal area of Ha Tinh province.

No.	Restoration area	Restoration rate from rainfall (%)
1	<i>Holocene</i>	35
2	<i>Pleistocene</i>	35
3	<i>Neogen, Triat, Ordovic-Silur</i>	15

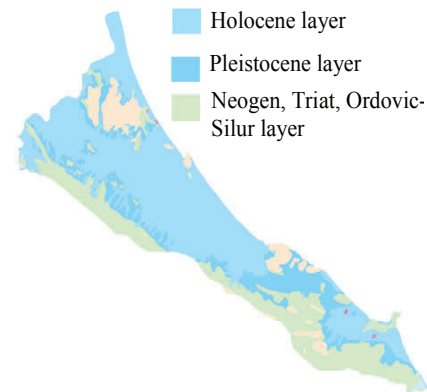


Fig. 3. Exposure area of geological layers in the research area.

- A 3-year period was used to warm up the model to reduce the effect of initial conditions.

- Computational time step: daily.

Calibration and validation

For model calibration, this research employs monitoring data from January 2014 to December 2016 from four groundwater level stations within research area (Table 5).

Table 5. Differences between the simulated water level and the water level measured at groundwater level monitoring wells in the research area.

No.	Station	Mean absolute difference	Root mean square deviation	Maximum difference (m)
1	QT2a-HT_0	0.05	0.004	0.15
2	QT5a-HT_0	0.13	0.023	0.32
3	QT6-HT_0	0.18	0.057	0.91
4	QT6b-HT_0	0.13	0.039	0.81

The results of the model calibration and validation show that the model parameters are reliable and can be applied to research on groundwater in the coastal area of Ha Tinh.

Salt-water reserve in terms of the climate change scenarios

The results of calculating the salt-water storage in the Holocene and Pleistocene layers in 2020 and 2030 in terms of scenarios RCP4.5 and RCP8.5 compared to the current situation (the average of the period 1986-2005) in the coastal area of Ha Tinh province are shown in Figs. 4A, 4B.

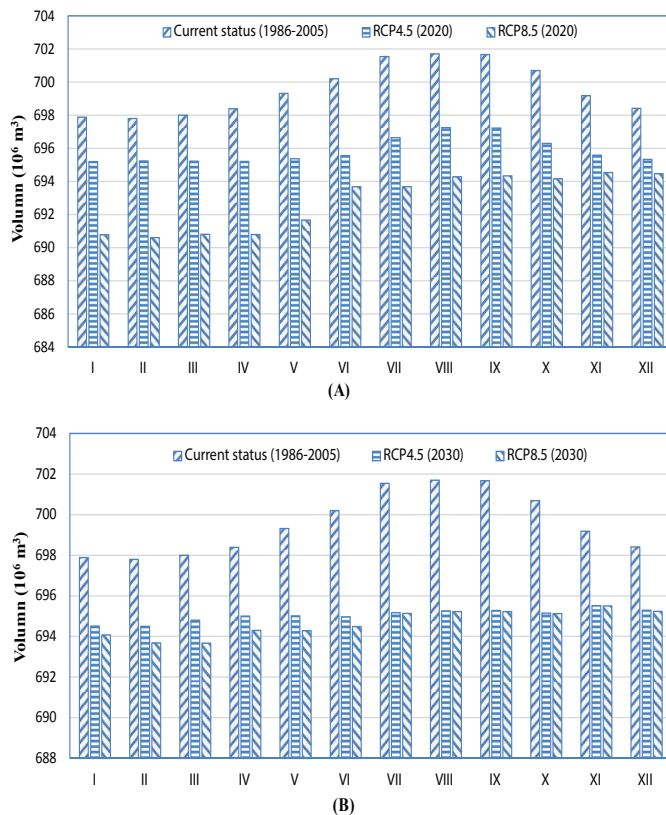


Fig. 4. Salt-water storage diagrams in the Holocene and Pleistocene layers in 2020 (A) and 2030 (B) in the research area according to scenarios RCP4.5 and RCP8.5.

The results in Fig. 4 show that groundwater salinity in the research area in 2020 in terms of scenarios RCP4.5 and RCP8.5 tends to decrease compared with the current situation. By 2020, the level of salinity intrusion tends to decrease in all months of the year in both the RCP4.5 and RCP8.5 scenarios. For both scenarios, the largest decrease occurs in July, and the smallest in January for RCP4.5, and in December for RCP8.5. This phenomenon occurs due to the hypothesis of the problem claims that the amount of groundwater extraction remains unchanged relative to the current situation; this change may primarily be due to the

change of rainfall and a sea level rise of 0.09 m. By 2030, in terms of scenarios RCP4.5 and RCP8.5, the storage tends to decrease relative to the current situation. In that year, the level of salinity intrusion tends to decrease in all the months of the year in terms of both RCP4.5 and RCP8.5 scenarios, with the largest decrease occurring in August. This change is primarily due to a change in rainfall by 2030, which is quite similar for both RCP4.5 and RCP8.5 scenarios and the scenario of a 0.13 m rise in sea level.

The magnitude of saline intrusion in 2020 and 2030 is less than that of the (current) baseline period due to a significant increase in rainfall in these years.

