



## ORIGINAL RESEARCH ARTICLE

## Vertical bacterial variability in oxidation ponds in the tropical zone

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## ABSTRACT

**BACKGROUND AND OBJECTIVES:** Community domestic wastewater contains organic substances that can be decomposed through natural processes. Treatment using oxidation pond systems is popular in tropical zones because these locations provide a climate suitable for the growth and organic decomposition activities of various bacteria that remove organic contaminants from wastewater. Given that bacteria play an important role in the biodegradation of organic substances in wastewater treatment plants, their degradation activity is used as an indicator of water quality. The purpose of this study is to examine the vertical variability of bacteria in natural treatment oxidation ponds in tropical zones.**METHODS:** Wastewater samples were collected from an oxidation pond at 3 different depths (0–0.6, 0.6–1.5 and more than 1.5 meter), and their chemical, physical and biological qualities were analysed. Next-generation sequencing techniques were used to identify bacterial diversity, and the water quality at each depth was applied as an indicator of bacterial degradation activities.**FINDINGS:** Community domestic wastewater contained 10 major bacterial phyla that differed at different depths. Amongst these phyla, the Actinobacteriota dominated (25.35 to 28.23 percent), followed by Cyanobacteria (19.49 to 21.57 percent), Planctomycetota (15.50 to 17.41 percent), Firmicutes (9.97 to 10.79 percent), Proteobacteria (9.73 to 10.79 percent), Verrucomicrobiota (6.47 to 7.69 percent), Chloroflexi (2.79 to 2.99 percent), Bacteriota (0.96 to 1.41 percent), Acidobacteriota (0.70 to 0.80 percent) and SAR324 clade (marine group B) (0.69 to 0.61 percent). Four organic substances were found in contaminated domestic wastewater. 1) Photosynthetic cyanobacteria and phytoplankton performed aerobic degradation and accounted for the dissolved oxygen levels of 7.76, 7.45 and 7.42 milligrams per liter, respectively at various depths along the vertical profile. 2) Bacteria and archaeans that participate in carbon compound treatment included Planctomycetes, Verrucomicrobiota, Bacteroidota and Euryarcheota. These bacteria exhibited a treatment efficiency for biochemical oxygen demand and low abundance at all depths. Biological oxygen demand increased to 23.11, 24.27 and 34.48 milligrams per liter with depth. 3) Nitrogen-cycling bacteria included nitrifying and denitrifying bacteria. They belonged to the Actinobacteriota, Planctomycetota, Firmicutes, Verrucomicrobiota, Chloroflexi, Bacteriota, Protobacteria and Acidobacteriota phyla. They exhibited an ammonia-nitrogen treatment efficiency of 91.73 percent. 4) Phosphorus cycling-related bacteria in the Actinobacteriota phylum presented the orthophosphorus treatment efficiency of 65.41 percent.**CONCLUSION:** The results of this study suggested that bacterial communities did not significantly differ along oxidation depth because they work together with chemotrophs, which participate in organic substance decomposition, and phototrophs, which are involved in oxygen generation and nutrient removal. All of the bacteria found in domestic wastewater benefit and support oxidation pond systems in tropical zones. Anaerobic bacteria can be found in wastewater and used in treatment systems due to their protective mechanisms against oxygen toxicity and self-repair mechanisms. The knowledge gained from this study can be used as a reference in future works on natural wastewater treatment systems.DOI: [10.22034/gjesm.2024.03.16](https://doi.org/10.22034/gjesm.2024.03.16)This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

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## INTRODUCTION

Water constitutes the majority of the earth's surface and is considered the fundamental compound that supports all living things in the environment. The growth of the global population and expansion of urban communities and agricultural and industrial areas have led to increased demands for water. While point sources for agricultural and industrial wastewater can be identified, domestic wastewater is complex. Rose et al. (2021) suggested that approximately 80 percent (%) of used water is converted into wastewater, which is mostly composed of degradable proteins, carbohydrates, lipids and cellulose. Self-purification occurs when domestic wastewater is released into natural water sources; however, in many cases, the loading capacity of natural water resources is the limiting factor for treatment. Effective treatment technologies have been implemented to address the above situation. They include aerated lagoons, activated sludge, trickling filters and oxidation ponds. Oxidation ponds are commonly preferred as treatment systems in tropical zones because the climate in these zones is best suited for biodegradation, nutrition conversion and pathogenic bacterial removal (Mahapatra et al., 2022). In general, oxidation pond systems consist of consecutive ponds with three main functions, namely, 1) sedimentation, 2) oxidation and 3) stabilisation. In oxidation pond systems, different biotic factors, such as bacteria and phytoplankton, together with abiotic factors, such as sunlight and wind, drive treatment, generating wastewater effluent that often meets the natural standards for discharge into nature. However, considering the complexity of domestic water, the appropriate recommended depth of treatment systems is unclear. Different pond depths also shape the normal bacterial flora that function in organic biodegradation and generate carbon dioxide (CO<sub>2</sub>) and nutrients, including ammonium-nitrogen (NH<sub>4</sub><sup>+</sup>-N), nitrate-nitrogen (NO<sub>3</sub><sup>-</sup>-N) and orthophosphate (PO<sub>4</sub><sup>3-</sup>). These nutrients can then be taken up by phytoplankton and cyanobacteria that use light energy in the photosynthetically active radiation (PAR) range of 400–700 nanometer (nm) for photosynthesis (Sukchinda et al., 2019). PAR energy is a limiting factor for optimal treatment because the lowest required photosynthesis photon flux density (PPFD) is 4–20 micromole per second per square meter (μmol/m<sup>2</sup>/s) (Ritchie, 2010), whereas the highest suggested PPFD should not exceed 1400–1900 μmol/m<sup>2</sup>/s (Ziganshina

