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Review

Antibiotics in surface water of East and Southeast Asian countries: A focused review on contamination status, pollution sources, potential risks, and future perspectives

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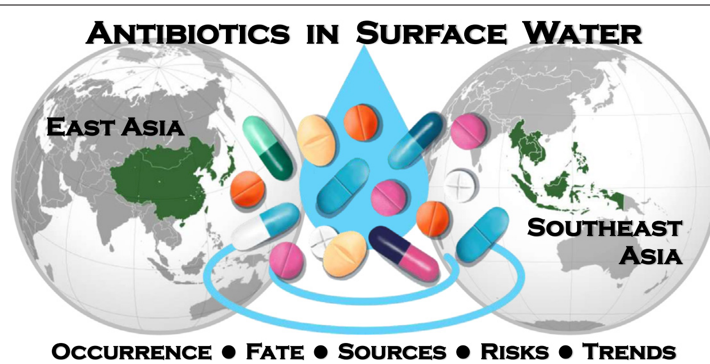
HIGHLIGHTS

- Antibiotic contamination status in East/Southeast Asia's surface water is reviewed.
- Antibiotics are ubiquitous in surface water, especially in aquaculture and urban areas.
- Antibiotic levels varied greatly from few ng/L to hundreds μg/L in surface water.
- Ecological risks and prevalence of antibiotic resistance were widely observed.
- Regional monitoring studies and environmental guidelines for antibiotics are needed.

GRAPHICAL ABSTRACT



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A B S T R A C T

This review provides focused insights into the contamination status, sources, and ecological risks associated with multiple classes of antibiotics in surface water from the East and Southeast Asia based on publications over the period 2007 to 2020. Antibiotics are ubiquitous in surface water of these countries with concentrations ranging from <1 ng/L to hundreds μg/L and median values from 10 to 100 ng/L. Wider ranges and higher maximum concentrations of certain antibiotics were found in surface water of the East Asian countries like China and South Korea than in the Southeast Asian nations. Environmental behavior and fate of antibiotics in surface water is discussed. The reviewed occurrence of antibiotics in their sources suggests that effluent from wastewater treatment plants, wastewater from aquaculture and livestock production activities, and untreated urban sewage are principal sources of antibiotics in surface water. Ecological risks associated with antibiotic residues were estimated for aquatic organisms and the prevalence of antibiotic resistance genes and antibiotic-resistant bacteria were reviewed. Such findings underline the need for synergistic efforts from scientists, engineers, policy makers, government managers, entrepreneurs, and communities to manage and reduce the burden of antibiotics and antibiotic resistance in water bodies of East and Southeast Asian countries.

Contents

1. Introduction

Since the discovery of penicillin by A. Fleming in 1929 (Fleming, 1929), antibiotics from multiple classes have been produced and used worldwide for the treatment human, animal, and plant diseases caused by pathogenic bacteria (Thakare et al., 2020). As a result, substantial amounts of antibiotics have been released into the environment through discharge from households, hospitals, pharmaceutical companies, wastewater treatment plants (WWTPs), and aquaculture and livestock farms (Kümmerer, 2009a; Danner et al., 2019; Kovalakova et al., 2020). Antibiotics have been considered as emerging classes of pollutants because of their omnipresence at elevated concentrations in surface water, groundwater, soil, sediment, and biota in virtually all parts of the world (Kümmerer, 2009a; Carvalho and Santos, 2016; Binh et al., 2018; Danner et al., 2019; Felis et al., 2020; Kovalakova et al., 2020; Lyu et al., 2020). The presence of multiple antibiotics in the water environment can pose ecological risks to aquatic organisms, and is responsible for the promotion of antibiotic resistance genes (ARG) and antibiotic-resistant bacteria (ARB) in the aquatic ecosystems (Kümmerer, 2009b; Carvalho and Santos, 2016; Danner et al., 2019; Kumar et al., 2019; Kovalakova et al., 2020). This situation raises serious concerns regarding antibiotic residues in the aquatic environment, especially in developing countries where management practices of antibiotic use and antibiotic-related waste have not been effectively performed (Segura et al., 2015; Lundborg and Tamhankar, 2017; Binh et al., 2018; Kumar et al., 2019).

The East and Southeast Asia consists of nineteen countries and special administrative regions with a total population of about 2.3 billion people, accounting for 30% of the global population in 2018 (United Nations, 2018). China is considered as the biggest producer and consumer of antibiotics in the world with a total national production and usage amounts of 248,000 and 162,000 tons in 2013, respectively (Zhang et al., 2015). The number of scientific publications (including original papers and critical reviews) about the contamination status and relevant risks of antibiotics in Chinese aquatic environment has gradually increased over the last decade, illustrating the interest in this issue in China (Z. Li et al., 2020; Lyu et al., 2020). Elevated releases of antibiotics and prevalence of ARG and ARB from WWTPs and aquaculture and livestock sectors in South Korea have been also addressed (Sim et al., 2011; Jang et al., 2018; Kim and Kim, 2016; Kim et al., 2019; Kim et al., 2020). Moreover, the production, use, and environmental residues of antibiotics are expected to increase in Southeast Asia region, as a result of rapid economic development and population growth together with the ubiquity of tropical infectious diseases and improper habits of drug usage (Shimizu et al., 2013; Lundborg and Tamhankar, 2017; Binh et al., 2018).

There are several review articles that provide comprehensive insights into the occurrence, fate, sources, and risks of antibiotics in water bodies of global (Kümmerer, 2009a; Danner et al., 2019; Felis et al., 2020; Kovalakova et al., 2020), regional (e.g., Europe, Carvalho and Santos, 2016; Szymańska et al., 2019; and Africa, Faleye et al., 2018), and national scales (e.g., China, W. Zhao et al., 2016; Guan et al., 2017; Li et al., 2018a; Liu et al., 2018; Z. Li et al., 2020; Lyu et al., 2020; and Vietnam, Thuy and Nguyen, 2013; Binh et al., 2018). However, to the best of our knowledge, no review paper has focused on antibiotics in surface water in the East and Southeast Asian countries. The review presented here, based on data compiled from peer-reviewed papers published mainly during 2007–2020, provides a compendious picture on the concentrations and profiles of antibiotics in receiving water and their potential sources. Relevant comparisons are made between several countries in the East and Southeast Asia. Target antibiotics comprise compounds of multiple classes such as β-lactams (BLs), fluoroquinolones (FQs), macrolides (MLs), sulfonamides (SFs), tetracyclines (TCs), and other classes. Basic information about major antibiotics described in this review is summarized in Table 1. Current understanding of the environmental behavior, fate, and negative impacts of antibiotics in surface water of these countries is included. In addition, general comments and future perspectives on different aspects regarding antibiotics in the aquatic environment of the investigated regions are provided, heading towards effective monitoring, assessment, management, and remediation schemes for these emerging pollutants in the East and Southeast Asia.

2. Production, import/export, and use of antibiotics in East and Southeast Asian countries

2.1. Production, import, and export

China is the largest producer and exporter of antibiotics in the world with total production and export amounts of 248,000 and 88,000 tons in 2013, respectively, while the imported amount was only 600 tons (Zhang et al., 2015). In Japan, antibiotic research and production started in the 1940s and a total of 117 antibiotics and bioactive microbial products being discovered during 1946–1995, among them 41 agents have been licensed internationally (Kumazawa and Yagisawa, 2002). In South Korea, the production value of antibiotics reached 1.6 trillion won ($1.4 billion) in 2010 (Kim, 2012). Some East and Southeast Asian countries, such as China (€660 million, 23%), Japan (€120 million, 4%), Singapore (€90 million, 3%), and South Korea (€80 million, nearly 3%), have become the most important antibiotic exporters to the European Union in 2018 (Eurostat, 2019). Statistical data about production, import, and export of antibiotics in other East and Southeast Asian countries are relatively limited.

2.2. Consumption

As the most populated country in the world, China is the biggest user of antibiotics with total usage of 162,000 tons in 2013, dominated by MLs (42,200 tons), BLs (34,100 tons), and FQs (27,300 tons) (Zhang et al., 2015). Proportion of total antibiotics used for humans in China was 48%, corresponding to 77,760 tons (Zhang et al., 2015). The annual consumption amounts of human antibiotics in Vietnam, South Korea, Japan, Philippines, Mongolia, and Brunei in 2015 were estimated to be 1086, 546.4, 524.9, 260.5, 133.2, and 1.13 tons, respectively (WHO, 2018; Carrique-Mas et al., 2020). The total human antibiotic consumption rates expressed as defined daily dose (DDD) per 1000 inhabitants per day derived for different East and Southeast Asian countries in 2015 (Klein et al., 2018; WHO, 2018) are shown in Fig. 1. Accordingly, Mongolia had the highest human antibiotic consumption rate, followed by Vietnam, South Korea, and Thailand. Relatively high consumption rate of antibiotics estimated for Mongolian people is possibly due to popular use of injection treatment, widespread and inappropriate antibiotic prescription, or even self-medication, and an outbreak of private pharmacy market in this country since 1990 (Togoobaatar et al., 2010; Nakajima et al., 2010). However, it should be noted that these estimations were based on data of imported and produced antibiotics, and more accurate studies conducted at the end-user level are needed (Dorj et al., 2019). There is no clear relationship between antibiotic consumption rates and population size and income level. The consumption patterns of antibiotic classes differ between countries. For example, the Japanese formulation was dominated by MLs and cephalosporins, followed by FQs and penicillins (Tsutsui et al., 2018), whereas the antibiotic prescriptions in South Korea and Vietnam contained higher proportions of penicillins and cephalosporins as compared to MLs and FQs (Yoon et al., 2015; Carrique-Mas et al., 2020).

For non-human use of antibiotics, China also served as a leading consumer with total veterinary usage of 84,240 tons in 2013, which were mainly administered to pigs (62%) and chickens (23%) (Zhang et al., 2015). The use of antibiotics in livestock production in China accounted for 23% of total amount applied in this sector globally in 2010, and it is projected to double by 2030 (Van Boeckel et al., 2015). In South Korea, about 984 tons of antibiotics were sold in 2018 for use in pigs (50%), fishery (25%), poultry (16%), and cattle (9%), with penicillines (259 tons) and tetrcyclines (249 tons) as the most dominant classes (MFDS, 2019). The Red River Delta in Vietnam and the northern suburbs of Bangkok, Thailand are considered as hotspots of veterinary antibiotic consumption in Southeast Asia (Van Boeckel et al., 2015). The usage of veterinary antibiotics in Vietnam was estimated to be 2751 tons in 2015, and was attributed to pig (58%), aquaculture (31%), and chicken (7%) production (Carrique-Mas et al., 2020). Some Southeast Asian countries such as Indonesia, Myanmar, and Philippines are expected to greatly increase their antibiotic usage for food animals in the upcoming years (i.e., 150% to 200% between 2010 and 2030; Van Boeckel et al., 2015).