The results of calculating salt-water storage in the Holocene and Pleistocene layers for the periods 2016-2035, 2046-2065, and 2080-2099 in terms of the RCP4.5 and RCP8.5 scenarios compared to the current situation in the coastal area of Ha Tinh province are shown in Figs. 5A, 5B.

Figure 5 shows the trends of salinity intrusion into groundwater in the research area, which are very complex. With scenario RCP4.5, in the early years of the 21st century (2016-2035) the level of salinity intrusion tends to decrease;

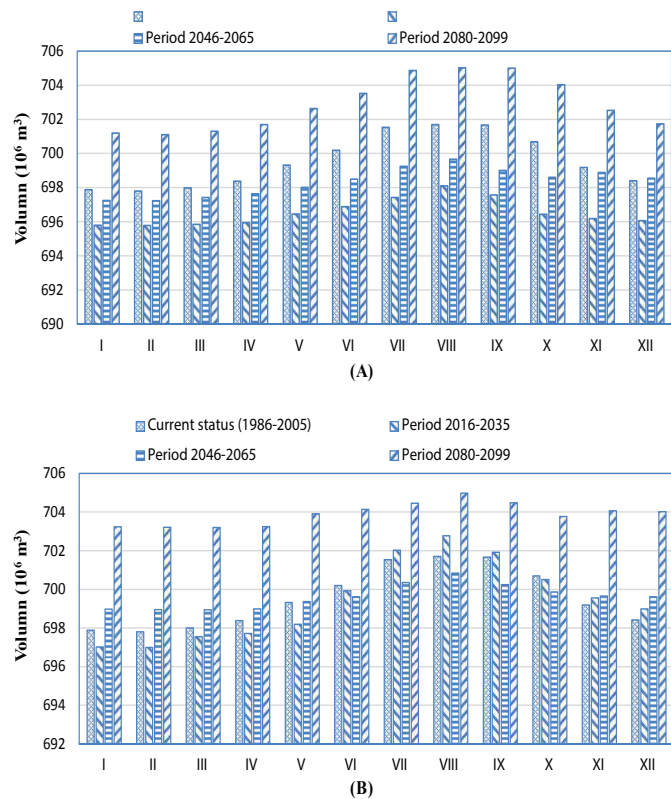


Fig. 5. Saltwater storage charts in the Holocene and Pleistocene layers of the research area for the periods 2016-2035, 2046-2065, and 2080-2099 in terms of scenarios RCP4.5 (A) and RCP8.5 (B).