et al., 2020) to avoid damaging bacterial cells. Algal–bacterial symbiosis under optimal conditions is the main component of treatment in oxidation ponds (Mahapatra et al., 2022). Changes in the depths of treatment ponds often affect PAR quantity and quality (Brunet et al., 2014) because they would also affect the rates of photosynthesis and production of oxygen (O<sub>2</sub>) in the lower layers of ponds (Menden-Deuer, 2012). In natural treatment ponds, *Cylindrospermopsis* shows the highest distribution at the depth of 150 cm below the surface. Moreover, water quality tends to increase with depth due to the increase in the density of *Cylindrospermopsis* at deep levels, wherein total nitrogen and NH<sub>4</sub><sup>+</sup>-N are significantly correlated with *Cylindrospermopsis* (Srichomphu et al., 2024). Changes in the depth of treatment ponds would attenuate aerobic degradation (dissolved oxygen [DO] < 1.0 mg/L) (Zhang and Zhang, 2018). As a result, oxidation ponds with inappropriate depths often promote anaerobic degradation (DO < 1.0 mg/L). The products of anaerobic degradation lead to the formation of sulphide compounds that are toxic to phytoplankton and aerobic bacteria, resulting in a decrease in the efficiency of the overall treatment system (Tudsanaton et al., 2021). The quality of the treated water is the result of interactions amongst bacteria, cyanobacteria and phytoplankton. The results of the current study suggest that the vertical variability of bacteria can be used to support the design and efficient function of municipal wastewater treatment systems in tropical zones. In oxidation ponds, the bacterial degradation of organic substances in wastewater is related to depth. The findings of this study could be used to establish guidelines for determining the appropriate depth of oxidation ponds for effective wastewater treatment. This study was performed by collecting wastewater samples at three water depths through aseptic techniques. The samples were then analyzed for Deoxyribonucleic acid (DNA) subjected to sequencing on the basis of 16s ribosomal ribonucleic acid (16s rRNA) and microbiome bioinformatics analysis to investigate the vertical variability of bacterial community distribution. This work hypothesised that the vertical variability of bacterial communities in oxidation ponds affects changes in the water quality and wastewater treatment efficiency of treatment systems, wherein the driving force is defined by the pattern of organic substance decomposition. This study aims to identify changes in bacterial vertical

distribution to determine the role and function of bacteria in community wastewater treatment systems in tropical zones in accordance with depth. The study site is the community domestic wastewater treatment system of The Kings Royally Initiated Laem Phak Bia Research and Development project (LERD Project), Phetchaburi, Thailand. This study has been carried out in 2022.

## MATERIALS AND METHODS

### Study site

The oxidation ponds investigated in this work are located at the LERD Project site, Phetchaburi, Thailand (13°02'53.3"North 100°05'15.3"East). They receive domestic wastewater from Phetchaburi Municipality. Wastewater is transferred through an 18.5-kilometer (km) High density polyethylene (HDPE) pipeline to the project site. The ponds at the treatment site are consecutively connected in a series of five to allow for natural oxidation. O<sub>2</sub> is added through natural phytoplankton thermosiphoning (under the assumption that O<sub>2</sub> diffuses along the temperature gradient) (Noikondee *et al.*, 2019) and photosynthesis to drive aerobic degradation by bacteria, thus producing nutrients and reducing wastewater contamination. The LERD climate station recorded an average daily ambient temperature of 27.9 ± 2.9 degrees Celsius (°C) over a 10-year period. The warmest month was April, which had a temperature of 30.4 ± 3.6 °C, whereas the coolest month was December, which had a temperature of 25.7 ± 3.3 °C. The daily average solar intensity was 228.63 Watts per square meter (W/m<sup>2</sup>). The highest intensity occurred in January (265.10 W/m<sup>2</sup>), whereas the lowest occurred in September (200.54 W/m<sup>2</sup>). In summary, the temperature and solar radiation at the study site remained relatively constant throughout the recorded period.

### Sample collection

Oxidation pond no. 1 was used for sample collection because it was the first pond to aerobic biotic degradation. Samples were collected in February (dry period) from 11:00 to 14:00 hours (h) (Srichomphu *et al.*, 2020) via composite sampling by using a water sampler at five different locations and three different depths: top layer at 0–0.6 meter (m), middle layer at 0.6–1.5 m and bottom layer at >1.5 m (Fig. 1). Noikondee *et al.* (2019) suggested that the temperature gradient in the oxidation ponds of the LERD project can be separated into three levels. Upon collection, the water samples were stored at 4 °C, and their chemical and biological parameters were later analysed. Plankton samples were then collected with 20 liters (L) of water by using a mesh net with a diameter of 36 micrometer (µm) at the same depth and sampling points. The samples were subsequently fixed with 4% formaldehyde (Nguyen and Nhien, 2020). PPF (model SQ-421, Apogee, the USA) was measured every 15 cm, and the data were stored with a CR-300 data logger (Campbell Scientific, the USA).

### Sample analysis

Water samples were collected from the oxidation pond with three replications per sample and analysed for physical and chemical parameters, including temperature, DO, potential of hydrogen (pH) (Multi 3510 IDS, WTW, the USA), biochemical oxygen demand (BOD), NO<sub>3</sub><sup>-</sup>-N, ammonia-nitrogen (NH<sub>3</sub>-N) and PO<sub>4</sub><sup>3-</sup> in accordance with the standard of APHA (2017). Next-generation sequencing was performed on the basis of 16s rRNA genes (Bellebcir *et al.*, 2023) to examine bacterial diversity. A total of 2 L of each sample was centrifuged at 8000 rpm for 10 min to obtain a pellet, which was subjected to DNA extraction. Bacterial 16S rRNA genes were amplified by using the 341F (5'-TCGT

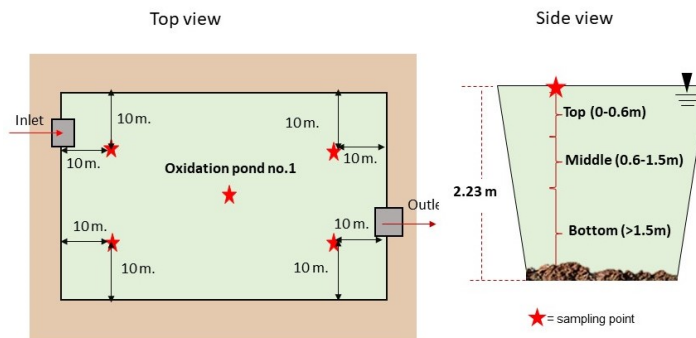


Fig. 1: Wastewater collection points in the oxidation ponds of the LERD project

CGGCAGCGTCAGATGTGTATAAGAGACAG-3') and 805R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG -3') primers targeting V3–V4 regions and 2X sparQ HiFi PCR master Mix (QuantaBio, the USA). Subsequently, 16s amplicons were purified by using sparQ Puremag Beads (QuantaBio, the USA). V3–V4 sequencing was performed on an Illumina Miseq system at Omics Sciences and Bioinformatics Center (Chulalongkorn University, Bangkok, Thailand). Microbiome bioinformatics analyses were conducted by using Quantitative Insights into Microbial Ecology 2 version 2020.8 (Bolyen *et al.*, 2019). Phytoplankton was counted by using a Sedgewick–Rafter chamber under microscopy, and phytoplankton density was calculated in terms of cells/cubic meter (cell/m<sup>3</sup>) by using Eq. 1 (Srichomphu *et al.*, 2024).