2.3. Stewardship and use practices

Inappropriate sale and use of antibiotics in humans has been, and continues to be, a great issue of concern in many East and Southeast Asian countries, largely due to the development of antimicrobial resistance (Lansang et al., 1990; Nakajima et al., 2010; Widayati et al., 2011; Quet et al., 2015; Holloway et al., 2017; Chanvatik et al., 2019; Kong et al., 2019; Suy et al., 2019; Miyazaki et al., 2020). The most critical limitations in an effective management and use of antibiotics in such countries have been: (1) self-medication (i.e., the use of antibiotics

Table 1
General information about major antibiotics described in this review.[a]

Class	Antibiotic	Acronym	Molecular formula	Molecular weight	$\log K_{OW}$	pK_a	Water solubility (mg/L)
β-Lactams (BLs)	Amoxicillin	AMO	$C_{16}H_{19}N_3O_5S$	365.40	0.87	3.2/11.7	3430 (25 °C)
	Ampicillin	AMP	$C_{16}H_{19}N_3O_4S$	349.40	1.35	2.5/7.3	10,100 (21 °C)
	Cephalexin	CEP	$C_{16}H_{17}N_3O_4S$	347.39	0.65	5.2/7.3	10,000
	Cefazolin	CEZ	$C_{14}H_{14}N_8O_4S_3$	454.51	−0.58	3.6	210 (25 °C)
	Penicillin	PEN	$C_{16}H_{18}N_2O_4S$	334.39	1.83	2.76	210
Fluoroquinolones (FQs)	Ciprofloxacin	CIP	$C_{17}H_{18}FN_3O_3$	331.34	0.28	6.09	30,000 (20 °C)
	Enrofloxacin	ENR	$C_{19}H_{22}FN_3O_3$	359.39	0.58	5.69	612
	Levofloxacin	LEV	$C_{18}H_{20}FN_3O_4$	361.37	−0.39	6.25	1440
	Lomefloxacin	LOM	$C_{17}H_{19}F_2N_3O_3$	351.35	−0.30	5.64	27,200
	Norfloxacin	NOR	$C_{16}H_{18}FN_3O_3$	319.33	0.46	6.34/8.75	280 (25 °C)
	Ofloxacin	OFL	$C_{18}H_{20}FN_3O_4$	361.37	−0.39	5.97/9.28	10,800 (25 °C)
Macrolides (MLs)	Azithromycin	AZI	$C_{38}H_{72}N_2O_{12}$	748.98	4.02	8.74	2.37 (25 °C)
	Clarithromycin	CLA	$C_{38}H_{69}NO_{13}$	747.95	3.16	8.99	1.69 (25 °C)
	Erythromycin	ERY	$C_{37}H_{67}NO_{13}$	733.93	3.06	8.88	4.2 (25 °C)
	Roxithromycin	ROX	$C_{41}H_{76}N_2O_{15}$	837.05	1.70	12.45	0.0189 (25 °C)
	Tylosin	TYL	$C_{46}H_{77}NO_{17}$	916.10	1.63	7.73	211
Sulfonamides (SFs)	Sulfadiazine	SDZ	$C_{10}H_{10}N_4O_2S$	250.28	−0.09	6.36	77 (25 °C)
	Sulfamerazine	SMR	$C_{11}H_{12}N_4O_2S$	264.31	0.14	6.99	202 (20 °C)
	Sulfamethazine	SMT	$C_{12}H_{14}N_4O_2S$	278.33	0.89	7.59	1500 (29 °C)
	Sulfamethizole	SMZ	$C_9H_{10}N_4O_2S_2$	270.33	0.54	6.71	1050 (37 °C)
	Sulfamethoxazole	SMX	$C_{10}H_{11}N_3O_3S$	253.28	0.89	6.16	610 (37 °C)
	Sulfapyridine	SPY	$C_{11}H_{11}N_3O_2S$	249.29	0.35	8.43	268 (25 °C)
	Sulfathiazole	STZ	$C_9H_9N_3O_2S_2$	255.32	0.05	7.24	373 (25 °C)
Tetracyclines (TCs)	Chlortetracycline	CTC	$C_{22}H_{23}ClN_2O_8$	478.88	−0.62	2.99	259 (25 °C)
	Doxycycline	DXC	$C_{22}H_{24}N_2O_8$	444.43	0.63	3.09	50,000 (25 °C, pH = 2.16)
	Oxytetracycline	OTC	$C_{22}H_{24}N_2O_9$	460.43	−0.90	3.37	313 (25 °C)
	Tetracycline	TET	$C_{22}H_{24}N_2O_8$	444.43	−1.37	3.30	231 (25 °C)
Antibiotics of other classes	Chloramphenicol	CHP	$C_{11}H_{12}Cl_2N_2O_5$	323.13	1.14	7.49	2500 (25 °C)
	Lincomycin	LIN	$C_{18}H_{34}N_2O_6S$	406.54	0.20	7.60	927 (25 °C)
	Trimethoprim	TMP	$C_{14}H_{18}N_4O_3$	290.32	0.91	7.12	400 (25 °C)

[a] Data retrieved from https://pubchem.ncbi.nlm.nih.gov and https://www.drugbank.ca.

without medical prescription) that leads to underuse or abuse and even misuse; (2) inadequate knowledge and inappropriate prescription behavior of doctors and healthcare staff; (3) improper stewardship of antibiotics in healthcare facilities and pharmacies, especially in rural areas; (4) underdeveloped health infrastructure and legislative systems; and (5) the lack of understanding and awareness of people regarding benefits and risks of antibiotics. Implementation strategies for antibiotic stewardship have been proposed by the World Health Organization (WHO, 2019), and have been approved in China (Xiao, 2018), Japan (The Government of Japan, 2017), South Korea (Kim et al., 2016), and some Southeast Asian countries (Zellweger et al., 2017).

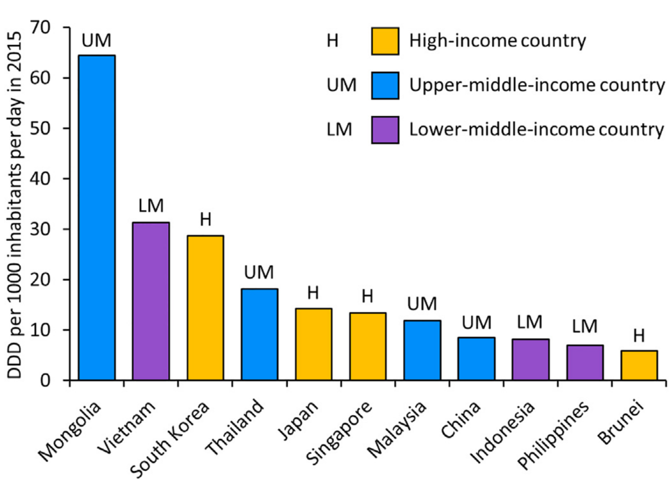

Fig. 1. Defined daily dose (DDD) per 1000 inhabitants per day of total human antibiotics estimated for different East and Southeast Asian countries in 2015. Data were retrieved from Klein et al. (2018) and WHO (2018).

In addition, the misguided application of veterinary antibiotics in livestock and aquaculture production can also pose a significant threat to environmental and human health through antibiotic residues and resistance in waste flows generated and food produced from various farms in East and Southeast Asia (Nhung et al., 2016; Goutard et al., 2017; Qiao et al., 2018). In order to control and minimize ecological risks and antibiotic resistance, the appropriate use, management, and total reduction of antibiotics used as veterinary medicines and growth promoters have been implemented in China (Hu and Cowling, 2020), Japan (The Government of Japan, 2016), and some Southeast Asian countries like Indonesia, Thailand, and Vietnam (Coyne et al., 2019).

3. Contamination status of antibiotics in receiving water

3.1. China

There has been a great number of studies on the occurrence of antibiotics in the aquatic environment of China. Our discussion is mainly based on systematic reviews and recent research papers on large-scale water systems with the goal of providing an up-to-date and comprehensive view regarding contamination status and trend of antibiotics in Chinese surface water. A total of >90 antibiotics belonging to five main classes (i.e., SFs, FQs, TCs, MLs, and BLs) were monitored in water samples from China during 2005–2016, showing concentration range of 0.1 to 1000 ng/L with the median <100 ng/L (Li et al., 2018a). W. Zhao et al. (2016) reviewed research papers published during 2012–2015 and found that TMP, SMX, ERY, and TET were the most predominant compounds in surface water of China (average concentrations >50 ng/L). Extremely high concentrations of TCs such as CTC (median 6820; maximum 68,900 ng/L), OTC (median 39.5; maximum 361,000 ng/L), and TET (median 26.0; maximum 25,500 ng/L) were detected in surface water from the Hai River system (Chen et al., 2018). Concentrations of OTC as high as 200 to 700 μg/L were found in surface water of the Xiao River, which received effluent from an OTC production

WWTP (Li et al., 2008). It was estimated that usage amounts of TET, OTC, and CTC in China in 2013 were 1450, 1360, and 262 tons, respectively (Zhang et al., 2015). The abundance of tetracyclines in surface water samples from some river systems in China is probably due to poor treatment efficiency of sewage, surface runoff, and direct discharge from livestock sectors (Chen et al., 2018). Besides TCs, SFs and FQs were relatively more abundant than other classes in surface water (Chen et al., 2018; Liu et al., 2018; Lyu et al., 2020). Despite the fact that BLs are readily hydrolyzed in environmental water, the dominance of this class (e.g., AMO, CEP, CEZ, and PEN) was documented in three major rivers (i.e., the Yangtze, Yellow, and Pearl Rivers) with median concentrations from 96.8 to 1520 ng/L and maximum concentration up to 3380 ng/L (Zhang et al., 2015; Guan et al., 2017).

Concentrations of antibiotics in seawater were about one order of magnitude lower than in river and lake water, largely due to effects of dilution, deposition, and degradation during riverine transportation (W. Zhao et al., 2016; Li et al., 2018a). In terms of geographical distribution, more elevated concentrations of antibiotics were found in rivers and lakes in North and East China (e.g., the Beijing–Tianjin–Hebei Region, Hai River Basin, Yangtze River Basin, Taihu Lake, and Baiyang Lake) (Chen et al., 2018; Li et al., 2018a; Liu et al., 2018; Lyu et al., 2020), which were consistent with higher population size and usage of antibiotics in North and East China when compared to other regions (Zhang et al., 2015). The principal sources of antibiotics in Chinese surface water were estimated to be wastewater from aquaculture and husbandry farms and insufficiently treated sewage (Chen et al., 2018; Lyu et al., 2020).

3.2. Japan

Macrolides (AZI, ERY, CLA, and ROX), sulfonamides (SPY and SMX), and TMP were frequently detected in water of the Tama River in the Tokyo metropolitan area at concentrations from 4 to 448 ng/L (Managaki et al., 2007). From the same river and its tributaries, Mano and Okamoto (2016) measured concentrations of 10 pharmaceuticals including antibiotics such as CLA (median 99; range 0.82–1200 ng/L), SMX (33; 0.89–110 ng/L), LEV (29; <0.71–600 ng/L), and AZI (17; <0.036–500 ng/L), with little change in concentrations of some antibiotics observed over the sampling period (i.e., 2005–2012). According to a nationwide monitoring study conducted in 37 major Japanese rivers during 2006–2007, total concentrations of 7 SFs, TMP, and 4 MLs ranged from below quantification limits to 626 (median 7.3) ng/L with CLA and SPY as the most abundant compounds (Murata et al., 2011). Generally speaking, antibiotic residues were higher in urban rivers than rural ones and were more dominated by human antibiotics from sewage effluent rather than veterinary medicines in livestock wastewater (Murata et al., 2011).

3.3. South Korea

Contamination degree of antibiotics in surface water from some major rivers in South Korea such as the Han River and the Nakdong River was not too severe with maximum concentrations of individual compounds not exceeding 1000 ng/L (Ji et al., 2010; Im et al., 2020). Some SFs such as SMZ, SMX, and STZ were detected in the Han River and the Nakdong River basins in 2007 at higher frequency and concentrations (<10.0–253 ng/L) as compared to compounds of other classes such as TMP, ENR, and ROX (<10.0–60.0 ng/L) (Ji et al., 2010). More recently, in the Han River watershed that covers both urban and rural areas of Seoul City and Gyunggi Province, CLA was detected at the highest frequency and concentrations (median 151; maximum 663 ng/L), followed by TMP (median 44; maximum 103 ng/L) and SMX (median 11; maximum 31 ng/L); while detection rate and levels of SMT and STZ were quite low (Im et al., 2020). Im et al. (2020) indicated that the most antibiotic-contaminated sites in tributaries of the Han River were associated with highly populated areas with extensive economic activities in Seoul, and that WWTP effluent was the most important sources of antibiotics and other pharmaceuticals in these areas.