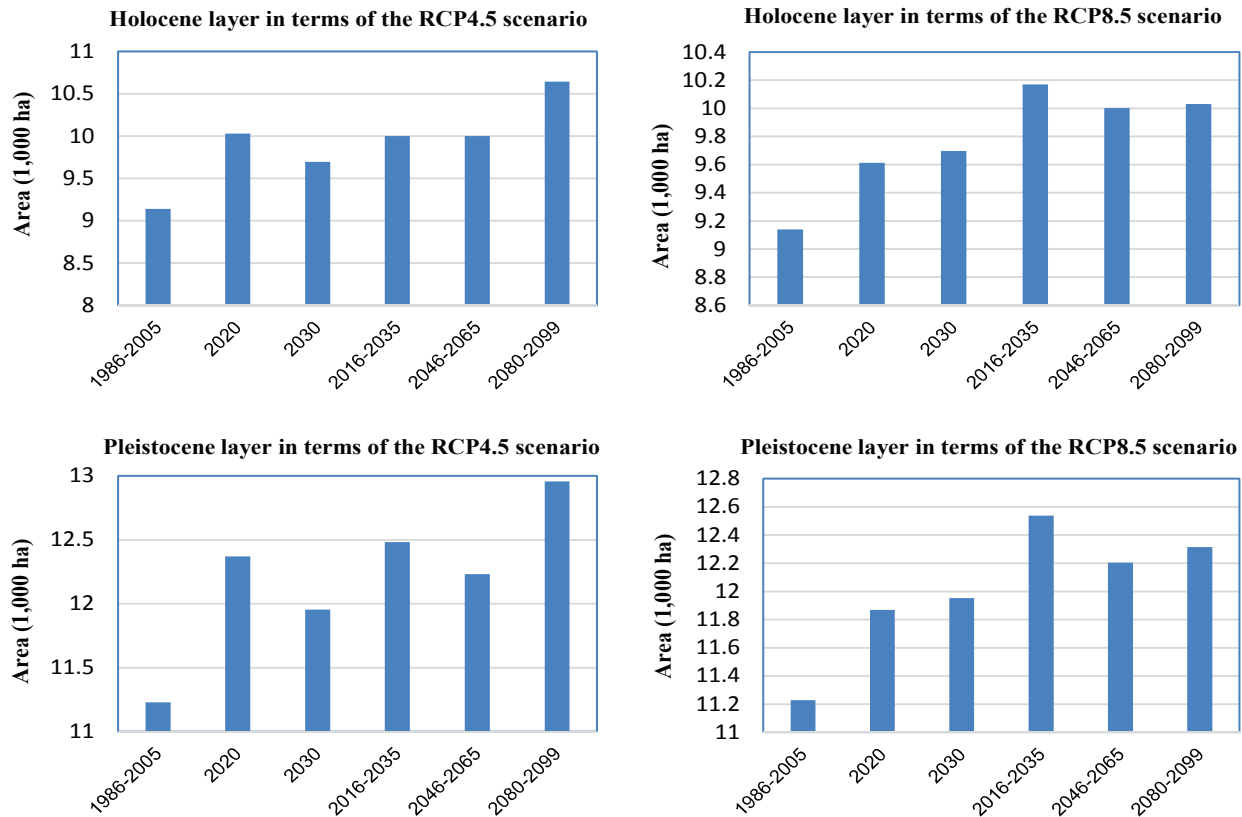


Fig. 6. Surface area of groundwater salinisation in terms of the climate change scenarios in the coastal area of Ha Tinh province.

then it increases gradually at the end of the century (2080-2099). With scenario RCP8.5, in the early years of the 21st century (2016-2035) groundwater salinity intrusion increases in July, August, September, November, and December compared to the current situation, and decreases in the remaining months. However, in the last years of the 21st century (2080-2099), saline intrusion into groundwater tends to increase sharply in comparison with that of all months of the year.

Area of salinity intrusion in terms of the climate change scenarios

As shown in Fig. 6, by 2020 and 2030, salinity will intrude into both the Holocene and Pleistocene layers in terms of both climate change scenarios, RCP4.5 and RCP8.5; however, in the 1986-2005 baseline period, intrusion only occurred near the river banks and rivermouth area.

The results in Fig. 6 show that, in terms of all climate change scenarios considered, the area of saline groundwater in both the Pleistocene and Holocene layers slightly varies from month to month during the year. The changing trends in the area of saline groundwater intrusion in the Holocene

and Pleistocene layers are similar in each climate change scenario. In terms of the RCP4.5 scenario, the area of saline groundwater is lower than the current one in the early and mid-21st century and is higher at the end of the century. In terms of the RCP8.5 scenario, the area of saline groundwater does not change substantially relative to the status in the early and mid-21st century, and increases at the end of the century.

As shown in Fig. 7, the area of salt-water intrusion in the Holocene layer is primarily in Ky Anh town and Nghi Xuan, Thach Ha, and Cam Xuyen districts, with area itself ranging from 1,500 ha to over 2,000 ha; while in the coastal districts, the area of saline intrusion into the groundwater ranges from 300 ha to over 600 ha. In the Pleistocene layer, the largest areas of saltwater intrusion are in Ky Anh district and Ky Anh town with over 2,000 ha. Nghi Xuan and Cam Xuyen districts experience 1,900 ha of intrusion and Thach Ha district approximately 1,500 ha. In the remaining districts, the area of saltwater intrusion is approximately equal to that which occurs in the Holocene layer. This trend of changes in the area of saline intrusion into groundwater is similar to that in the other areas in coastal Ha Tinh.

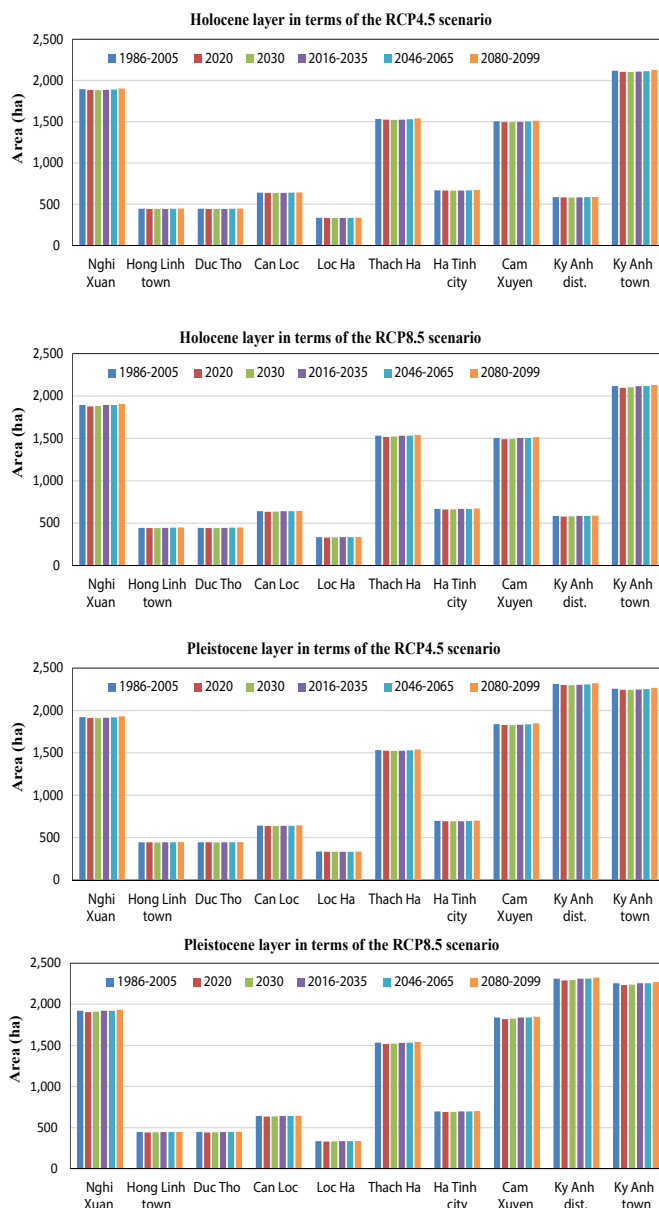


Fig. 7. Surface area of groundwater salinisation in terms of the climate change scenarios in the coastal districts of Ha Tinh province.