$$C = \frac{NV_2}{V_1} \quad (1)$$

Where,

C = plankton density in cells/L (conversion [×1000] was conducted to obtain the value in cells/m<sup>3</sup>)

N = average density per 1 milliliter (mL) of plankton

V<sub>1</sub> = volume of water filtered through the plankton filter bag (L).

V<sub>2</sub> = volume of sample water contained in the sample bottle (mL)

#### Statistical analysis

Analysis of variance (ANOVA) was used to analyse water quality with three replicates per parameter and depth. The data were then subjected to Duncan's multiple range test. (DMRT) for each replication was used in analysis with confidence intervals of 95% (*p* < 0.05). Analysis was performed with Statistical Package for the Social Sciences software version 26.0 (SPSS Inc., Chicago, Illinois, the USA) (Abbasi *et al.*, 2023).

## RESULTS AND DISCUSSION

### Wastewater treatment performance

Symbiosis between different groups of bacteria and phytoplankton at different depths under the appropriate conditions allow for the degradation of organic substances in wastewater. In this work, water quality was used as an indicator of treatment efficiency. Analysing the physical quality of water at three sampling levels revealed that the temperatures at the top, middle and bottom layers of the sampled pond were 27.0 °C, 26.9 °C and 26.9 °C, respectively,

which are suitable for bacterial and phytoplankton habitats and supporting degradation (Dias *et al.*, 2017). Tejaswini *et al.* (2019) stated that the optimal temperature for biological wastewater treatment in tropical zones falls within 25 °C–30 °C. DO slightly decreased with depth to 7.76, 7.45 and 7.42 mg/L, thus supporting treatment (Table 1). This result may be attributed to oxygenic photosynthesis by phototrophic phytoplankton. High total phytoplankton cell densities of 10<sup>7</sup> cells/m<sup>3</sup> were observed at every depth (Nguyen and Nhien, 2020). DO concentration was related to increased pH levels; the pH range of 8.73–8.92 is favourable for bacterial activities in aerobic and anaerobic purification (Bouchaala *et al.*, 2021). The consumption of CO<sub>2</sub> by phytoplankton in the water via photosynthesis resulted in the breakage of carbonate ions (HCO<sub>3</sub><sup>-</sup>) and increase in hydroxyl ions (OH<sup>-</sup>). BOD concentrations in influent and effluent were 29.70 ± 0.79 and 18.30 ± 0.05 mg/L, respectively, and were associated with the treatment efficacy of 38.38%, which passed Thailand's water quality standard (20.0 mg/L). This reduction of BOD was attributed to biological mechanisms involving the bacterial release of extracellular enzymes that are specific to substrates, which are then broken down into small components and taken up by cells for growth and energy production (Liu *et al.*, 2021). For example, enzymes, such as amylase, participate in the hydrolysis of carbohydrates and organic substances with high carbon content (measured in terms of BOD), ultimately forming sugars. Organic carbon is broken down and CO<sub>2</sub> is produced as a by-product during decomposition in the presence of O<sub>2</sub>. CO<sub>2</sub> can serve as a carbon source for cyanobacteria. Under anaerobic conditions, organic substances are decomposed and eventually converted into methane (CH<sub>4</sub>) through methanogenesis. CH<sub>4</sub> then serves as a carbon source for methanotrophs, thus reducing BOD concentrations at different depths. BOD significantly increased (*p* < 0.05) to 23.11 ± 0.62, 24.27 ± 0.06 and 34.48 ± 0.13 mg/L at depths of 0–0.6, 0.6–1.5 and <1.5 m, respectively. The increase in BOD at the lower depths of the pond is supported by the increase in the deposition of dead bacteria, phytoplankton cells and other organic sediments (Bartosiewicz *et al.*, 2021) from bacterial cell walls. Negatively charged phytoplankton and some species that can produce extracellular polymeric substances (EPS) processing the ability to promote cell aggregation, leading to the formation of large cell clusters that settle by gravity

Table 1: Water quality of the influent; top, middle and bottom layers; and effluent of the sampled LERD oxidation pond

Parameters	Units	Std*	Input	Layers			Output	F-test	Efficiency (%)
				Top	Middle	Bottom			
Temperature	°C	23–32	27.4	27.0	26.9	26.9	27.4	-	-
pH	-	-	8.10	8.92	8.81	8.73	8.90	-	-
DO	mg/L	>3.00	4.03	7.76	7.45	7.42	7.34	-	-
BOD	mg/L	<20	29.7 ± 0.79	23.11 ± 0.62 <sup>c</sup>	24.27 ± 0.06 <sup>b</sup>	34.48 ± 0.13 <sup>a</sup>	18.3 ± 0.05	**	38.38
NO <sub>3</sub> <sup>-</sup> -N	mg/L	5.00	0.56 ± 0.05	2.25 ± 0.16 <sup>a</sup>	1.13 ± 0.08 <sup>b</sup>	0.65 ± 0.09 <sup>c</sup>	2.73 ± 0.27	**	-387.50
NH <sub>3</sub> -N	mg/L	0.50	2.54 ± 0.15	1.55 ± 0.12	1.44 ± 0.19	1.33 ± 0.18	0.21 ± 0.09	NS	91.73
PO <sub>4</sub> <sup>3-</sup>	mg/L	≤0.1	1.85 ± 0.77	0.66 ± 0.15 <sup>b</sup>	0.63 ± 0.06 <sup>b</sup>	1.04 ± 0.07 <sup>a</sup>	0.64 ± 0.13	**	65.41
Total phytoplankton	cell/m <sup>3</sup>	-	-	10 <sup>7</sup> × 2.99	10 <sup>7</sup> × 2.97	10 <sup>7</sup> × 2.73	-	-	-