In contrast to the major rivers of South Korea with relatively low antibiotic residues, small streams near to point sources have been heavily affected by antibiotics (Ok et al., 2011; Awad et al., 2014; Kim et al., 2019). Elevated concentrations of three sulfonamides (e.g., SMT, SMX, and STZ; range 10–10,570 ng/L), one macrolide (TYL: 230–2190 ng/L), and three tetracyclines (TET: 1420–254,820; CTC: <10–44,420; and OTC: <10–1410 ng/L) were recorded in surface water receiving discharge from a local swine manure composting facility (Ok et al., 2011; Awad et al., 2014). The high levels of these veterinary antibiotics were largely attributed to releases from a local swine manure composting facility (Ok et al., 2011; Awad et al., 2014). The maximum concentrations of some antibiotics such as SMT, STZ, OTC, SMX, and CTC of 21.3, 17.4, 16.9, 3.91, and 3.33 μg/L, respectively were detected in local streams, as a result of concentrated animal feeding operations (Kim et al., 2019).

3.4. Vietnam

The presence of antibiotics in Vietnamese surface water was first described in 2005 in the Mekong Delta, southern Vietnam (Managaki et al., 2007). Individual antibiotics like SMX, SMT, TMP, and ERY were found in water samples from the Mekong River with concentrations ranging from 7 to 33 ng/L (Managaki et al., 2007). Another study in this delta with larger sample size (i.e., time series sampling) also reported similar contamination levels of SMX, TMP, ENR, and SDZ in canal and river water with median values of 21, 17, 12, and 4 ng/L, respectively (Nguyen et al., 2015). Concentrations of total seventeen antibiotics in the Hau River, a distributary of the Mekong River, ranged from 17 to 91 (mean 43) ng/L (Shimizu et al., 2013). Meanwhile, extremely low concentrations of DXC, OTC, LIN, SMX, and SMZ (not detected to 1.1 ng/L) were found in water samples from two rivers and two canals in the Mekong Delta (Do et al., 2020). These studies emphasized the possible contributions of aquaculture, husbandry, and domestic wastewater to antibiotic residues of the Mekong Delta water resources (Managaki et al., 2007; Shimizu et al., 2013; Nguyen et al., 2015). Studies on antibiotic residues in northern part of Vietnam are relatively limited. Ngo et al. (2020) detected relatively low concentrations of antibiotics in surface water of the Cau River collected from eight sites in Bac Kan, Thai Nguyen, and Bac Ninh Provinces. In the Cau River water samples, LIN (range 0.30–24.9 ng/L) and SMX (mean 21.6; maximum 66.6 ng/L) were the most frequently detected compounds (Ngo et al., 2020). Higher antibiotic concentrations were found in surface water of some urban lakes in Hanoi City with median values of 255, 37, 36, 34, and 26 ng/L for SMX, LIN, OFL, SMZ, and TMP, respectively, pointing towards untreated sewage and stormwater runoff as antibiotic sources (Tran et al., 2019).

3.5. Thailand

Information about antibiotic residues in receiving water in Thailand is relatively scarce. OTC and ENR were found at concentrations as high as 3050 and 1590 ng/L, respectively, in water samples from the Tha Chin River, indicating the influence of antibiotic application in tilapia cage farming (Rico et al., 2014). In six canals from Bangkok, concentrations of CIP (median 53; range 9–194 ng/L) and STZ (50; 29–107 ng/L) were higher than other antibiotics such as ROX, SMX, SMT, and TMP (range 2–52 ng/L) (Tewari et al., 2013). Lower concentrations of these antibiotics were found in water of the Chao Phraya River (i.e., generally <10 ng/L; Tewari et al., 2013). Similarly, ERY, CIP, and LIN were detected at low frequency and concentrations (average 12.8, 20.2, and 21.7 ng/L, respectively) in downstream water samples from Bangkok (Li et al., 2012). SMX (0.95–575 ng/L) and CLA (0.82–25.8 ng/L) were among the most frequently detected compounds in receiving water from three hospital WWTPs in Bangkok (Sinthuchai et al., 2016). Some FQs such as CIP, LOM, NOR, and OFL were not quantified in water from a lake without any inflow in Khon Kaen (Takasu et al., 2011). In general, concentrations of many antibiotics in receiving water in Thailand were relatively low partly due to effective wastewater treatment (Li et al., 2012); however, contributions from sources other than WWTP effluent (i.e., untreated discharges) were also considered (Tewari et al., 2013; Sinthuchai et al., 2016).

3.6. Other Southeast Asian countries

So far, little is known about the occurrence of antibiotics in ambient water from other Southeast Asian countries. Concentrations of total seventeen antibiotics in the Laguna Lake, the Pasig River, and the Manila Bay in Philippines ranged from 14 to 192 (median 49) ng/L with major compounds as SMX (median 33; range 8–94 ng/L), SMT (9; 2–73 ng/L) (Shimizu et al., 2013). Very low concentrations (e.g., from not detected to <10 ng/L) of antibiotics such as SMT, SMX, SDZ, SMR, LIN, and CHP were measured in surface water from nine mangroves in Singapore (Bayen et al., 2016). Urban surface water in Singapore was also slightly contaminated with antibiotics at average concentrations ranging from 0.5 ± 0.2 ng/L (CHP) to 6.4 ± 3.0 ng/L (SMT) (Yi et al., 2019). Yi et al. (2019) found that concentrations of some human antibiotics such as AZI, CLA, LIN, and SMX positively correlated with population density, while levels of veterinary medicines such as SMT and SMR did not correlate with population density and were higher in agricultural areas. Low et al. (2020) analyzed multi-residues of twenty-two antibiotics in riverine estuarine water from the Larut River and the Sangga Besar River in Perak, Malaysia, and found low detection frequency (up to 23%) and concentrations (up to 18 ng/L) with the most considerable compounds as ERY, OFL, and CIP. Antibiotic levels in the water samples from the Larut River were higher than those found in the Sangga Besar River because the Larut River receives wastewater from hospital, zoo, and poultry slaughterhouse, which were contaminated with several antibiotics such as ERY, OFL, CIP, TET, and OTC (Low et al., 2020). The available data indicated that the degree of antibiotic contamination in surface water of these countries was not severe; however, more comprehensive monitoring studies (e.g., detailed analytical protocols with larger sample sizes and wider geographical scales) are needed to provide better insights into the spatial distribution and compositional profiles of antibiotics in water environments of such countries.

3.7. General discussions

Antibiotics have been extensively detected in surface water from different aquatic systems such as seas, bays, rivers, lakes, streams, and canals of the East and Southeast Asian countries. Concentrations of antibiotics varied greatly between studies, showing a very wide range from <1 ng/L to hundreds μg/L with median values of individual compounds mainly in the range of 10 to 100 ng/L. Concentrations and profiles of total antibiotics in surface water depend on various factors such as geography, hydrology, meteorology, anthropogenic activities (e.g., antibiotic usage patterns and wastewater treatment efficiency), and the nature of the specific classes and compounds. To facilitate comparison, we selected data of four frequently monitored antibiotics such as OTC, SMX, CLA, and CIP, representing four main classes of TCs, SFs, MLs, and FQs, respectively (Fig. 2). The data showed wider ranges and higher maximum concentrations of antibiotics in surface water from the East Asian countries as compared to the Southeast Asian ones. Previous studies have revealed some principal rules of antibiotic distribution in surface water of this region: (1) higher concentrations of human antibiotics were found in highly populated areas; (2) aquaculture and livestock production strongly affected receiving water by elevated amounts of veterinary medicines; and (3) a decreasing trend of freshwater–seawater in antibiotic concentrations was observed. Except for some potential outliers of certain antibiotics in near-source sites due to sampling bias, the overall antibiotic levels in surface water of the East and Southeast Asian countries were within the ranges documented for other parts of the world (Kümmerer, 2009a; Carvalho and Santos, 2016; Szymańska et al., 2019; Kovalakova et al., 2020). Besides, long-term monitoring studies on antibiotics in surface water of these regions are still limited, which prevent understanding towards temporal trend of these contaminants.

4. Environmental behavior and fate of antibiotics

4.1. Phase distribution characteristics

The distributions of antibiotics between dissolved and particulate phases, and between water and sediment are important factors that affect contamination levels and fate of these substances. Most of previous studies used membrane filtration (e.g., usually 0.45-μm filter and sometimes together with 0.22-μm filter) as a sample pretreatment step, therefore, measured concentrations represented dissolved phase concentrations basically. To date, few studies have discussed the occurrence and partition of antibiotics between dissolved and particulate phases in surface water of the countries covered by the present review. Dong et al. (2019) estimated partition coefficients (K_P) between water and suspended particulate matter (SPM) of TCs in surface water of the Meijiang River, China, and found that DXC (average $\log K_P = 4.74$) was more associated with SPM rather than other TCs such as TET, OTC, and CTC ($\log K_P = 4.18$–4.32). These observations were consistent with the fact that hydrophobicity of DXC was higher than other TCs with greater K_{OW} (see Table 1). Apart from hydrophobic partitioning onto organic carbon of SPM, overall interactions between antibiotics existing as zwitterion or monoanion forms and SPM can also be contributed by hydrogen bonds, cation exchange, and cation bridging (Dong et al., 2019). K_P and organic carbon-water partition coefficients (K_{OC}) of several SFs and MLs were estimated for surface water from East River, southern China, showing higher affinity of MLs and TMP to particulate matter as compared to SFs (Zhang et al., 2017). Adsorption behavior of antibiotics onto SPM is also affected by particle size, for example, SPM of 63–150-μm fraction has stronger adsorption capacity to TET and NOR in water columns of the Meiliang and Gonghu Bays, China (Luo et al., 2019).

The distribution of antibiotics between water and sediment is also driven by the nature of compounds (e.g., hydrophobicity and functional groups) and sediment properties (e.g., particle size and composition), resulting in highly variable sediment-water partition coefficients (K_P and K_{OC}) of antibiotics (Chen and Zhou, 2014; S. Zhao et al., 2016; Chen et al., 2017; Zhang et al., 2017; Li et al., 2018b). In general, MLs, FQs, TMP, and CHP are more readily deposited to the sediment bed than SFs and TCs. Chen and Zhou (2014) and Chen et al. (2017) found significant correlations between $\log K_{OC}$ with $\log K_{OW}$ and log molecular weight for most antibiotics in their studies, except for some compounds such as thiamphenicol, ENR, OFL, and TET. Meanwhile, Li et al. (2018b) did not observe any relationship between $\log K_{OC}$ and $\log K_{OW}$, implying the variation in sediment and water properties. In addition, Zhang et al. (2017) indicated that SPM may have higher capacity to adsorb antibiotics than sediment because SPM has a greater interaction surface with entire water columns while sediment mainly equilibrates with the bottom water layer, which may contain lower contaminant levels than surface layer. Understanding the distribution of antibiotics between water–SPM–sediment phases can provide in-depth insights into environmental behavior and fate of these pollutants, which may help in implementing appropriate management and remediation strategies regarding residual antibiotics in the aquatic environment. To our knowledge, available studies about this important topic were mainly conducted in China, therefore, large information gaps in other East and Southeast Asian countries are necessarily filled.