Conclusions

Saline water intrusion tends to decrease in terms of the two climate change scenarios considered - RCP4.5 and RCP8.5 - by 2020 and 2030: salt-water storage will decrease by 0.53 to 0.96% and by 0.65 to 0.70% by 2020 and 2030, respectively.

In terms of both RCP4.5 and RCP8.5 scenarios, the average for the future periods 2016-2035, 2046-2065, and 2080-2099 shows that the development of groundwater salinity in the research area is quite complex. In the early and mid-century, the level of saline intrusion tends to

decrease slightly, and thereafter it increases gradually at the end of the century.

According to the climate change scenarios, at the beginning of the century, rainfall in Ha Tinh increased and so did the reserve of underground water in the province; at the end of the century, the sea level in Ha Tinh will rise (by 68 cm), saline intrusion into groundwater will increase, and groundwater saline storage will tend to decrease slightly relative to the beginning of the century.

These results are calculated based on the averages for periods of heavy and light rainfall, so the trend of an increase in levels of salinity in groundwater is not clear. In fact, salt-water intrusion frequently occurs during the years of light rainfall, especially in the months of the dry season. It is therefore necessary to undertake a more detailed examination for each year, especially those of lighter rainfall in order to obtain a more specific assessment of the impact of climate change on groundwater. The results of this study provide the premise and basis for further research.

ACKNOWLEDGEMENTS

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The authors declare that there is no conflict of interest regarding the publication of this article.

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Assessment of storm surge risk in aquaculture in the Northern coastal area of Vietnam

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Abstract:

This research utilizes the disaster risk concept developed by the Intergovernmental Panel on Climate Change (IPCC) to determine and assess the storm surge risk in aquaculture in the coastal area from Quang Ninh to Ninh Binh province. The results indicated that the highest level of risk occurred in Thai Thuy district (Thai Binh province) and Quang Yen town (Quang Ninh province). The second highest level of risks occurred in Tien Hai district (Thai Binh province), Mong Cai city and Hai Ha district (Quang Ninh province). The lowest level of risk transpires in Uong Bi city (Quang Ninh province) and Kien An district (Hai Phong city). The results provide a scientific basis to support local government in establishing proactive response plans to storm surges, reduce and prevent storm surge damage to aquaculture, assist policy making and establish suitable development priorities for the coastal areas in the Northern region.

Keywords: aquaculture, coastal area, risk assessment, storm surge.

Classification number: 5.2

Introduction

Aquaculture is the fastest-growing food sector globally, with an average annual growth rate of 6% over the past decade. According to the Food and Agricultural Organization (FAO), global aquaculture production has tripled from 1995 to 2014 and reached 74 million tons in 2014. Produce from Asia accounts for approximately 89% of total worldwide production [1]. In Vietnam, aquaculture is an important economic sector, which has a high export value; aquaculture contributes to the improvement of the livelihood of people, especially in coastal areas. According to the Vietnam Directorate of Fisheries, aquaculture production increased fourfold over a 10-year period between 2001 and 2011 from more than 700,000 tons to nearly 3 million tons, with an average annual growth rate of 15.7%. The volume of coastal aquaculture production (saline, brackish) is roughly 29% of the total aquaculture production [2].

The industry is heavily dependent on weather conditions and natural environments. The dependency poses a risk to millions of employees who are directly or indirectly involved in the sector. This attribute is engendered by the complexities of weather events, natural disasters, and environmental problems such as pollution. Such conditions create high-risk profiles and pose significant damages not only to property but also to the livelihood of people. This case is especially true for individuals living in the Northern coastal area that is directly affected by a large number of natural disasters such as storms, floods, extreme waves and storm surges. In particular, aquaculture is highly vulnerable to storm surges. Water level that increases to a certain point and overflows into aquaculture ponds could alter the salinity profile of these ponds, hence affecting the growth and production of aquatic species. Additionally, storm surges that occur rapidly (associated with higher tides) could inundate the area and eventually causing loss [3].

The volume of research on the topic of storm surge risk is abundant. The National Oceanic Information Service

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Center of India [4] identified the elements that affect the height of storm surges, such as wind speed, maximum wind radius, storm trajectory, centre pressure and shoreline elevation. The agency indicated three levels of disaster risk for coastal areas based on the height of storm surges, namely very high (>5 m), high (3-5 m) and medium (1.5-3 m). Storm surge risk in the coastal areas of India has since been classified. However, this method of determining risk caused by storm surges merely considered the effects of natural factors, including the height of the surge without any regard for socioeconomic and human aspects. ToRii and KaTo (Japan) appraised the risk of storm surge using four main approaches, namely 1) evaluation of the probability of tide and wave velocity, 2) assessment of sea dykes, 3) simulation of flood and 4) risk assessment based on evacuation and home safety; flood risk due to storm surges is evaluated according to flood simulation results [5].