\*Std = standard. The standard of control drain effluent water from the community wastewater treatment system set by the Ministry of Natural Resource and Environment, Thailand

\*\*Statistical variances at a 95% confidence level, ns = no significant differences at a 95% confidence level  
a–c indicate high-to-low statistical values from Duncan's multiple range test.

and accumulate in the water bed, allowing sediments to absorb and release nutrients effectively (Chan *et al.*, 2022). Organic nitrogen is degraded by protease enzymes, which break down proteins into amino acids (Liu *et al.*, 2021). These amino acids serve as a crucial nitrogen source for bacteria and phytoplankton. In the presence of O<sub>2</sub>, nitrogenous organic substances are decomposed into inorganic substances, such as NO<sub>3</sub><sup>-</sup>-N, through nitrification. Under anaerobic conditions, nitrogen compounds can be found in the form of NH<sub>3</sub>-N. NH<sub>3</sub>-N treatment efficacy reached 91.73% and did not significantly differ along the vertical profile (1.55 ± 0.12, 1.44 ± 0.19 and 1.33 ± 0.18 mg/L). The conversion of NH<sub>3</sub>-N into NO<sub>3</sub><sup>-</sup>-N through the biological process of nitrification accounted for the low NO<sub>3</sub><sup>-</sup>-N treatment efficacy (Table 1), which nonetheless exceeded the national standard (5.0 mg/L). The changes in NO<sub>3</sub><sup>-</sup>-N along the vertical profile revealed that NO<sub>3</sub><sup>-</sup>-N concentration was significantly negatively correlated with depth and decreased to 2.25 ± 0.16, 1.13 ± 0.08 and 0.65 ± 0.09 mg/L as a result of denitrification, which is based on the use of PO<sub>4</sub><sup>3-</sup> by bacteria and phytoplankton through mineralisation. PO<sub>4</sub><sup>3-</sup> was increased with pond depth (0.66 ± 0.15, 0.63 ± 0.06 and 1.04 ± 0.07 mg/L) because it was released from sedimented organic substances. The properties of the sediments mentioned above and high pH levels also support chemical precipitation (Li *et al.*, 2022). The interaction of chemical and biological processes resulted in a PO<sub>4</sub><sup>3-</sup> treatment efficacy of 65.41%,

suggesting that the vertical profile of the pond is under aerobic conditions, wherein O<sub>2</sub> is an important factor in the water quality changes that occur in the system and processes supported by temperature and pH.

#### Light under water

Light is a physical factor that affects the photosynthesis of phototrophs. O<sub>2</sub>, which is measured in the form of DO, is produced in the system (Marzetz *et al.*, 2020). The process is crucial for aerobic degradation, wherein aerobic bacteria utilise O<sub>2</sub> as a terminal electron acceptor in energy generation and biological nutrient removal in domestic wastewater treatment systems, and highlights the supporting role of light in aerobic degradation. The highest PPFD of 1,939.5 ± 1.00 μmol/m<sup>2</sup>/s was found at the water surface. However, PPFD decreased in accordance with depth. PPFD at the top, middle and bottom layers of the pond ranged from 1,939.5 ± 1.00 μmol/m<sup>2</sup>/s to 81.5 ± 1.00 μmol/m<sup>2</sup>/s, 81.5 ± 1.00 μmol/m<sup>2</sup>/s to 1.44 ± 0.01 μmol/m<sup>2</sup>/s and 1.44 ± 0.01 to 0.00 μmol/m<sup>2</sup>/s. The average absorption values at the top, middle and bottom layers were 97.67%, 3.05% and 0.10%, respectively (Fig. 2).

#### Microbial community patterns

The bacteria and archaeans found in this study included photosynthetic and BOD-, nitrogen- and phosphorus-removing bacteria. A total of 26 bacterial phyla were identified. The 10 main phyla were found at all depths and did not significantly differ amongst samples

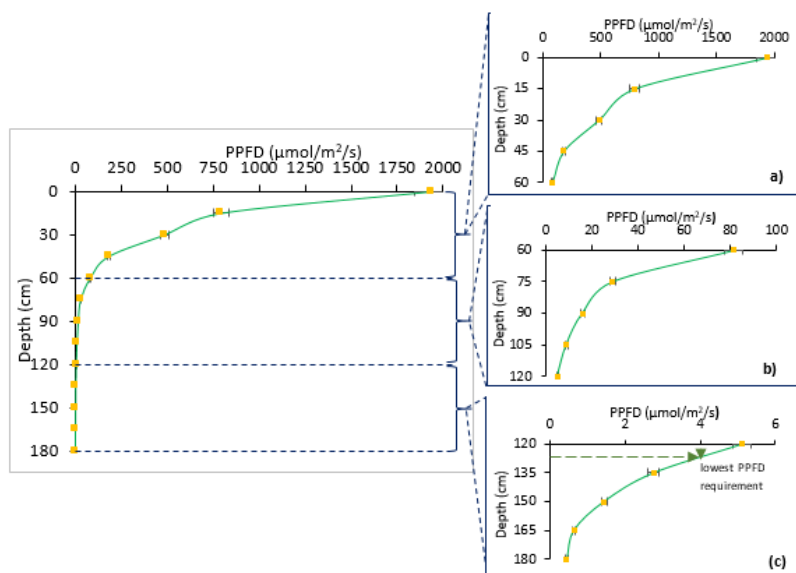


Fig. 2: Light profile (PPFD:  $\mu\text{mol}/\text{m}^2/\text{s}$ ) at the (a) top, (b) middle and (c) bottom layers

taken at different depths. Actinobacteriota dominated amongst the phyla, followed by Cyanobacteria, Plactomycetota, Firmicutes, Proteobacteria (Alpha and Gammaprotobacteria), Verrucomicrobiota, Chloroflexi, Bacteriota, Acidobacteriota and SAR324 clade (marine group B) (Fig. 3a). More than 42 families were identified. The bacteria showed similar relative abundances, with the top three dominant families consisting of Cyanobiaceae, Pirellulaceae and Clostridiaceae, and uncultured bacteria were found at all levels with abundances of 3.2%–3.5% (Fig. 3b). Anaerobic bacteria/archaeans showed certain patterns or differences at different depths and were grouped in accordance with  $\text{O}_2$  demands: 18.99%, 16.38% and 18.48%. Six phyla, namely, Euryarchaeota (Methanobacteriaceae), Firmicutes (Clostridiaceae), Peptostreptococcaceae and Erysipelotrichaceae), Verrucomicrobiota (Akkermansiaceae and Terrimicrobiaceae), Proteobacteria (Rhizobiales incertae sedis), Planctomycetota (Rubinisphaeraceae) and SAR324 clade (marine group B), were identified (Fig. 3) (Tudsanaton et al., 2023).