4.2. Transformation and degradation of antibiotics

Antibiotics from their primary sources (e.g., untreated sewage and wastewater or surface runoff) and secondary sources (e.g., WWTP

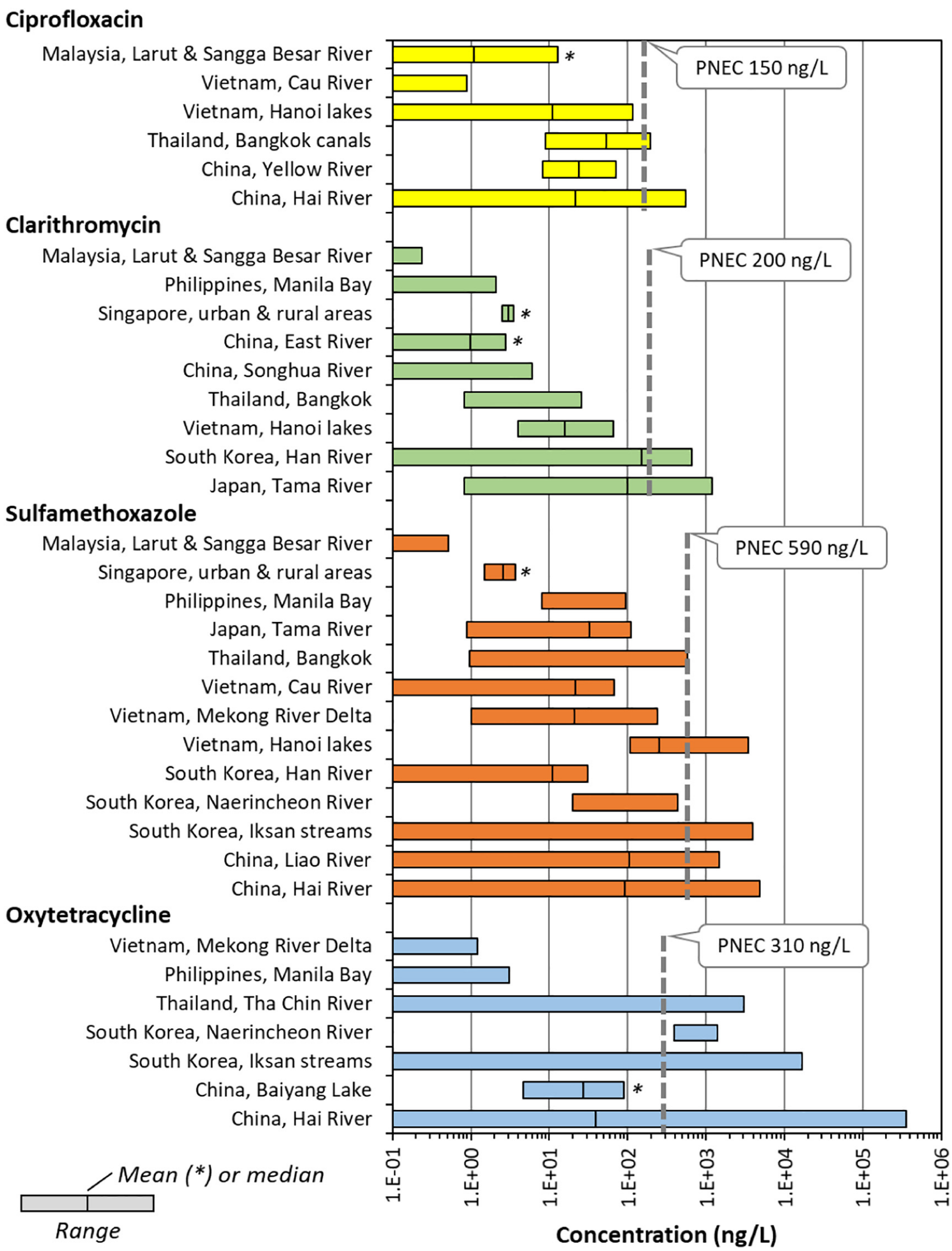

Fig. 2. Comparison of concentrations of selected antibiotics in surface waters from East and Southeast Asian countries. Data were retrieved from: China (Bai et al., 2014; Cheng et al., 2014; S. Zhao et al., 2016; Wang et al., 2017; Zhang et al., 2017; Chen et al., 2018), Japan (Mano and Okamoto, 2016), South Korea (Ok et al., 2011; Kim et al., 2019; Im et al., 2020), Vietnam (Nguyen et al., 2015; Tran et al., 2019; Do et al., 2020; Ngo et al., 2020), Thailand (Tewari et al., 2013; Rico et al., 2014; Sinthuchai et al., 2016), Singapore (Yi et al., 2019), Philippines (Shimizu et al., 2013), and Malaysia (Low et al., 2020). The predicted no effect concentrations (PNEC) derived from ecotoxicity data in the literature (see Supplementary data for more details) were also included.

effluent) discharged into ambient water are involved in a series of partitioning (as described in Section 4.1 above) and degradation/transformation processes (Kümmerer, 2009a; Carvalho and Santos, 2016; Danner et al., 2019). Antibiotic decay can be regulated by biotic (i.e., biodegradation) and/or abiotic (e.g., photolysis and hydrolysis) activities (Kümmerer, 2009a). Seasonal monitoring studies have indicated significant decline in concentrations of many antibiotics during hot seasons due to photodegradation and strong microbial activities. However, studies on degradation products of antibiotics in environmental water from the East and Southeast Asian countries remain scarce. Sy et al. (2017) determined the occurrence of 2-hydroxy-3-phenylpyrazine (HPP), a degradation product of BLs such as AMP and CEP, in surface water of two major deltas in Vietnam. HPP was found in 60 out of 98 river and pond water samples at concentrations ranging from 1.3 to 413.3 ng/L, reflecting readily degradable nature of BLs and the role of degradation products as indicators of their parent compounds in monitoring studies (Sy et al., 2017). Li et al. (2008) detected high concentrations of OTC metabolites such as 4-epi-OTC (11,800–34,200 ng/L), α-apo-OTC (5130–12,900 ng/L), and β-apo-OTC (1780–15,100 ng/L) in Xiao River water, although levels of these metabolites were <5% of OTC. In another study conducted in the Meijiang River in China, several degradation products of TET and OTC were detected, showing significant degradation rate of 35% and 98%, respectively (Dong et al., 2019). For example, concentration of OTC in these samples was about 0.21 ng/L, whereas concentration as high as 18.0 ng/L was found for α-apo-OTC (Dong et al., 2019). Additional field studies about the existence of antibiotic degradation/transformation products are needed because their environmental abundance and/or toxicity may surpass those of their parent compounds (Li et al., 2008; Sy et al., 2017; Dong et al., 2019).

4.3. Seasonal variations

Concentrations and profiles of antibiotics in specific areas may change significantly during different sampling periods. Such seasonal variation phenomena mainly depend on rainfall amounts and flow conditions (e.g., high flows in rainy season and low flows in dry season), temperature of ambient water and its effect on aquatic microbial activities, solar radiation intensity and sunshine duration, the nature of compounds, and seasonal usage patterns of antibiotics. Several studies have indicated that higher water concentrations of many antibiotics were found in dry and cold seasons than in wet and hot seasons (Tewari et al., 2013; Awad et al., 2014; Dong et al., 2016; S. Zhao et al., 2016; Li et al., 2018b; Kim et al., 2019; Im et al., 2020; Ngo et al., 2020). Wet and hot seasons (e.g., summer) have higher rainfall, ambient temperature, and sunlight intensity, which may decrease antibiotic concentrations by dilution, biodegradation, and photodegradation, respectively. Similarly, Wang et al. (2017) found higher levels of antibiotics in water samples from Songhua River, northeastern China during icebound season than non-icebound seasons due to lower flow and surface runoff, cold temperature, and the role of snow and ice cover on the river surface. In contrast, higher detection frequency and concentrations of some antibiotics were recorded in surface water in wet seasons elsewhere (Rico et al., 2014; Yu et al., 2019). These observations can be explained by the increasing of surface runoff, desorption and resuspension from sediment due to high flow conditions, and higher water turbidity that may implement antibiotic stability. In addition, the roles of local and seasonal usage patterns of antibiotics have also been emphasized, implying that residues of some antibiotics were higher in seasons when they were used intensively (Rico et al., 2014; Dong et al., 2016; S. Zhao et al., 2016; Li et al., 2018b; Yu et al., 2019; Im et al., 2020; Ngo et al., 2020).

5. Pollution sources of antibiotics

The pollution sources of antibiotics in the aquatic environments of East and Southeast Asia are, as anywhere, quite complex and can be classified into five main groups: (1) effluent from hospitals and pharmaceutical companies; (2) effluent from aquaculture and livestock farms; (3) untreated municipal sewage; (4) effluent from WWTPs, and (5) landfill leachates. Concentrations and profiles of antibiotics in their sources from some East and Southeast Asian countries are summarized in Table 2. In general, concentrations of individual antibiotics varied greatly from few ng/L to mg/L. It is obvious that concentration levels and compositional profiles of antibiotics in their sources differ significantly between studies due to the discrepancy in source types, characteristics of input and output flows, treatment technologies in investigated facilities, and study methods (e.g., lists of target compounds, sampling strategies, and analytical performance). As discussed in Section 3.7, median concentrations of individual antibiotics in receiving water mostly distributed in a range of 10 to 100 ng/L. However, markedly higher median values were found for antibiotics in their sources (e.g., 1000 to 10,000 ng/L). The decline of antibiotic levels from point sources to ambient water can be explained by natural remediation activities such as hydrolysis, photolysis, microbiolysis, and deposition to sediment (see details in Section 4). Further discussions for each type of antibiotic sources are provided below.

5.1. Effluent from hospitals and pharmaceutical companies

Hospital wastewater is considered as significant pollution sources due to its complex composition that contains several toxic components including coliforms, pathogens, heavy metals, radioelements, and chemical and pharmaceutical residues (Oliveira et al., 2018; Khan et al., 2020). Wastewater from several hospitals and health care facilities in big cities in Vietnam such as Hanoi and Ho Chi Minh City contained elevated concentrations of CIP, NOR, OFL, ERY, SMX, and TMP with median or mean values from about 1000 to 34,300 ng/L (Duong et al., 2008; Vo et al., 2016; Thai et al., 2018). The occurrence of many human antibiotics (e.g., CEZ, CEP, OFL, ROX, LOM, NOR, SMX, and TMP) at μg/L levels was also reported for hospital wastewater in China (Lin et al., 2008; Chang et al., 2010). Concentrations of some major antibiotics and their metabolites (e.g., ERY-H_2O, TET, CIP, OFL, and TMP) in raw wastewater from a hospital in Perak, Malaysia ranged from 66.9 to 1262 ng/L (Low et al., 2020), lower than those measured in Vietnam and China. The concentration ranges of antibiotics found in hospital wastewater from these countries were comparable to or still lower than levels documented for similar sources in India (Diwan et al., 2009, 2010) and some European countries (Ohlsen et al., 2003; Lindberg et al., 2004). Except for some extremely high concentrations of SMX and TMP (up to 100–250 μg/L) that were detected in few wastewater samples from one pharmaceutical manufacturer in Hanoi, Vietnam, median values of most antibiotics in pharmaceutical factories' effluent fell in the range of 100 to 1000 ng/L (Lin et al., 2008; Thai et al., 2018). The dominance of some FQs, SFs, and TMP in wastewater from hospitals and drug companies is likely due to their high production rate and/or environmental persistence (Chang et al., 2010; Felis et al., 2020).

5.2. Effluent from aquaculture and livestock production

The abundance of antibiotics in wastewater from aquaculture (e.g., fish and shrimp) and livestock (e.g., pig and poultry) production has been widely reported in Vietnam, Thailand, and China (Table 2). Concentrations of some antibiotics such as TMP, SMX, NOR, and oxolinic acid in a range of 10 μg/L to above 6 mg/L were found in water samples from shrimp ponds and surrounding canals in four Vietnamese mangrove areas (Le and Munekage, 2004). These levels were several orders of magnitude higher than those measured in other areas in Vietnam (Takasu et al., 2011; Shimizu et al., 2013) and Thailand (Rico et al., 2014; Takasu et al., 2011; Shimizu et al., 2013). For wastewater from pig and poultry farms, concentrations of antibiotics were found to vary in a wide range from few ng/L to about 170 μg/L (Lin et al., 2008; Takasu et al., 2011; Jiang et al., 2013; Shimizu et al., 2013; Shi et al., 2020). In livestock wastewater, predominant compounds detected were LIN and OTC (Lin et al., 2008; Shimizu et al., 2013; Shi et al., 2020), together with considerable levels of compounds from other classes such as FQs and SFs (Takasu et al., 2011; Jiang et al., 2013; Shimizu et al., 2013). Some compounds such as OTC, ENR, CIP, OFL, and ERY-H_2O were observed in wastewater from a zoo in Perak, Malaysia, however, with quite low concentrations (<MDL–109 ng/L; Low et al., 2020). Negative impacts on receiving water of antibiotic residues from aquaculture and livestock sectors have been demonstrated by many local studies conducted in Vietnam (Le and Munekage, 2004), Thailand (Rico et al., 2014), China (Jiang et al., 2013; Selvam et al., 2017), and South Korea (Ok et al., 2011; Awad et al., 2014; Kim et al., 2019).