In Vietnam, a large number of studies have also assessed the risk of storm surges. Viet Lien Nguyen (2010) classified storm surge risk into 15 levels, with frequencies of 1, 2, 5, 10 and 20%. Storm surge risk were then further evaluated by exploring sea level rise of 0 cm, 30 cm and 75 cm representing different impact levels of climate change. The research provided an overview of the level of risk, of which economic and social factors have been evaluated in addition to hazard-related aspects [6]. In their study on “Assessing the risks of climate change and sea level rise in Binh Thuan province”, Xuan Hien Nguyen, et al. (2013) overlapped hazard maps with potential damage to assess the risks of Binh Thuan to natural disasters, including flood, agricultural drought, water shortage and sea level rise in the context of climate change. The study categorized risk into five levels, namely very high, high, medium, low and very low, corresponding to the possible effects and various degrees of potential damage [7]. The Vietnam Institute of Meteorology, Hydrology and Climate Change (IMHEN) built the programme on “Updating the disaster risk disaster, mapping disaster warning, especially disaster related to storms, storm surges, floods, flash floods, landslides, droughts, saline intrusion”, including the content of disaster risk assessment and disaster warning surge mapping [8].

As for aquaculture in Vietnam in general and the coastal area of the Northern region in particular, the potential impact and risk of storm surges have not been fully evaluated. Only a limited number of studies have investigated the effect of climate change on aquaculture. These studies include “Assessing the economic impact of climate change on fisheries in the North and proposing solutions to mitigate damages caused by climate change” by Ngoc Thanh Nguyen (2015) [9], “Impact of climate change on agricultural and fishery production” (for two selected provinces of Phu Tho

and Hoa Binh province) by Quang Ha Pham (2011) [10] and “Impact of salinity intrusion and adaptation in aquaculture in the Mekong Delta” (under the impact of climate change and sea level rise) by Thi Phuong Mai Le (2017) [11]. Moreover, research on extreme weather events, especially storm surges, in aquaculture in coastal areas is lacking.

The implementation of the risk assessment of storm surges associated with aquaculture in the coastal area of the Northern region is therefore necessary to minimize the damage caused by this natural hazard on aquaculture. The objective of this study is to determine the magnitude of storm surges and risk assessment and to develop storm surge risk maps for aquaculture in the coastal area from Quang Ninh to Ninh Binh province.

Method and procedure for assessing the storm surge risk in aquaculture in the coastal area from Quang Ninh to Ninh Binh province

Data sources

The evaluation of storm surge risk in aquaculture is based on two major sources. The first source consists of storm surge data, including “Updating partition storm, storm risk assessment, storm surges and wind division for inland areas when the heavy storm, super storm landed” in 2016 [12] and “Flooding risk caused by strong storm, super storm surges for coastal provinces” from Quang Ninh to Ninh Binh in 2016 [13]. These data are used in calculating hazard and exposure. The second source comprises Societal, economic and aquaculture data, especially aquaculture data, including Quang Ninh Statistical Yearbook 2016 [14], Hai Phong Statistical Yearbook 2016 [15], Thai Binh Statistical Yearbook 2016 [16], Nam Dinh Statistical Yearbook 2016 [17] and Ninh Binh Statistical Yearbook 2016 [18]. These data are utilized in calculating exposure and vulnerability.

Method

The storm surge risk in aquaculture is appraised based on IPCC’s risk assessment approach to natural disasters (Fig. 1). The risk index of this approach is determined based on the following equation [19]:

$$R = f(H, E, V)$$

In particular, hazard (H) connotes the occurrence probability of storm surge with adverse effects on vulnerable objects within the area affected by this natural phenomenon. Exposure (E) refers to the geographical presence of individuals, livelihood activities, natural resources, infrastructure and economic, social and other forms of property at locations that may be adversely affected by storm surge hazards, and hence deal with potential damage, loss or damage in the future. Vulnerability (V) refers to the

susceptible tendency of the elements of storm surge hazards and comes in various forms such as human, economic and social vulnerability. Vulnerability is a function of sensitivity and resilience.

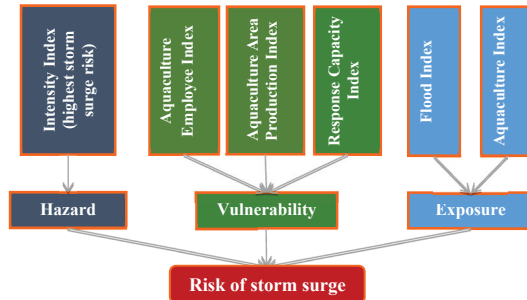


Fig. 1. Methodology for disaster risk assessment with $R = f(H, E, V)$.

To calculate the storm surge risk in aquaculture, a set of criteria for H, E and V components is established. These criteria are presented in Table 1.