#### Main microbial drivers of aerobic wastewater treatment

The two main groups of bacteria were identified and classified in accordance with  $\text{O}_2$  demand: 1) aerobic and 2) anaerobic bacteria, which included Archaea, an ancient bacterial lineage that does not

require  $\text{O}_2$  to grow. Archaeans can exist in domestic wastewater because they can decompose organic substances (protein, carbohydrate and lipids) by working together with various bacteria in the form of a consortium under suitable conditions (Mathew et al., 2022). Archaeans and bacteria in the top, middle and bottom layers of the pond separated into two groups: 1) chemotrophic and 2) phototrophic bacteria. DO is an important driver in aerobic wastewater treatment systems. In this study, the highest DO concentration was observed during midday (11:00–14:00 h). It was influenced by phytoplankton photosynthesis. PPFD is an abiotic factor. It is light energy with an important role in the treatability of oxidation ponds because it influences the addition of  $\text{O}_2$  via thermosiphoning and photosynthesis, wherein light is used as energy by phytoplankton and photoautotrophic cyanobacteria. Although phototrophs are dependent on light as their PPFD preference varies depending on their species (Theus et al., 2022). However, in oxidation ponds, depth influences photoinhibition because photosynthesis by phytoplankton and cyanobacteria requires a maximum PPFD of 1400–1900  $\mu\text{mol}/\text{m}^2/\text{s}$  (Ziganshina et al., 2020). In this study, PPFD had a negative relationship with phytoplankton and DO (Table 1). However, the vertical DO content was sufficient for aerobic degradation because the PPFD measured at lower pond depths was adequate for photosynthesis by phytoplankton and cyanobacteria, which require a minimum PPFD



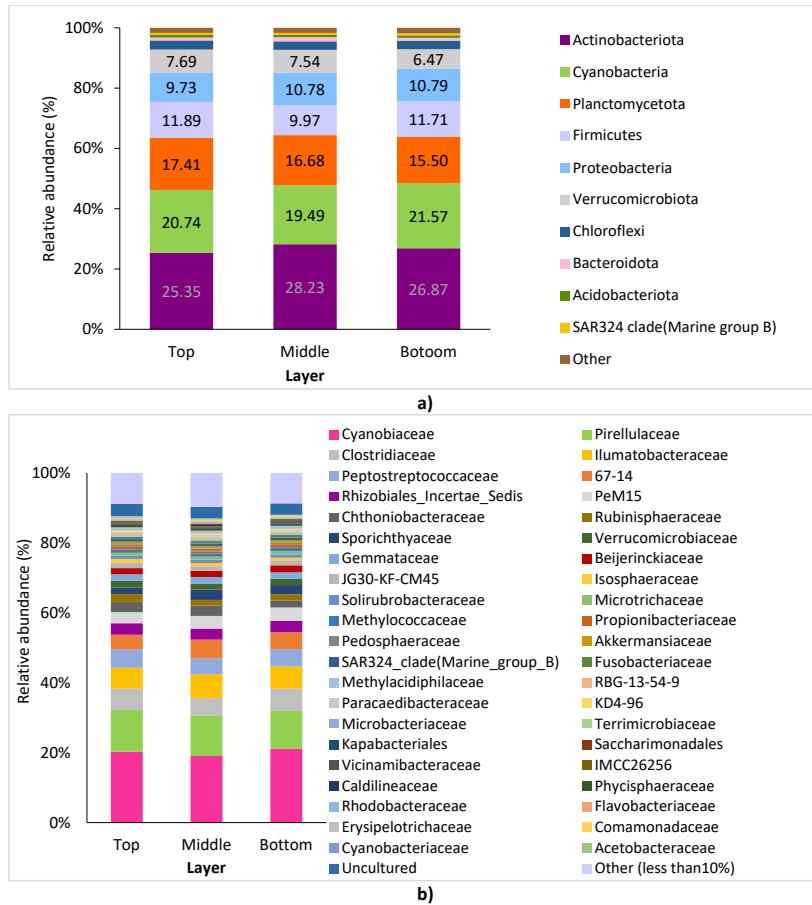


Fig. 3 : Relative abundance of dominant bacterial (a) phyla and (b) families at three different layers of the sampled oxidation pond

of 4–20  $\mu\text{mol}/\text{m}^2/\text{s}$  (Ritchie, 2010). The oxygenic photosynthetic bacteria found at all depths originated from one photolithoautotrophic cyanobacterial phylum and separated into four families, including 1) the dominant family Cyanobiaceae (*Cyanobium* PCC-6307), 2) Cyanobacteriaceae, 3) Nostocaceae and 4) Synechococcaceae. These families are the main  $\text{O}_2$  producers and contributed more than 80% of the  $\text{O}_2$  measured in the water (Yin et al., 2021). *Cyanobium* spp. were found at all depths because they contain chlorophyll pigments and carotenoid and phycobiliprotein accessory pigments as well as have the ability to absorb green or blue light, which can penetrate deep levels (Bernet et al., 2021). For this reason, *Cyanobium* spp. use accessory pigments instead of chlorophyll to absorb light energy (PPFD) and have the optimal irradiance of 200–260  $\mu\text{mol}/\text{m}^2/\text{s}$  (Pagels et al., 2020), accounting for their high abundance of 20.33% at the pond surface (Fig. 3) The

above phenomena indicate that in the investigated wastewater treatment system, oxygenic photosynthetic bacteria played a main role in the photosynthesis process which adds  $\text{O}_2$  during daylight hours, thus supporting the organic degradation activities of aerobic and facultative anaerobic bacteria. In this study, anoxygenic photosynthetic bacteria, which do not photosynthesize and produce  $\text{O}_2$  as a product, were found at every water depth and identified as members of the Chloroflexi phylum (2.8%–3.0%). They were classified as filamentous green bacteria that do not require  $\text{O}_2$  and mediate the formation of flocs that house various bacteria (consortium), thus supporting the fermentation of carbohydrates and complex polymeric organic carbon compounds (Speirs et al., 2019)