Table 2
Concentrations (ng/L) and profiles of antibiotics in their sources from East and Southeast Asian countries.

Source type	Country	Sampling sites	Concentrations (ng/L) and profiles of antibiotics	References
Hospital wastewater	Vietnam	Hanoi, 6 hospitals	Concentrations of CIP and NOR in untreated wastewater from six hospitals ranged from 1100 to 44,000 and 900 to 17,000, respectively.	Duong et al., 2008
		Hanoi, 1 hospital	Total antibiotic concentrations ranged from 26,280 to 83,790 (median 50,090) with major compounds as CIP (median 34,300), OFL (9440), SMX (2910), and TMP (1010).	Thai et al., 2018
		Ho Chi Minh City, 39 health care facilities	Average concentrations of antibiotics in raw wastewater were 10,900 ± 8100 (OFL), 9600 ± 9800 (NOR), 5300 ± 4800 (CIP), 2500 ± 1900 (SMX), 1200 ± 1200 (ERY), and 1000 ± 900 (TMP).	Vo et al., 2016
	China	Chongqing, 4 hospitals	OFL was the most predominant antibiotic (1600–4240). Other antibiotics with concentrations up to >1000 were ROX, LOM, NOR, and SMX.	Chang et al., 2010
		6 hospitals	Median concentrations of the major antibiotics were 6221 (CEZ), 2457 (CEP), 1591 (metronidazole), 1088 (OFL), 1040 (TMP), 721 (CLA), 676 (ERY-H_2O)[a], and 647 (SMX).	Lin et al., 2008
	Malaysia	Perak, 1 hospital	Concentration ranges of major antibiotics in raw wastewater were 394–1262 (ERY-H_2O), 66.9–1092 (TET), 388–578 (CIP), 93.9–253 (OFL), and 75.6–93.1 (TMP).	Low et al., 2020
Pharmaceutical company wastewater	Vietnam	Hanoi, 4 companies	Total antibiotic concentrations ranged from <MDL[b] to 348,900 (median 11,220) with major compounds as OFL (median 1370), TMP (765), SMX (320), and CIP (257).	Thai et al., 2018
	China	3 companies	Median concentrations of the major antibiotics were 853 (OFL), 396 (CIP), 107 (ERY-H_2O), and 87 (CLA).	Lin et al., 2008
Aquaculture wastewater	Vietnam	Mangrove areas, 16 shrimp ponds	Concentration ranges of antibiotics in shrimp ponds and surrounding canals were 80,000–2,030,000 (TMP); 40,000–5,570,000 (SMX); 60,000–6,060,000 (NOR); and 10,000–2,500,000 (oxolinic acid).	Le and Munekage, 2004
		Hanoi, shrimp ponds	Total antibiotics ranged from <MDL to 999 with frequently detected compounds as SMX, OTC, and ERY.	Shimizu et al., 2013
		Coastal shrimp ponds	Concentrations of OFL ranged from <MQL[c] to 190 ± 48.6. Other antibiotics like NOR, CIP, and LOM were less frequently quantified.	Takasu et al., 2011
	Thailand	Tha Chin River, tilapia farm	Average concentrations of OTC inside the cages after 15 min, 1 h, and 15 h after antibiotic administration were 37,000; 400; and <MDL, respectively.	Rico et al., 2014
		Khon Kaen, shrimp ponds	Total antibiotics ranged from 6 to 187 with frequently detected compounds as OTC and ERY.	Shimizu et al., 2013
		Khon Kaen, shrimp and tilapia ponds	NOR (16.2 ± 17.6–287 ± 15.2) and LOM (<MQL–179 ± 137) were found at higher frequency and concentrations than OFL and CIP.	Takasu et al., 2011
	China	Aquacultures	Median concentrations of the major antibiotics were 229 (SMX), 85 (TMP), and 70 (LIN).	Lin et al., 2008
Livestock wastewater	Thailand	Khon Kaen, pig farms	Total antibiotics ranged from 6151 to 180,678 (average 57,371) with major compounds as LIN (47,345; 77–174,759), OTC (3172; <MDL–6290), and SMT (1260; 62–4605).	Shimizu et al., 2013
		Khon Kaen, pig farms	Concentrations of OFL (91.5 ± 70.1–2560 ± 736), CIP (46.2 ± 20.5–1050 ± 706), and NOR (83.9 ± 12.8–465 ± 92.3) were higher than LOM (<MQL–143 ± 47.3).	Takasu et al., 2011
	China	Longyan City, pig farms	Total concentrations of TCs and SFs (including their metabolites) were 1450–10,590 and 180–78,100, respectively.	Jiang et al., 2013
		Chongqing, pig and poultry farms	Total antibiotics of raw sewage from pig farms and poultry farms were 10,219–39,195 and 1419–36,027, respectively. Major compounds were LIN, LOM, sulfanilamide, NOR, OFL, SMT, and CIP.	Shi et al., 2020
		Animal husbandries	Median concentrations of the major antibiotics were 56,760 (LIN); 8318 (OTC), 4844 (STZ), 2821 (CTC), 1129 (TET), and 1001 (TYL).	Lin et al., 2008
	Vietnam	Hanoi, Ho Chi Minh City, Can Tho, livestock farms	Total antibiotics ranged from 6 to 19,465 (average 3587) with major compounds as SMT (3087; <MDL–19,153) and SMX (388; <MDL–2715).	Shimizu et al., 2013
Urban sewage	Vietnam	Hanoi, urban canals	Concentrations (median and range) of major compounds were SMX (7631; 310–15,591); ERY (5542; <MQL–48,517); AMO (3162; <MQL–20,608); and LIN (952; 67–1968).	Tran et al., 2019
		Hanoi, Ho Chi Minh City, Can Tho, canals	Total antibiotics ranged from 306 to 9343 (average 3220) with major compounds as SMX (1718; 103–4330), LIN (1053; 132–2666); and ERY (550; 28–2246).	Shimizu et al., 2013
	China	Regional discharges	Median concentrations of the major antibiotics were 5892 (CEZ), 1065 (OFL), 803 (TMP), 705 (ERY-H_2O), 610 (CEP), and 515 (CLA).	Lin et al., 2008
		Guangzhou, urban rivers	Major compounds were NOR (median 1000; range <MDL–2702); CIP (242; <MDL–415); SMT (33.6; <MDL–331); and SPY (26.2; <MDL–60.2).	Huang et al., 2019
	Philippines	Manila, urban canals	Total antibiotics ranged from 1160 to 2000 (average 1576) with major compounds as SMX (802; 580–1066), ERY (332; 246–453); and TMP (258; 169–369).	Shimizu et al., 2013
	Indonesia	Jakarta, urban canals	Total antibiotics ranged from 17 to 1489 (average 607) with major compounds as SMX (282; 11–779), LIN (185; <MDL–399); ERY (62; 6–133); and TMP (59; 2–141).	Shimizu et al., 2013
	Malaysia	Kuala Lumpur, urban canals	Total antibiotics ranged from 46 to 334 (average 259) with major compounds as ERY (129; 29–166), SMX (76; 14–102); and TMP (29; 1.2–38.3).	Shimizu et al., 2013
WWTP effluent	South Korea	24 WWTPs of 4 categories	Concentrations of total pharmaceuticals (including antibiotics) ranged from 51 to 44,128,000. The most predominant antibiotic was LIN (240–43,909,000).	Sim et al., 2011
		5 livestock WWTPs	Concentrations (mean and range) of antibiotics were: STZ (28,200; <MDL–159,000); SMT (20,820; <MDL–115,000), CTC (11,470; 730–33,520); and OTC (<MDL).	Kim et al., 2020
	Vietnam	Hanoi, an urban hospital WWTP	Average concentrations in wastewater after treatment were 14,800 (metronidazole), 7900 (CIP), 5000 (OFL), 3000 (SMX), 1300 (TMP), and 400 (spiramycin).	Lien et al., 2016
		Hanoi, a rural hospital WWTP	Average concentrations in wastewater after treatment were 21,500 (CIP), 6400 (SMX), 2100 (OFL), 1500 (TMP), 1400 (metronidazole), and 500 (spiramycin).	Lien et al., 2016
		Hanoi, Huu Nghi Hospital WWTP	Average concentrations in wastewater after treatment were 3700 ± 1300 (CIP) and 1500 ± 300 (NOR).	Duong et al., 2008
	China	Sewage treatment plants	Median concentrations of the major antibiotics were 695 (ERY-H_2O), 321 (TMP), 283 (CEP), 226 (SMX), 178 (nalidixic acid), and 172 (CLA).	Lin et al., 2008
		Chongqing, WWTPs	Concentration ranges of antibiotics were 6.52–246 (SFs), 89.8–249 (SF metabolites), 25.3–333 (FQs), 13.1–172 (NOR), 11.9–91.5 (OFL).	Jiang et al., 2013
		Chongqing, municipal	Mean concentrations of individual antibiotics ranged from 1.24 to 516 with major	Shi et al., 2020

(continued on next page)

Table 2 (*continued*)

Source type	Country	Sampling sites	Concentrations (ng/L) and profiles of antibiotics	References
		WWTPs	compounds as marbofloxacin, climbazole, ENR, CIP, NOR, and OFL.	
		Beijing, municipal WWTP	Secondary effluent: 1869 (FQs) > 1053 (SFs) > 353 (MLs). Tertiary effluent: 123 (FQs) > 25.9 (SFs) > 24.7 (MLs). Major compounds were OFL, NOR, ERY, SMX, SDZ, and ROX.	Li et al., 2013
	Thailand	Bangkok, hospital WWTPs	Concentrations (mean and range) were 1.50 (<MDL–5.99) for CEP to 420 (27.0–1499) for SMX. Major compounds were SMX, CIP, CLA, and metronidazole.	Sinthuchai et al., 2016
		Bangkok, WWTPs	Levels (mean and range) were 14.8 (<MDL–85.4) for ERY to 50.4 (5.9–173) for ROX.	Li et al., 2012
		Bangkok, WWTPs	Concentration ranges were 2.4–25 for TMP to 12–231 for CIP.	Tewari et al., 2013
	Singapore	WWTP	Major antibiotics in secondary effluent (activated sludge system): CTC (1757; 1472–1986); OTC (1469; 840–2014); TET (766; 691–1536); CLA (532; 387–637); CIP (495; 321–524). Major antibiotics in microfiltration permeate (membrane bioreactor system): CTC (807; 505–1732); CLA (425; 159–635); OTC (387; 335–1069); SMX (336; 290–562); CIP (333; 5–421).	Tran et al., 2016
Landfill leachates	China	Shanghai, municipal landfill sites	Concentration ranges of major antibiotics were: ERY (1276–39,800); OFL (205–12,311); SMX (6.4–8488); NOR (<MDL–5319); and CIP (60.2–4482).	Wu et al., 2015
		Chongqing, municipal landfill sites	Median concentrations of major antibiotics were 13,403 (danofloxacin), 7997 (pefloxacin), 5455 (NOR), and 3436 (marbofloxacin).	Shi et al., 2020
		Zhejiang, municipal landfill sites	Concentration ranges of major antibiotics were: OTC (<MDL–21,700); SMX (<MDL–4030); and ERY (<MDL–1330).	Fang et al., 2020
		3 landfill sites	Concentrations ranged from 9 to 3410 and decreased in order: CIP > ERY > CEP > TMP > SMX > ROX > CHP.	Chung et al., 2018
	Singapore	Closed landfill site	Concentrations of most antibiotics were <MQL, except for SMT (339; 62–438) and LIN (10.7; <MQL–23.7).	Yi et al., 2017

[a] ERY-H_2O refers to dehydrated ERY, a major metabolite of ERY.
[b] Concentrations lower than method detection limits.
[c] Concentrations lower than method quantification limits.