Table 1. Indicators and the types of relationship to the levels of risk.

No.	Criterion	Risk assessment indicator	Relationship type with R
(1)	Hazard		
1	Hazard index (H)	Maximum storm surge risk (H)	↑
(2)	Exposure		
2	Flood index (E1)	Flooding ratio due to storm surges in typhoon level 14 (E1-1)	↑
3		Flooding ratio due to storm surges in typhoon level 13 (E1-2) during high tide	↑
4		Flooding ratio due to storm surges in typhoon level 13 (E1-2) during average tide	↑
5	Aquaculture index (E2)	Aquaculture area by administrative units (E2-1)	↑
6		Number of aquaculture farms by administrative unit (E2-2)	↑
(3)	Vulnerability		
7	Aquaculture employee index (V1)	Number of people employed in aquaculture (V1-1)	↑
8		Number of people working additional jobs (V1-2)	↑
9		Number of non-aquaculture workers engaged in aquaculture activities (V1-3)	
10	Aquaculture index (V2)	Aquaculture area ratio (V2-1)	↑
11		Aquaculture development index (V2-2)	↑
12		Aquaculture production (V2-3)	↑
13		Output/1 ha of aquaculture (V2-4)	↑
14	Response ability index (V3)	Monthly income per capita (V3-1)	↓

Note: all the sub-indices use the 2016 data.

The set of indices used to estimate storm surge risk in aquaculture is summarized in Table 1. Data standardization entails the conversion of the collected raw data with different units to the dimensionless values ranging from 0 (minimum value) to 1 (maximum value) to facilitate the comparison of administrative units. The unequal weighted method proposed by Iyengar and Sudarshan (1982) is applied to weight the indicators [20]. The final result is an average quantitative (risk index) that allows for relative comparisons between coastal districts and creates a storm surge risk map for aquaculture in the coastal area from Quang Ninh to Ninh Binh province.

Risk assessment procedure for storm surges

The assessment of storm surge risk in aquaculture consists of the following steps:

Step1: standardizing the data

In this step, data are standardized by converting the different value and unit indicators to dimensionless values within the range of 0 to 1 to compare the various administrative units. Standardization is conducted for each individual indicator. Prior to standardization, the relationship between each indicator and the risk index should be determined based on the reference, expert input or community experience. According to the study, the majority of hazards (H), exposures (E) and sensitivities in vulnerability (V) are positively associated with risk (R), whereas response indicators (in V) are inversely related to the (R) risk index.

The following standardized formula is applied if the relationship between the indicator and the risk index is covariates:

$$x_{ij} = \frac{X_{ij} - \text{Min}\{X_{ij}\}}{\text{Max}\{X_{ij}\} - \text{Min}\{X_{ij}\}} \quad (1)$$

If the relationship is inverse, the normalized formula is:

$$y_{ij} = \frac{\text{Max}\{X_{ij}\} - X_{ij}}{\text{Max}\{X_{ij}\} - \text{Min}\{X_{ij}\}} \quad (2)$$

where: X_{ij} is the value of the i indicator in the j administrative unit in the matrix of the data set; $\text{Max}\{X_{ij}\}$ and $\text{Min}\{X_{ij}\}$ are the maximum and minimum values of the i indicator in the whole administrative unit of the study area, respectively.

Step 2: determining the weights of indicators

This study selected the unequal weighted method proposed by Iyengar and Sudarshan (1982), in which each

indicator receives weight based on the standard deviation per indicator. A brief formulation of the method is as follows:

The weight of each indicator is determined by:

$$w_j = \frac{c}{\sqrt{\text{Var}(x_j)}} \quad (3)$$

where: w_j is the weight of the j indicator of component H/E/V, and $\text{Var}(x_j)$ is the variance of the j indicator defined by:

$$\text{Var}_{x_j} = \sum_{i=1}^n \frac{(x_{ij} - \bar{x}_j)^2}{(n-1)}$$

c : is determined by the following formula:

$$c = \left[\sum_{j=1}^m \frac{1}{\sqrt{\text{Var}(x_j)}} \right]^{-1}$$

where: m is the number of indicators of the indicator group (criteria).

The total weight of the indicator group must be 1, $0 < w_j < 1$.

After determining the weight, the value of indicator groups (criteria) for each administrative unit is calculated according to the following formula:

$$M_{i,j} = \sum_{j=1}^m w_j x_{ij} \quad (4)$$

where: $i = 1 \div n$, number of administrative units of the computed area, $M_{i,j}$ is the value of the j indicator group of the i administrative unit, and W_j is the weighted value of the j indicator.

Step 3: developing the risk index

After determining the weights and values of the indicator group (criteria), the value of each key component of the risk index per administrative unit is identified. An example of the value of the exposure level component E is as follows:

$$E_i = (\sum_{i=1}^n M_{i,j} \times m_{M_{i,j}}) / m \quad (5)$$

where: E_i is the value of the exposure component for the i unit; $m_{M_{i,j}}$ is the number of indicators of the indicator group $M_{i,j}$, and m is the total indicator of component E.