*Role of bacteria in oxidation ponds*

BOD indicates the contamination of water by

organic carbon substances. This study found that the abundances of Planctomycetes, Bacteroidota (Zhang *et al.*, 2020) and Verrucomicrobiota (Jia *et al.*, 2019), which have major roles in carbon and nitrogen cycling in aquatic environments, decreased from 17.41% to 15.50%, 7.69% to 6.47 and 0.97% to 0.96% (Fig. 3a) with depth, respectively. These three phyla are responsible for organic carbon degradation (Suominen *et al.*, 2021). The reduction in the abundance of heterotrophic bacteria with depth corresponded to an increase in BOD to  $23.11 \pm 0.62$ ,  $24.27 \pm 0.06$  and  $34.48 \pm 0.13$  mg/L at the top, middle and bottom layers of the pond, respectively. The heterotrophic bacteria identified in this work consisted of six families, including Pirellulaceae, the dominant family; Rhodobacteraceae; Flavobacteriaceae; Saprospiraceae; Crocinitomicaceae; and Mircobacteriaceae (Fig. 3b). These families are aerobic bacteria whose main functions are to degrade BOD in the treatment system under aerobic conditions (Table 1). The abundances of these BOD-degrading bacteria tended to decrease with depth (12.95%, 12.76% and 11.67%). This phenomenon contributed to the increase in BOD given that the amount of decomposers decreased. In addition, organic matter increased with depth due to the sedimentation of bacterial cells and phytoplankton. The accumulation of sediment also promoted the anaerobic decomposition of organic matter. Nevertheless, natural oxidation by methanogens in the pond treatment system can degrade the organic carbon in the form of BOD into  $\text{CH}_4$  under anaerobic conditions. These methanogens are members of the Archaea domain and accounted for approximately 1% of the total bacteria. They were classified as members of the phylum Euryarcheota (Methanobacteriaceae), and their population increased with pond depth. This study found bacteria from two methanogen families: 1) Steroidobacteraceae and 2) Methanobacteraceae (*Methanobacterium*). These families are class I methanogens that produce  $\text{CH}_4$  through strict hydrogenotrophic methanogenesis (Lyu and Lu, 2018), which is supported by Clostridiaceae (sensu stricto 1 and 13). Clostridiaceae, which was found at all depths, produce hydrogen gas ( $\text{H}_2$ ) as a substrate (Cao *et al.*, 2022). All the above processes support the ability to remove organic compounds from carbohydrates in wastewater through carbohydrate utilisation. This study also found aerobic and anaerobic methanotrophic bacteria (methanotrophs), including Methylococcaceae (*Methyloparacoccus*

and Rubinishaeraceae (*SH-PL14*), that use  $\text{CH}_4$  produced by methanogens as a carbon source and energy for growth. This Organic carbon degradation and inorganic carbon reduction phenomenon also contributed to the reduction in carbon compounds in wastewater and indicates that the treatment of organic substances, particularly carbon compounds or BOD, in municipal wastewater relies on the interaction of aerobic bacteria with anaerobic bacteria/archaeans. The  $\text{O}_2$  concentration is related to a reduction in methanogenesis because  $\text{O}_2$  is toxic to anaerobic bacteria. Reactive oxygen species (ROS), which consist of the superoxide radical ( $\text{O}_2^{\cdot-}$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and the hydroxyl radical ( $\text{OH}\cdot$ ), can damage genetic material and cell structures. However, anaerobic bacteria, such as Clostridiaceae, have been suggested to mount antioxidative defense responses by producing the enzymes 1) superoxide dismutase (SOD), which changes  $\text{O}_2^{\cdot-}$  into  $\text{H}_2\text{O}_2$  and  $\text{O}_2$ , and 2) catalase, which eliminates  $\text{H}_2\text{O}_2$  toxicity by converting  $\text{H}_2\text{O}_2$  into  $\text{H}_2\text{O}$  and  $\text{O}_2$  (Brioukhanov and Netrusov, 2004). Moreover, the methanogens found in this study can produce more SOD during the log phase and catalase during the stationary phase than during other phases (Brioukhanov *et al.*, 2002). The enzymes that play a role in reducing ROS toxicity are alkyl hydroperoxide reductase, thioredoxin reductase and thioredoxin. They are produced by the phyla Planctomycetota, Verrucomicrobiota, Bacteroidota, Acidobacteriota, Chloroflexi, Actinobacteriota, SAR324 clade (marine group B) and Firmicutes (Johnson and Hug, 2019), which were found at all pond depths. Changes in nitrogen are related to nitrification by 1) nitrifying bacteria, which consist of ammonia-oxidising bacteria (AOB) and nitrite-oxidising bacteria (NOB), and 2) denitrification by denitrifying bacteria, which considerably reduce  $\text{NO}_3^-$ -N concentration with depth (Table 1). The nitrifying bacterium Comamonadaceae (Jia *et al.*, 2019) was found at all depths. The AOB families Nitrosomonadaceae (Ellin6067), Pirellulaceae and Alphaproteobacteria were also found. The NOB Gammaproteobacteria was also identified. AOB and NOB phyla consisted of Actinobacteriota (25.4% to 26.9%), Planctomycetota (15.5% to 17.4%), Firmicutes (10.0% to 11.9%), Chloroflexi (2.8% to 3.0%), Bacteriota (1.0% to 1.4%) and Acidobacteriota (0.7% to 0.8%). Baskaran *et al.* (2020) suggested that bacteria in the aforementioned phyla participate in nitrification. All of these phyla are the main phyla found in this study



Table 2: Bacterial communities at the phylum level in other wastewater treatment systems