5.3. Untreated municipal sewage

Drainage or combined sewer systems receive discharges from households and commercial sectors as well as surface runoff (e.g., stormwater and snowmelt) and transfer them to WWTPs; however, serious pollution can occur during heavy storm events in which excess stormwater and sewage enter receiving water due to limited hydraulic capacity of sewers and WWTPs (Krejci, 1996; Muller et al., 2020). The occurrence of antibiotics in sewage from various East and Southeast Asian countries summarized in Table 2 showed a wide concentration range of individual antibiotics from few ng/L to nearly 50 μg/L. The available data indicated that levels of antibiotics in urban canals and rivers in Vietnam (Shimizu et al., 2013; Tran et al., 2019) and China (Lin et al., 2008; Huang et al., 2019) were generally higher than those observed in other countries like Philippines, Indonesia, and Malaysia (Shimizu et al., 2013). Antibiotics that were frequently detected at high concentrations in sewage of these countries include SMX, LIN, ERY, TMP, and other FQs, SFs, and MLs to some extent. BLs have not been usually monitored in surface water due to their low stability and undetectable concentrations; however, AMO and some cephalosporin antibiotics like CEZ and CEP were found at μg/L levels in drainage water in urban areas elsewhere (Lin et al., 2008; Tran et al., 2019), providing evidence for their substantial usage and continuous discharges. Concentrations of some antibiotics such as SMX and ERY in Vietnamese sewage may exhibit an increasing trend over time (Shimizu et al., 2013; Tran et al., 2019).

5.4. Wastewater treatment plant effluent

Many studies have investigated the antibiotic removal efficiency of different types of hospital (H), pharmaceutical manufacturing (P), livestock (L), and municipal (M) WWTPs in South Korea, China, Vietnam, Thailand, and Singapore over the last decade (Table 2). These studies usually reported concentrations of antibiotics in influents and effluent, however, only information on effluent concentrations was summarized here due to their direct impacts on receiving water. Concentrations of individual antibiotics varied greatly from few ng/L to 44 mg/L. Sim et al. (2011) reported that LIN was found as major compounds in WWTP effluent with maximum concentration reaching 44 mg/L and median values from 2.43 μg/L (M-WWTPs) to 568 μg/L (P-WWTPs). Concentrations of STZ, SMT, and CTC as high as 159, 115, and 33.5 μg/L, respectively were measured in effluent of L-WWTPs (Kim et al., 2020). Concentrations of antibiotics in the range of 400 to 21,500 ng/L were reported for H-WWTP effluent in Hanoi, Vietnam (Duong et al., 2008; Lien et al., 2016), which were much higher than those measured in H-WWTPs in Bangkok, Thailand (about 1–1500 ng/L; Sinthuchai et al., 2016). Various antibiotics of multiple classes were also frequently detected at concentrations from 1 to <2000 ng/L in M-WWTP effluent in China (Lin et al., 2008; Jiang et al., 2013; Li et al., 2013), Thailand (Li et al., 2012; Tewari et al., 2013), and Singapore (Tran et al., 2016). Concentrations and patterns of antibiotics in effluent differ between studies, locations, and plants, as a result of differences in input flows and treatment technologies and the fact that removal pathways and efficiencies are compound-specific. Previous studies revealed that advanced treatment technology such as membrane bioreactors exhibited higher antibiotic elimination performance than conventional activated sludge processes (Tran et al., 2016; Kim et al., 2020).

5.5. Landfill leachates

So far, little is known about release behavior and environmental loads of antibiotics from landfill sites in most East and Southeast Asian countries. Concentrations of individual antibiotics in leachates of several municipal solid waste landfill sites in China fell in the range of few ng/L to nearly 40 μg/L (Wu et al., 2015; Chung et al., 2018; Fang et al., 2020; Shi et al., 2020). According to Fang et al. (2020), concentrations of antibiotics in fresh leachates were higher than in aged and middle-aged leachates. Concentrations of most antibiotics in leachates from a 16-year old closed landfill site in Singapore were lower than the method quantification limits, except for SMT (median 339; range 62–438 ng/L) and LIN (10.7; <MQL–23.7) (Yi et al., 2017), which were markedly lower than antibiotic levels in Chinese landfill leachates. Levels and profiles of antibiotics in raw leachates depend on disposal activities, waste types and compositions, landfill matrix, seasonal factors, and variations in properties and fate of specific compounds (Wu et al., 2015; Yi et al., 2017; Chung et al., 2018; Fang et al., 2020; Shi et al., 2020). The need for appropriate treatment methods aimed at reducing antibiotic residues in landfill leachates (e.g., aerobic biological treatment or constructed wetlands) has been highlighted (Yi et al., 2017; Chung et al., 2018).

6. Potential risks of antibiotics in water environments

6.1. Ecological risks

The occurrence of antibiotics and their degradation products in the aquatic ecosystems can pose a threat to non-target organisms of different trophic levels including bacteria, algae, plants, invertebrates, and fish (Carvalho and Santos, 2016; Danner et al., 2019; Kumar et al., 2019; Felis et al., 2020). Most of the available studies on antibiotic contamination of surface water included ecological risk assessments using risk quotient (RQ) schemes. RQs of an antibiotic in water to certain species are estimated by dividing measured environmental concentrations (MEC) by predicted no effect concentrations (PNEC). The PNEC values are based on toxicological data, for example, acute half maximal effective concentrations (EC_{50}) or half maximal lethal concentrations (LC_{50}) from toxicological testing, and uncertainty or safety assessment factors (e.g., 100, or 1000) (see Supplementary data for more details). Levels of risk are generally classified into four groups: no risk ($RQ < 0.01$), low risk ($0.01 \leq RQ < 0.1$), medium risk ($0.1 \leq RQ \leq 1$), and high risk ($RQ > 1$) (Hernando et al., 2006; Verlicchi et al., 2012; Rodiguez-Mozaz et al., 2020). In general, FQs, MLs, and SFs exhibited higher ecotoxicity than BLs and TCs (Liu et al., 2018; Felis et al., 2020); and cyanobacteria and algae are most sensitive to antibiotics among organisms of different trophic levels (W. Zhao et al., 2016; Felis et al., 2020; Kovalakova et al., 2020). Significant ecological risks (e.g., inhibition of algae and cyanobacteria growth) of some antibiotics such as OFL, CIP, ENR, ERY, SMX, and SMT have been widely reported elsewhere in China (Dong et al., 2016; W. Zhao et al., 2016; Deng et al., 2018; Liu et al., 2018), South Korea (Kim et al., 2019), Malaysia (Low et al., 2020), and Vietnam (Tran et al., 2019; Ngo et al., 2020). In certain areas, significant risks from BLs (e.g., AMP and AMO) and TCs (e.g., TET and OTC) were also estimated with the maximum RQs reaching 10^1 to 10^3 (Rico et al., 2014; Kim et al., 2019; Tran et al., 2019; Low et al., 2020). Few studies evaluated the toxicity of antibiotic mixtures in field water samples by using toxicity tests. Mano and Okamoto (2016) conducted 72-h algal growth inhibition tests by exposing *Raphidocelis subcapitata* to extracts from Tama River water. The results exhibited weak toxicity of river water to algae (largely due to low contamination degree) with significant contribution of CLA, AZI, and triclosan (an antibacterial agent) (Mano and Okamoto, 2016). Clearly, further toxicity tests on a range of species/communities that are exposed to a cocktail of multiple antibiotics are needed, especially for heavily contaminated surface water. Overall, elevated antibiotic residues may negatively alter the balance of aquatic ecosystems through deleterious effects on natural microbial communities (in particular, beneficial microorganisms) and on algae, the primary producers of aquatic food webs (Kümmerer, 2009a; Carvalho and Santos, 2016; Binh et al., 2018; Felis et al., 2020; Kovalakova et al., 2020).

6.2. Risks of antibiotic resistance development

Antibiotic residues in the environment are related to the development of antibiotic resistance genes (ARG) and antibiotic-resistant bacteria (ARB), which have been considered as the most critical risks regarding these micro-pollutants (Kümmerer, 2009b; Kumar et al., 2019; Felis et al., 2020). Due to high consumption demands and improper use of antibiotics as well as inadequate control and abatement technology of antibiotic-containing wastes, serious concern about the prevalence of ARG and ARB has been raised in most East and Southeast Asian countries (Lundborg and Tamhankar, 2017; Zellweger et al., 2017; Qiao et al., 2018). In this review, we focus on the occurrence of ARG and ARB in surface water and wastewater from various countries in the two Asian regions (Table 3). The detection of ARG and ARB encoding different antibiotic classes such as BLs, SFs, FQs, MLs, TCs, etc., as well as multidrug resistance, in wastewater and receiving water of various aquatic systems (e.g., seas, rivers, lakes, ponds, and canals) was reported in China, Japan, South Korea, Singapore, Thailand, and Vietnam. Insufficiently treated hospital wastewater, livestock discharge, and combined sewer overflows and stormwater in highly populated areas have been considered as major sources of ARG and ARB in these countries (Takasu et al., 2011; Ham et al., 2012; Rho et al., 2012; Boonyasiri et al., 2014; Le et al., 2016; Haller et al., 2018; Hwang and Kim, 2018; Liu et al., 2019). This situation suggests the need for appropriate wastewater management and treatment schemes in order to control and reduce the abundance and risks of antibiotic resistance in their sources, before these harmful components can enter receiving water. Previous studies have indicated that the prevalence and diversity of ARG and ARB in surface water are regulated by multiple factors including water properties (e.g., temperature, pH, SPM, etc.), weather conditions (i.e., seasonal variations), and especially chemical pollution from anthropogenic activities (e.g., heavy metals and antibiotic residues) (Hoa et al., 2011; Ham et al., 2012; Chen et al., 2020; W. Li et al., 2020). The ecological and public health risks related to ARG and ARB in environmental water have also been discussed elsewhere and will not be treated here (Ham et al., 2012; Boonyasiri et al., 2014; Hwang and Kim, 2018; Liu et al., 2019).

7. Future perspectives

7.1. Academic and technical aspects

As described above, there are many information gaps about the occurrence, distribution, and risks of antibiotics in surface water from East and Southeast Asia. To our knowledge, so far, available data on concentrations and patterns of antibiotics in surface water and in pollution sources for several countries in these two regions (e.g., Mongolia, North Korea, Brunei, Cambodia, Laos, Myanmar, and Timor-Leste) are still limited. Furthermore, except for China where a huge number of studies have been conducted over the last decade, additional investigations across broadened geographical scales, with larger target lists of antibiotics, and adequate detection of pollution sources are necessary in the remaining countries. In addition, in-depth studies about phase distribution (i.e., water-particle, particle-sediment, and water-sediment equilibrium), long-term temporal trend analysis, determination of antibiotic degradants, and ecotoxicity of antibiotic mixtures in environmental matrices are needed. The establishment of national, regional, or inter-regional monitoring networks for antibiotics and antibiotic resistance in the aquatic environment is highly recommended. The designation, construction, and operation of proper sewer systems, especially in big cities, plays an important role in development of infrastructure in general, and in the control of municipal wastewater that contains a variety of micro-pollutants (including antibiotics) and harmful microbes (e.g., ARB) in particular. Concerns about negative effects of antibiotics and/or antibiotic resistance in sewage have been raised in metropolitan areas of not only developing countries like China, Vietnam, and Thailand, but also developed countries like Japan (Takasu et al., 2011; Ham et al., 2012; Boonyasiri et al., 2014; Huang et al., 2019; Tran et al., 2019). The improvement of wastewater treatment efficiency with advanced technologies, strict monitoring of effluent quality, and timely detection of untreated discharges from commercial and industrial sectors can effectively help to prevent serious pollution incidents and long-term toxic impacts. Such research efforts have also been underlined for other developed countries (Carvalho and Santos, 2016; Felis et al., 2020; Kovalakova et al., 2020; Rodiguez-Mozaz et al., 2020).

7.2. Management and educational aspects

The management of antibiotics and their associated impacts on human and environmental health in each country or region are generally overseen by governmental agencies responsible for medicine and health, agriculture, environment, etc., and by advisory panels of professional authorities like the World Health Organization (WHO), Food and Agriculture Organization of the United Nations (FAO), and United Nations Environment Programme (UNEP), etc. The WHO published

Table 3
Antibiotic resistance genes and antibiotic-resistant bacteria in surface water and wastewater from East and Southeast Asian countries.