A similar procedure is applied to determine the hazard (H) and vulnerability (V) components. Finally, the risk index per the i administrative unit is:

$$R_i = \frac{(H_i + E_i + V_i)}{3} \quad (6)$$

Results and discussion

H_{ij} , E_{ij} and V_{ij} for each coastal district administrative unit are calculated and standardized according to Formula (1) or (2), respectively. The weight of each indicator is given by

Formula (3). H_i , E_i and V_i are calculated according to Formula (4). The values of components H, E and V are calculated by Formula (5), whereas R is calculated according to Formula (6). Calculation results for H_i , E_i , V_i and R risk indicators for the coastal districts of Bac Bo are presented in Table 2.

Table 2. Storm surge risk index on aquaculture for the coastal area from Quang Ninh to Ninh Binh province.

City/District/Town	H	E	V	R
Ha Long	0.569	0.218	0.129	0.306
Mong Cai	0.941	0.225	0.267	0.477
Uong Bi	0.005	0.117	0.103	0.075
Quang Yen	0.559	0.582	0.465	0.536
Tien Yen	0.490	0.095	0.144	0.243
Dam Ha	0.777	0.168	0.208	0.384
Hai Ha	1.000	0.133	0.247	0.460
Van Don	0.584	0.133	0.276	0.331
Co To	0.510	0.001	0.263	0.258
Hai An	0.540	0.558	0.069	0.389
Kien An	0.282	0.043	0.047	0.124
Do Son	0.495	0.165	0.248	0.303
Duong Kinh	0.525	0.032	0.273	0.277
Thuy Nguyen	0.277	0.192	0.347	0.272
Kien Thuy	0.480	0.147	0.227	0.285
Tien Lang	0.406	0.247	0.207	0.287
Cat Hai	0.554	0.217	0.135	0.302
Thai Thuy	0.356	0.696	0.558	0.537
Tien Hai	0.183	0.699	0.566	0.483
Nghia Hung	0.059	0.258	0.363	0.227
Giao Thuy	0.134	0.424	0.457	0.338
Hai Hau	0.000	0.248	0.413	0.221
Kim Son	0.094	0.532	0.444	0.357

Note: H, E, V and R are standardized and divided into five levels: very low, low, medium, high and very high.

Calculated results indicated that with typhoon category 13, 14, the highest risk of water level rise decreases from Quang Ninh's districts to Ninh Binh's districts. Simulated storm surges ranges from 2.68 m to 4.70 m. In the coastal area of Quang Ninh province, large water storage areas such as Hai Ha, Mong Cai and Dam Ha districts exhibit the highest storm surge levels of 4.70 m, 4.58 m and 4.25 m, respectively. The districts of Nam Dinh province and Kim Son district of Ninh Binh province demonstrate very low levels of risk. Uong Bi city, Kien An district and Thuy Nguyen district are also at very low risk levels because they are not directly adjacent to the sea.

One of the criteria for assessing aquaculture's exposure to storm surge is the inundation rate of coastal districts, which

corresponds to storm category scenarios. For example, with typhoon category 13, Thai Thuy district of Thai Binh province exhibited the highest proportion of area flooded (46.05%); by contrast, Co To district displayed very little flooding, and Duong Kinh district did not demonstrate any flooding. In addition, criteria related to aquaculture should be given consideration. The largest aquaculture areas are located in the districts/towns, namely Quang Yen, Giao Thuy, Tien Hai, Kim Son and Thai Thuy. On the contrary, the smallest aquaculture areas are situated in Co To and Kien An districts. At the same time, Tien Hai and Giao Thuy districts also host the largest number of aquaculture farms, followed by Quang Yen town, Nghia Hung district, Mong Cai city, Cat Hai district and Hai Hau district. Accordingly, the districts with a very high level of exposure are Tien Hai, Thai Thuy and Quang Yen town; by contrast, the exposure values for Hai An, Kim Son and Giao Thuy district are at a high level. Meanwhile, the districts of Co To, Duong Kinh, Kien An, Tien Yen, Hai Ha and Van Don have a very low level of exposure.

In terms of vulnerability, very high levels are displayed at Tien Hai, Thai Thuy districts and Quang Yen town. Giao Thuy, Kim Son, Hai Hau and Nghia Hung districts exhibit high levels of vulnerability. The districts with very low levels of vulnerability include Kien An, Hai An, Uong Bi, Ha Long, Cat Hai and Tien Yen. Areas of high sensitivity and low response capacity are likely to be highly vulnerable. For example, Tien Hai and Thai Thuy districts have the largest number of workers in aquaculture, the highest aquaculture output in all districts and a relatively high proportion of aquaculture area; however, the average monthly income (response capacity) is low, thus resulting in a very high level of vulnerability. The converse is true with districts with low levels of vulnerability. Hai An and Kien An districts have the lowest number of employees in the aquaculture sector and a very low percentage of aquaculture area and aquaculture production; however, the average monthly income per capita is high, which consequently results in a very low level of vulnerability. As Uong Bi city is not directly adjacent to the sea, the aquaculture indices comparatively low and the monthly income per capita is relatively high; hence, the city's aquaculture sector is less vulnerable to storm surges. The vulnerability of Hai Phong's coastal districts is very low, as the indicators related to aquaculture are not high; moreover, monthly income per capita is higher than other areas.