Biological process	Wastewater treatment system/Method	Bacteria at the phylum level		Sources
		Other research	The current study (oxidation pond)	
1. O <sub>2</sub> production	Oxidation pond	Cyanobacteria	Cyanobacteria	<a href="#">Sukchinda et al., 2019</a> ; <a href="#">Srichomphu et al., 2024</a>
2. BOD removal	Oxidation ditch	Chloroflexi	Planctomycetes, Verrucomicrobiota, Bacteroidota, Euryarcheota	<a href="#">Xu et al., 2017</a> ; <a href="#">Nascimento et al., 2018</a> ; <a href="#">Cyzdik-Kwiatkowska and Zielinska, 2016</a>
	Activated sludge	Proteobacteria		
3. Nitrogen removal	Activated sludge	Proteobacteria	Proteobacteria, Actinobacteriota, Planctomycetota, Firmicutes, Verrucomicrobiota, Chloroflexi, Bacteriota, Acidobacteriota	<a href="#">Zhong et al., 2023</a>
	Oxidation pond	Verrucomicrobiota, Firmicutes, Chloroflexi, Bacteroidota		<a href="#">Saneha et al., 2023</a>
	Anaerobic-anoxic-aerobic method	Firmicutes, Proteobacteria		<a href="#">Zhao et al., 2019</a>
	Anaerobic-anoxic-aerobic method	Proteobacteria		<a href="#">Gu et al., 2022</a>
4. Phosphorus removal	Activated sludge	Proteobacteria	Actinobacteriota, Proteobacteria	<a href="#">Liu et al., 2016</a>
	Anaerobic-anoxic-aerobic method	Proteobacteria		<a href="#">Zhao et al., 2019</a> ; <a href="#">Gu et al., 2022</a> ;
	Oxidation ditch	Actinobacteriota		<a href="#">Xu et al., 2017</a>
	Enhanced biological phosphorus removal	Actinobacteriota, Proteobacteria		<a href="#">López-Vázquez et al., 2008</a>

(Fig. 3a). In particular, the Planctomycetes family plays an important role in the nitrogen cycle ([Zhang et al., 2020](#)). These findings account for the nonsignificant differences in NH<sub>3</sub>-N concentration along the vertical profile ( $1.55 \pm 0.12$ ,  $1.44 \pm 0.19$  and  $1.33 \pm 0.18$  mg/L) and indicate that the nonsignificant change in NH<sub>3</sub> at basic pH (pH of 8.73 to 8.92) ([Table 1](#)) resulted from the growth of phytoplankton and cyanobacteria ([Zang et al., 2011](#)). This study found that bacteria in the Proteobacteria and Chloroflexi phyla and Rhizobiales incertae sedis and Steroidobacteraceae families were present. Moreover, it discovered that the concentration of nitrogen contaminants, especially NO<sub>3</sub><sup>-</sup>-N, decreased with depth ([Table 1](#)). Denitrifying bacteria are anaerobic heterotrophic and chemolithotrophic denitrifiers ([Zhang et al., 2020](#)) that reduce NO<sub>3</sub><sup>-</sup>-N into nitrogen gas (N<sub>2</sub>) through the activation of the nitrous oxide reductase gene ([Rose et al., 2021](#)). Phosphorus removal is supported by 1) bacteria that produce carbon-phosphorus (C-P) lyase, Alpharotobacteria, Gammaproteobacteria and Actinobacteriota ([Sosa et al., 2019](#)) and 2) polyphosphate-accumulating bacteria (POB) with the pitA, pstABCS, phoU and ppk genes ([Kristensen et al., 2021](#)) in the Acidobacteriota phylum

and Vicinamibacteraceae and Comamonadaceae families. The results suggest that phosphorus-removing bacteria did not differ with depth ([Fig. 3b](#)). However, PO<sub>4</sub><sup>3-</sup> concentration significantly differed with depth ([Table 1](#)). PO<sub>4</sub><sup>3-</sup> accumulated at depths greater than 1.5 m ( $0.66 \pm 0.15$ ,  $0.63 \pm 0.06$  and  $1.04 \pm 0.07$  mg/L) because the pH values at these depths exceeded 8.5 ([Lee et al., 2015](#)). Moreover, the deposition of dead bacterial and phytoplankton cells released PO<sub>4</sub><sup>3-</sup> from nucleic acids or phospholipid bilayers in microbial cell membranes, and phosphonate sourced from washing agents oxidised into PO<sub>4</sub><sup>3-</sup> ([Rott et al., 2018](#)). These compounds are used as nutrients for the growth of phytoplankton, cyanobacteria and bacteria through uptake into the cells by the biochemical processes involved in growth, resulting in a decrease in phosphorus in wastewater. This work found that community wastewater treatment systems in tropical zones exhibited a diverse range of bacteria at the phylum level. These bacteria play a crucial role in the production of O<sub>2</sub> and treatment of organic substances containing carbon, nitrogen and phosphorus groups, exhibiting a distinct advantage over other community wastewater treatment systems ([Table 2](#)).

## CONCLUSION

This work determined the change in bacterial vertical variability along with depth in community domestic wastewater treatment systems in tropical zones on the basis of bacterial communities combined with physical and chemical water quality. The results of this study showed that water quality reflected the biodegradation activities that occurred in the treatment system and that the diversities of aerobic and anaerobic bacteria did not differ. The 10 main bacteria phyla were Actinobacteriota, Cyanobacteria, Planctomycetota, Firmicutes, Proteobacteria (Alpha- and Gammaproteobacteria), Verrucomicrobiota, Chloroflexi, Bacteriota, Acidobacteriota and SAR324 clade (marine group B). These bacterial phyla also supported the degradation of organic carbon, nitrogen and phosphorus compounds in domestic wastewater in tropical zones. The bacteria that were found at all three depths were classified into four main groups. 1) Phototrophic bacteria included oxygenic and anoxygenic photosynthetic bacteria. Cyanobiaceae (*Cyanobium* PCC-6307) was the dominant oxygenic photosynthetic bacterium (19.08% to 21.19%) at each depth and main O<sub>2</sub> producer in the investigated wastewater treatment system. Sunlight is an important factor for O<sub>2</sub> production through photosynthetic activity and measured in the form of PPFD. PPFD decreased from 1939.5 ± 1.00 μmol/m<sup>2</sup>/s at the surface of the pond to 0.00 μmol/m<sup>2</sup>/s at the bottom layer of the pond. However, O<sub>2</sub> in the vertical profile was sufficient for aerobic degradation by bacteria (7.76 to 7.42 mg/L). The anoxygenic photosynthetic bacterium in this study was identified as Chloroflexi (2.8% to 3.0%), which can form filaments to support the growth of other bacteria. 2) Bacteria that played a role in the treatment of the three groups of carbon compounds in the treatment system included BOD-removing heterotrophic bacteria with a major role in BOD degradation. They included Planctomycetes; Verrucomicrobiota; and Bacteroidota, which consisted of the Pirellulaceae, Rhodobacteraceae, Flavobacteriaceae, Saprospiraceae, Crocinitomicaceae and Mircobacteriaceae families. They also included methanogenic and archaean bacteria, namely, Steroidobacteraceae (bacteria) and Methanobacteraceae (archaean), which convert organic carbon into CH<sub>4</sub> through the hydrogenotrophic methanogenesis pathway. This pathway is also supported by hydrogen-producing bacteria (Clostridiaceae sensu stricto 1 and 13). Methanotrophic bacteria in the Methylococcaceae and Rubinishaeraceae families