Country	Location	Matrix	Observations of antibiotic resistance	References
China	Beijing, Wenyu River, WWTPs, and hospital	River water, influent, effluent	9 ARG encoding SFs, TCs, FQs, and MLs detected with relative abundance decreased by *sul*1 (1.77×10^{-3}–1.18×10^{-1}), *sul*2 (6.04×10^{-5}–7.11×10^{-2}), *erm*B (2.53×10^{-5}–4.19×10^{-2}), *tet*W (1.76×10^{-5}–1.23×10^{-2}), *tet*A (6.46×10^{-5}–1.08×10^{-2}), *tet*C (1.34×10^{-4}–5.35×10^{-3}), *tet*M (9.83×10^{-5}–7.97×10^{-3}), *qnr*S (1.67×10^{-6}–9.25×10^{-4}), and *tet*B (4.31×10^{-7}–3.92×10^{-4})	Liu et al., 2019
	Yitong River	River water	ARB resistant to TET, TMP, and sulfanilamide isoxazole	Yu et al., 2019
	Lake Tai	Lake water	4 ARG detected with dominant *sul*1 and *bla*TEM genes with number of copies ranging from 4.8×10^5 to 1.5×10^8 copies/mL	Stange et al., 2019
	Qingcaosha Reservoir	Drinking source water	12 ARG detected with dominant *sul*1, *sul*2, *tet*C, *bla*TEM-1, *mph*A, *str*A, and *qnr*S genes at 1.12×10^3 to 1.01×10^9 copies/L	Xu et al., 2020
	Yangtze River Estuary	Estuary water	ARG including *sul*1, *sul*2, *sul*3, *tet*A, *tet*W and *aac*(6′)-Ib genes in the range of 3.41×10^{-4} to 2.79×10^{-2} copies/16S rRNA gene[a]	Chen et al., 2020
	Shenzhen, Dapeng Cove	Seawater	10 ARG detected (total 1.27×10^2 to 1.26×10^6 copies/mL) with dominant *flo*R (7.84×10^5), *sul*1 (3.82×10^5), and *cml*A (2.04×10^5) genes	W. Li et al., 2020
Japan	Tokyo, Tama River	River water	Antibiotic-resistant and multidrug-resistant *E. coli* to TMP/SMX, AMP, TET, etc., and their combinations, concentrations ranging from 2 to 195 (mean 8) cfu[b]/100 mL	Ham et al., 2012
	Miyazaki, Yae River	River water	Enterococci resistant to ERY, TET, CIP, etc., and their combinations; vancomycin-resistant genes (*van*C1 and *van*C2/C3)	Nishiyama et al., 2017
South Korea	Seoul, hospital	Hospital effluent	Meropenem- and multidrug-resistant bacteria detected in effluent of a hospital at concentrations of 8 ± 3 cfu/mL	Hwang and Kim, 2018
	South Jeolla and Jeju, olive flounder farms	Untreated farm effluent	ARG encoding TCs, SFs, FQs, BLs, florfenicol, and multidrug resistance, with the dominance of TC-resistant genes (*tet*B and *tet*D). Total ARG number ranged from 4.24×10^{-3} to 1.46×10^{-2} copies/16S rRNA gene.	Jang et al., 2018
	Daejeon, animal farms and fish markets	Contaminated water	ARB resistant to CEZ (22.5×10^0–11.3×10^5), TET (0–17.5×10^5), gentamycin (10.0×10^0–12.5×10^5), NOR (55.0×10^0–92.5×10^0), ERY (16.5×10^1–50.1×10^4), vancomycin (13.5×10^1–10.8×10^5), and multidrug resistance (5–27,090) cfu/mL	Rho et al., 2012
	Keum River, Nakdong River, Soyang Lake, Juam Reservoir	Surface water	PEN- (average 2700 ± 2100 cfu/mL) and TET-resistant bacteria (average 23 ± 7.4 cfu/mL) also resistant to other antibiotics like hygromycin, AMP, ERY, TMP, kanamycin, etc.	Kim et al., 2015
Singapore	Two major hospitals	Untreated wastewater	Cephalosporin- and carbapenem-resistant bacteria and multidrug resistance	Haller et al., 2018
	Two hospitals	Hospital effluent	ARB resistant to amikacin (average 1.06×10^6), clindamycin (1.37×10^6), ERY (1.24×10^6), CIP (1.14×10^6), TET (1.30×10^6), meropenem (4.79×10^5), ceftazidime (8.22×10^5), vancomycin (9.19×10^5), CHP (6.08×10^5), and co-trimoxazole (2.54×10^5) cfu/mL; 16 relevant ARG detected with dominant *sul* and *aac*(6′)-Ib genes	Le et al., 2016
	Coastal fish farms	Coastal farm water	ARB resistant to AMP, CEZ, ceftazidime, cefepime, TMP/SMX, etc., and multidrug resistance; 12 ARG detected with dominant *sul*2, *dfr*A, and *aac*(6′)*-Ib* genes	Ng et al., 2018
Thailand	Bangkok, urban canals	Canal water	ARB resistant to ceftriaxone, cefoxitin, imipenem, ertapenem, gentamycin, amikacin, nalidixic acid, and CIP	Boonyasiri et al., 2014
	Two provinces, animal farms	Stagnant water	ARB resistant to ceftriaxone, cefoxitin, gentamycin, nalidixic acid, colistin, and CIP	Boonyasiri et al., 2014
	Khon Kaen, hospital WWTPs	WWTP effluent	ARB resistant to OFL (<DL[c]–1.3×10^5), NOR (<DL–1.2×10^5), and CIP (<DL–1.2×10^5) cfu/mL	Takasu et al., 2011
Vietnam	Mangrove areas, shrimp ponds	Pond water	ARB resistant to NOR, TMP, SMX, and oxolinic acid	Le et al., 2005
	Hai Phong, coastal shrimp ponds	Pond water	ARB resistant to SMX (1.10×10^3–7.97×10^4) and ERY (20–600) cfu/mL	Hoa et al., 2011
	Hanoi, shrimp ponds and pig farms	Pond water	ARB resistant to NOR (1.1×10^3–4.1×10^4) and CIP (2.4×10^2–4.1×10^3) cfu/mL	Takasu et al., 2011
	Hanoi, pharmaceutical companies	Wastewater	ARB resistant to TMP/SMX, CIP, and NOR	Thai et al., 2018
	Hanoi, Huu Nghi Hospital	Wastewater	ARB resistant to CIP and NOR in raw wastewater	Duong et al., 2008
	Hanoi, urban canals	Canal water	ARB resistant to SMX (8.92×10^4–5.00×10^6) and ERY (2.80×10^4–7.00×10^5) cfu/mL	Hoa et al., 2011
	Hanoi, urban canals	Canal water	ARB resistant to NOR (3.7×10^3–7.4×10^3) and CIP (3.2×10^3–3.7×10^3) cfu/mL	Takasu et al., 2011

[a] 16S ribosomal ribonucleic acid gene.
[b] Colony forming unit.
[c] Lower than detection limits.

the "Global Action Plan on Antimicrobial Resistance" along with practical toolkit for implementing antimicrobial stewardship, heading towards better knowledge and practices on antibiotics (WHO, 2015, 2019). Several countries in East and Southeast Asia have accelerated their efforts to combat antibiotic abuse and misuse as well as antibiotic resistance (Kim et al., 2016; Goutard et al., 2017; Zellweger et al., 2017; Singh et al., 2019; Hu and Cowling, 2020). However, Kraemer et al. (2019) noted that such action plans and policies have failed to specifically address the issues of antibiotic pollution and antibiotic resistance evolution in the environment. There are still several gaps in current policies regarding these issues, for example, the missing of specific quality guidelines for antibiotic residues, ARG, and ARB in effluent from pharmaceutical manufacturing facilities and WWTPs (Kraemer et al., 2019). According to the German Environmental Agency, the full range of options for action on reducing the input of antibiotics and ARB into surrounding environments must cover multiple aspects such as prevention, communication, authorization, WWTPs, water and soil standards, and agriculture (German Environment Agency, 2018). Nevertheless, environmental regulations and comprehensive monitoring databases in many East and Southeast Asian countries are still lacking. In the near future, the proposal of environmental quality standards and technical standards for antibiotic residues, ARG, and ARB in receiving water, wastewater, and WWTP effluent is thus an urgent need. In addition, knowledge, awareness, and responsibility for reducing the environmental burden of antibiotics and antibiotic resistance of all social sectors including decision makers, governmental managers, scientists, engineers, doctors, pharmacists, entrepreneurs, and people who may use antibiotics, should be improved by suitable education and propaganda methods (Om and McLaws, 2016; Haenssgen et al., 2018; Kong et al., 2019; Singh et al., 2019).

8. Summary

The occurrence of antibiotics and antibiotic resistance in surface water and wastewater from East and Southeast Asia was reviewed to evaluate their spatial distribution, behavior and fate, pollution sources, and risks, and to address research gaps and future requirements. Antibiotics belonging to multiple classes that applied to treat both human and animal infection have been widely detected in receiving water and wastewater of these countries with individual concentrations varying from ng/L to mg/L levels. Concentrations and compositional profiles of antibiotics in the studied areas or enterprises varied greatly and were regulated by many factors such as antibiotic consumption rate and usage behavior, efficiency of wastewater treatment systems, meteorological conditions, and the nature of the compounds. Although the highest concentrations of many antibiotics were found in surface water and wastewater from some countries like China, South Korea, and Vietnam, it is difficult to depict a distinct spatial trend of antibiotic distribution in East and Southeast Asia due to the lack of comprehensive and synchronous monitoring and assessment studies, and to the fact that information about aquatic residues of antibiotics in various countries of these regions is still unknown. The ecological risks of antibiotics and the prevalence of ARG and ARB have been documented in several East and Southeast Asian countries, suggesting the need of effective legislation, management, and abatement systems regarding antibiotics and waste flows containing them. As a concluding remark, we would like to refer to the Nobel Lecture "Penicillin" of A. Fleming given in December 11, 1945 (Fleming, 1945). In that speech, Fleming mentioned two prospects and one piece of advice on the use of penicillin and now both of them have come true: "The time may come when penicillin can be bought by anyone in the shops"; "negligent use of penicillin changed the nature of the microbe"; and "If you use penicillin, use enough" (Fleming, 1945). Indeed, the implementation of proper antibiotic use and stewardship is a critically important task in East and Southeast Asian countries, home of the major antibiotic markets and consumers in the world, in order to maintain the effectiveness and to minimize the deleterious effects of these medicines on human and ecosystem health. In addition, inadequate collection and treatment sewage systems are responsible for the abundance of antibiotics and their resistance in water ecosystems, raising the need for improvement of wastewater treatment efficiency and strict control of illicit discharges, especially in developing countries.

Declaration of competing interest

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

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Appendix A. Supplementary data

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Review

A Review on the Use of Microalgae for Sustainable Aquaculture

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Abstract: Traditional aquaculture provides food for humans, but produces a large amount of wastewater, threatening global sustainability. The antibiotics abuse and the water replacement or treatment causes safety problems and increases the aquaculture cost. To overcome environmental and economic problems in the aquaculture industry, a lot of efforts have been devoted into the application of microalgae for wastewater remediation, biomass production, and water quality control. In this review, the systematic description of the technologies required for microalgae-assisted aquaculture and the recent progress were discussed. It deeply reviews the problems caused by the discharge of aquaculture wastewater and introduces the principles of microalgae-assisted aquaculture. Some interesting aspects, including nutrients assimilation mechanisms, algae cultivation systems (raceway pond and revolving algal biofilm), wastewater pretreatment, algal-bacterial cooperation, harvesting technologies (fungi-assisted harvesting and flotation), selection of algal species, and exploitation of value-added microalgae as aquaculture feed, were reviewed in this work. In view of the limitations of recent studies, to further reduce the negative effects of aquaculture wastewater on global sustainability, the future directions of microalgae-assisted aquaculture for industrial applications were suggested.