The levels of storm surge risk in aquaculture in the Northern coastal area are identified based on the calculation of H, E, V and R components. These risk levels are summarized in Table 3.

Table 3. Levels of storm surge risk in aquaculture in the coastal districts in the Northern region.

No.	Standardized value	Level of risk	District/City
1	0.0 - ≤0.2	Very low	Uong Bi, Kien An
2	>0.2 - ≤0.4	Low	Hai Hau, Nghia Hung, Tien Yen, Co To
3	>0.4 - ≤0.6	Medium	Thuy Nguyen, Duong Kinh, Kien Thuy, Tien Lang, Do Son, Cat Hai, Ha Long City, Van Don, Giao Thuy
4	>0.6 - ≤0.8	High	Kim Son, Dam Ha, Hai An
5	>0.8 - ≤1.0	Very high	Hai Ha, Mong Cai, Tien Hai, Quang Yen, Thai Thuy

The hazard, exposure, vulnerability and risk maps for the aquaculture sector of the coastal area from Quang Ninh to Ninh Binh are subsequently developed. These maps are depicted in Fig. 2.

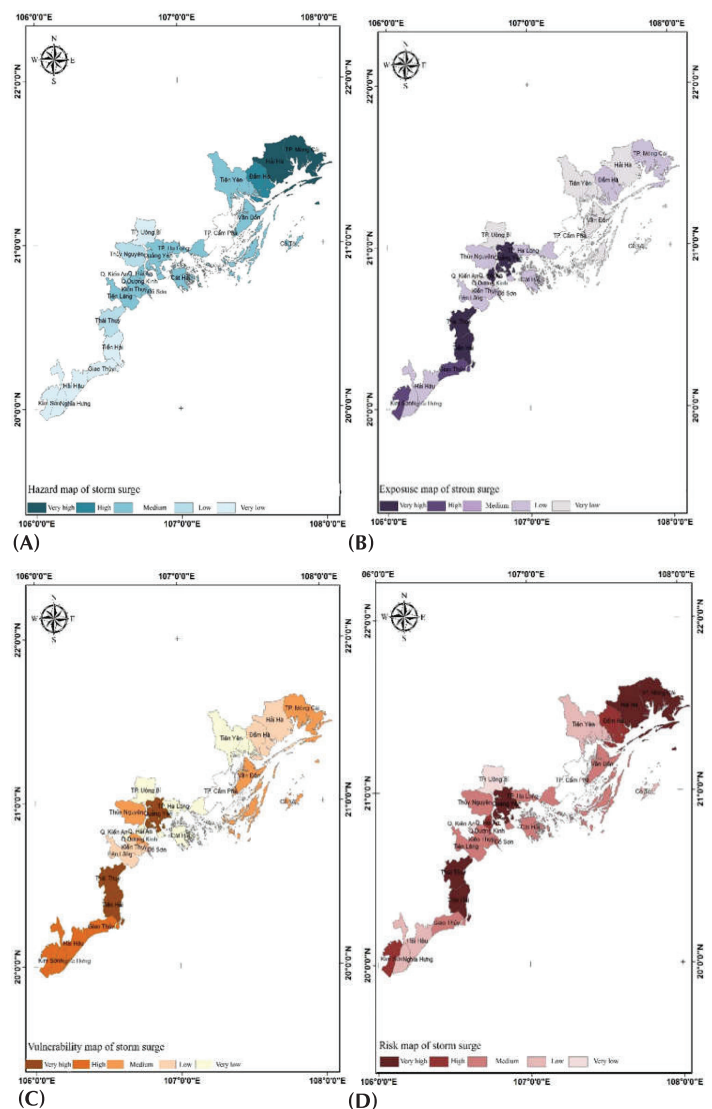


Fig. 2. Storm surge risk maps for the aquaculture sector of the coastal area from Quang Ninh to Ninh Binh: (A) hazard map, (B) exposure map, (C) vulnerability map and (D) risk map.

Conclusions and recommendation

The study presented a methodology for assessing the storm surge risk in aquaculture in the coastal area from Quang Ninh to Ninh Binh province in Vietnam. The method is applied in Vietnam and is based not only on the nature of the storm surge but also on the exposure level and the storm surge's capacity to affect life in economic, social and human terms. The components that contribute to the risk level of storm surges have been evaluated comprehensively; the natural and human factors have been appraised simultaneously. The study collected data, combined with the development of computational models, to ensure the accuracy of the results. The report indicates that data sources (completeness and reliability) play an important role in the risk calculation process. As a result, this study recommends the updating of statistical data every five years. Moreover, taking into account a number of specific indicators that are related to aquaculture is necessary for a more comprehensive assessment.

Local authorities could develop a response plan from hazard, exposure, vulnerability and risk maps. This approach could prevent and mitigate the damage caused by storm surges on aquaculture in the coastal area from Quang Ninh to Ninh Binh. In addition, research results provide a scientific basis to support policy making and rational development priorities for the Northern coastal area.

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