used CH<sub>4</sub> produced by methanogens in the treatment system for growth. All of these processes reduced the BOD concentration in the wastewater treatment system to 38.38%. 3) Nitrifying and denitrifying bacteria play a role in the treatment of nitrogen compounds. Nitrifying bacteria (AOB and NOB) in the Actinobacteriota, Planctomycetota, Firmicutes, Chloroflexi, Bacteriota and Acidobacteriota phyla were identified. They converted NH<sub>3</sub>-N into NO<sub>3</sub><sup>-</sup>-N, thus decreasing NH<sub>3</sub>-N in the treatment system by 91.73%. Denitrifying bacteria included Chloroflexi and Proteobacteria in the Rhizobiales incertae sedis and Steroidobacteraceae families. They reduce NO<sub>3</sub><sup>-</sup>-N into N<sub>2</sub>. This process caused NO<sub>3</sub><sup>-</sup>-N to decrease with depth (23.11 ± 0.62, 1.13 ± 0.08 and 0.65 ± 0.09 mg/L). 4) Bacteria for the treatment of phosphorus compounds included those that can produce C-P lyase. These bacteria include Proteobacteria, Actinobacteria and POB in the Acidobacteriota phylum. The high PO<sub>4</sub><sup>3-</sup> concentration at depths exceeding 1.5 m (1.04 ± 0.07 mg/L) resulted from chemical precipitation due to high pH. PO<sub>4</sub><sup>3-</sup> treatment efficacy reached 65.41%. Anaerobic bacteria/archaeans possess a self-repairing system that initiates the repair of DNA and cell structural damage caused by O<sub>2</sub> toxicity. Bacterial communities did not significantly differ along the vertical profile of oxidation ponds in tropical zones. In the treatment system, the products of anaerobic bacteria had no negative effects because of the balanced interaction of aerobic and anaerobic bacteria. This study was conducted in a real area with depth limitations. Therefore, future studies should investigate deep ponds. However, the depth of 2.23 m is suitable for treating community wastewater in tropical zones.

## AUTHOR CONTRIBUTIONS

C. Tudsanaton established the experimental design and performed material and manuscript preparation and data collection, analysis and interpretation. T. Pattamapitoon contributed to study conception, experimental design, material preparation, data collection and analysis and manuscript preparation and editing. O. Phewnil contributed to the experimental design and conducted data collection and interpretation. W. Wararam performed data collection, analysis and interpretation. K. Chunkao contributed to the experimental design and manuscript editing. P. Maskulrath performed data collection and manuscript preparation. M. Srichomphu conducted laboratory

analysis and material preparation

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### CONFLICT OF INTEREST

The authors declare no conflict of interests regarding the publication of this manuscript. In addition, ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission and redundancy have been completely addressed by the authors.

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### ABBREVIATIONS

16s rRNA	16s ribosomal ribonucleic acid
%	Percent
°C	Degree Celsius
μm	Micrometer

$\mu\text{mol}/\text{m}^2/\text{s}$	Micromole per second per square meter
ANOVA	Analysis of variance
AOB	Ammonia-oxidising bacteria
BOD	Biochemical oxygen demand
cell/m <sup>3</sup>	Cell per cubic meter
cell/L	Cell per liter
CH <sub>4</sub>	Methane gas
cm	Centimeter
CO <sub>2</sub>	Carbon dioxide
C-P	Carbon-phosphorus
DMRT	Duncan's multiple range test
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
EPS	Extracellular polymeric substance
H <sub>2</sub>	Hydrogen gas
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HCO <sub>3</sub> <sup>-</sup>	Carbonate ions
HDPE	High density polyethylene
h	Hours
km	Kilometer
L	Liter
LERD project	The King's Royally Initiated Laem Phak Bia Research and Development Project
m	Meter
m <sup>3</sup>	Cubic meter
mg/L	Milligrams per liter
mL	Milliliter
N <sub>2</sub>	Nitrogen gas
NGS	Next-generation sequencing
NH <sub>3</sub> -N	Ammonia-nitrogen
NH <sub>4</sub> <sup>+</sup> -N	Ammonium-nitrogen
nosZ	Nitrous oxide reductase gene
nm	Nanometer
NO <sub>3</sub> <sup>-</sup> -N	Nitrate-nitrogen
NOB	Nitrite-oxidising bacteria
O <sub>2</sub>	Oxygen gas
O <sub>2</sub> <sup>-•</sup>	Superoxide radical
OH <sup>-</sup>	Hydroxyl ion
OH•	Hydroxyl radical
PAR	Photosynthetically active radiation
pH	Potential of hydrogen
phoU	Gene encoding for phosphate accumulation

<i>pitA</i>	Gene encoding for phosphate accumulation
$PO_4^{3-}$	Orthophosphate
<i>POB</i>	Polyphosphate accumulating bacteria
<i>ppk</i>	Gene encoding for phosphate accumulation
<i>PPFD</i>	Photosynthesis photon flux density
<i>pstABCS</i>	Gene encoding for phosphate accumulation
<i>QIIME</i>	Quantitative Insights into Microbial Ecology
<i>ROS</i>	Reactive oxygen species
<i>rpm</i>	Revolutions per minute
SAR324 Clade	Bacteria phylum
<i>SOD</i>	Superoxide dismutase
SPSS	Statistical package for the social sciences

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