Keywords: aquaculture; microalgae; wastes treatment; resource utilization; sustainability

1. Introduction

In recent years, the depletion of wild fishery resource is driving the fast development of aquaculture worldwide [1]. In some countries, the farming of aquatic animals has surpassed the yield of wild fisheries [2]. It is expected that in the coming future, aquaculture will become the main industry providing aquatic products to human beings. However, with the continuous expansion of the scale of aquaculture and the increased production, water pollution has become a serious problem posing threats to the environmental protection and hindering the sustainable development of aquaculture [3,4]. Moreover, in aquaculture practice, with the water deterioration, high incidence of diseases would also increase the commercial risks of the whole industry [5].

To overcome the aforementioned problems, a lot of efforts were devoted into aquaculture to control wastewater pollution and improve survival efficiency of aquatic animals. The most common and straightforward method to control the pollution of aquaculture wastewater pollution is using traditional environmental remediation technologies, such as aeration, filtration, and anaerobic-anoxic-oxic (A^2O) system, to remove nutrients in wastewater [3,6,7]. In a real-world application, the treatment of wastewater by these technologies with high energy consumption or investment improves the total cost of aquaculture and increases the financial burden of industry [3,8]. By traditional technologies, nutrients, including nitrogen, phosphorus, and carbon, in wastewater could not be fully utilized and recycled as resources. Some technologies may produce a large amount of carbon dioxide and sludge, causing secondary environmental pollution [9]. In aquaculture, antibiotics and medicine are commonly used as a feasible way to prevent animals' diseases and reduce aquaculture risks. However, the overuse of antibiotics or medicine may negatively impact the meat quality of aquatic animals and cause food safety problems [10]. The accumulation of residual antibiotics or medicine in the water body may also become a trigger of antibiotics-resistance and even some ecological disasters [11]. Therefore, in recent years, interests in the development of economically feasible and environmentally friendly technologies to deal with the problems occurring in aquaculture are growing worldwide.

Microalgae, which could efficiently assimilate nutrients in a eutrophic water body, have been proven to be a good way for wastewater remediation [12,13]. The great performance of microalgae for nutrients assimilation has been widely observed in the remediation of food industry effluent, agricultural waste stream, municipal wastewater, and many other types of wastewater [14–16]. In recent years, more and more studies confirmed the beneficial role of microalgae in aquaculture wastewater treatment [17,18]. In addition to treating wastewater, microalgae could synthesize value-added components, including protein, lipid, and natural pigments. Previous studies have successfully applied various microalgal species, such as *Chlorella* sp., *Dunaliella* sp., and *Scenedesmus* sp., for the production of value-added biomass, which could be exploited to partly replace aquaculture feed and enhance the immunity of aquatic animals [15,18,19]. Last but not the least, microalgae with a high capacity of generating oxygen could act like a bio-pump for aeration in aquaculture and adjust the microbial community in a water body [20]. Thus, the water quality in aquaculture practice could be properly controlled to avoid algal bloom or oxygen depletion. Owing to aforementioned benefits, the use of microalgae for aquaculture wastewater remediation has recently emerged into the limelight.

In recent years, the concept of using microalgae in aquaculture has been proposed and a lot of efforts are devoted to promote the industrial implementation of microalgae-assisted aquaculture [19,21]. This work provides a state-of-the-art review on the use of microalgae, which plays important roles in water quality control, aquaculture feed production, and nutrients recovery, for the sustainable development of the aquaculture industry. This paper also focuses on the technologies and mechanisms related with microalgae-assisted aquaculture. Finally, challenges and prospects of the integration of microalgae with aquaculture are discussed. It is expected that the industrial implementation of microalgae technology in the near future could be a promising way to overcome problems in traditional aquaculture and upgrade the whole aquaculture industry for global sustainability.

2. Progress of Traditional Aquaculture

Although traditional aquaculture made a great contribution to the supply of aquatic products, problems associated with environmental protection and food safety seriously limited its sustainable development in the future. Problems commonly occurred in the traditional aquaculture industry include water deterioration and antibiotics abuse [9].

2.1. Problems in Aquaculture

Water deterioration, which refers to oxygen depletion, harmful algal bloom, and eutrophication of water body in aquaculture, may lead to the failure of aquatic animals rearing and even cause serious environmental disasters [9,22,23]. As shown in Figure 1, the water deterioration mainly occurs in three aspects: (1) The addition of an excessive amount of traditional aquaculture feed, which consists of biomass rich in protein and lipid, may not be fully eaten by aquatic animals and the residual feed would be converted to soluble nutrients, driven by some bacterial activities, partly contributing to the eutrophication in water body. (2) The water deterioration is attributed to the wastes secreted by aquatic animals. In the aquaculture with high stocking density, this could be the main reason for water deterioration. The mechanisms of water-deterioration induced animals' diseases or death have been widely documented by previous studies [24,25]. One of the key mechanisms is that elevated NH_4^+ comes into cells and displaces K^+, causing the depolarization of neurons and the activation of the N-methyl-D-aspartate (NMDA) receptor, which leads to an influx of excessive Ca^{2+} and subsequent cell death in the central nervous system [26]. (3) In the closed aquaculture system, eutrophication would accelerate the microbial reproduction and cause harmful algal bloom. A previous study discovered that some harmful algal bloom species, particularly cyanobacteria, could consume oxygen, at the same time, produce toxins [23]. As a result, owing to the oxygen depletion, bacterial reproduction, and toxin accumulation, survival and health of aquatic animals would be seriously threatened. Even if the water deterioration does not cause the failure of aquaculture, aquatic animals with diseases or toxins may cause serious food safety problems and negatively impact human health. Therefore, the negative effects of aquaculture problems on environmental protection and human health merit attention from both academia and the industry.

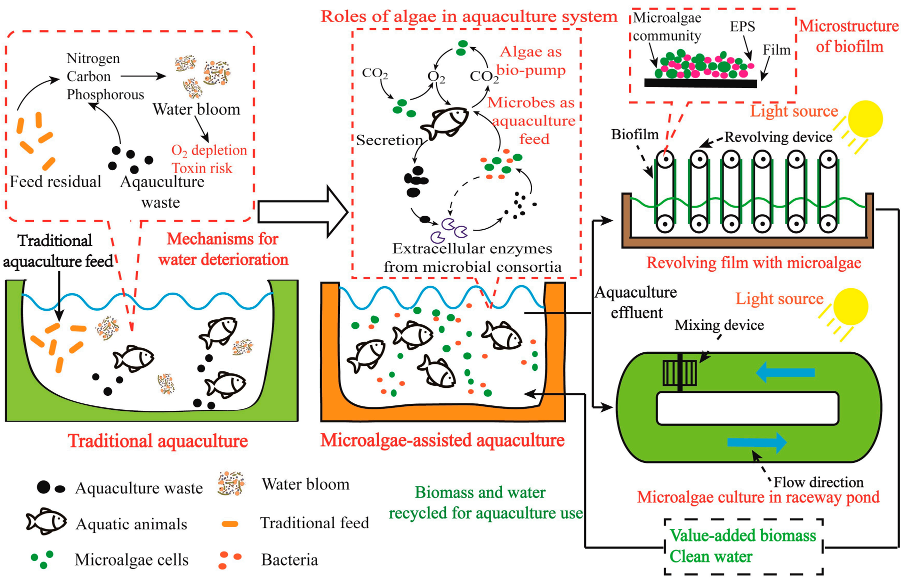

Figure 1. Comparison of conventional aquaculture and microalgae-assisted aquaculture.

2.2. *Conventional Technologies and Solutions*

2.2.1. Control of Water Quality

The most straightforward methods to control water quality in aquaculture include reducing the stocking density and frequent water replacement. However, in the aquaculture industry, owing to the low profitability and the high cost, the applications of these two methods are limited.

Wastewater treatment for water reuse is a possible way to reduce water replacement frequency and control the operation cost of the aquaculture system [7,27]. Technologies commonly applied for the aquaculture wastewater treatment include anaerobic treatment and aerobic treatment, which performed well in wastes removal and water purification. For example, Boopathy et al. (2007) reported that removal efficiency of chemical oxygen demand (COD) of sequencing batch reactor (SBR) under aerobic condition reach 97.34% in eight days [27]. In the study of Mirzoyan et al. (2008), aquaculture sludge was subjected to anaerobic treatment for methane production and up to 70% sludge-mass reduction was demonstrated [28]. However, from the perspective of nutrients recovery, these traditional treatment technologies are not highly recommended [9]. For example, organic carbon in aquaculture wastewater is converted to CO_2 and CH_4 by aerobic and anaerobic treatment. As a result, the aquaculture wastewater is treated at the expense of greenhouse gas emissions and the resources in wastewater could not be efficiently reused. An artificial wetland was considered as an environmentally friendly way to recover nutrients from aquaculture wastewater and improve water quality, however, its commercialization is hindered by the large ground occupation area and the high management cost. Longo et al. (2016) reported that the maintenance of a high content of dissolved oxygen (DO) by aeration is a technically feasible way to improve the stocking density, but the high electricity consumption of the aeration process increases the aquaculture cost [8]. Therefore, until now, the eco-friendly and economically promising technologies for water quality control have not been widely applied in aquaculture.

2.2.2. Use of Antibiotics or Medicines

Due to the unfavorable environment caused by water deterioration, in aquaculture practice, antibiotics or medicines are used to control the diseases of aquatic animals [29]. Commonly used antibiotics and medicines for aquaculture include ampicillin, oxacillin, penicillin, ceftazidime, cefazolin, and so forth [30]. However, the diseases in aquaculture are controlled by antibiotics at the expense of human health, environmental protection, and ecological stability. First, one of the problems created by the abuse of antibiotics is the presence of residual antibiotics in commercialized aquaculture products, of which the consumption is increasing continually in recent years. Second, antibiotics-resistance caused by the residual antibiotics has been considered as a trigger of ecological disasters. According to the survey of Hossain et al. (2012), in some cases antibiotics are no longer effective in treating bacterial diseases as the aquaculture pathogens have become antibiotics-resistant. Therefore, it is not a sustainable and eco-friendly way to control aquaculture diseases by the overuse of antibiotics or medicines. Previous studies mainly focused on the removal of antibiotics or medicines by conventional technologies, such as anaerobic and aerobic biological treatment processes [31,32], while did not devote much effort in the source reduction of antibiotics or medicines in aquaculture.

3. Microalgae-Assisted Aquaculture

3.1. Principles of Microalgae-Assisted Aquaculture

3.1.1. Principles

The concept of microalgae-assisted aquaculture is to convert organics in eutrophic effluents to biomass by microalgae growth and exploit value-added biomass to partly replace aquaculture feed and enhance aquatic animals' immunity. The construction of microalgae system also could accelerate the carbon dioxide fixation and promote oxygen release, acting like a bio-pump and creating a good environment for aquatic animals.

The specific scheme of microalgae-assisted aquaculture is shown in Figure 1. First, microalgae, which are inoculated into the fish rearing tank or pond, could enhance the self-purification capacity of aquaculture system by digesting some wastes secreted by aquatic animals and also act like a bio-pump to maintain the content of dissolved oxygen in water body. Second, microalgae cultivation system is constructed to assimilate nutrients in aquaculture effluent. Third, harvesting technology, which is suitable for the aquaculture system, is employed to obtain the microalgae biomass in an environmentally friendly and cost-saving way. Fourth, harvested fresh biomass is used as value-added aquaculture feed to reduce the fish rearing cost and treated effluent is recycled to the aquaculture system.

3.1.2. Advantages

According to the previous study, in a real-world application, the integration of microalgae with fish rearing not only brings technical advantages, but also creates economic benefits: (1) Oxygen production by microalgae alleviates the risks of oxygen depletion and reduces the energy consumption of traditional aeration devices. (2) Survival of microalgae in a fish rearing tank or pond may limit the growth of unfavorable or toxic microorganisms, creating a good environment for aquatic animals. Consequently, the water replacement frequency of a fish tank or pond would be minimized and the related cost would be reduced significantly. (3) As the immunity of aquatic animal is enhanced by the microalgae feed, overuse of antibiotics or medicines in aquaculture could be avoided, increasing the safety of aquaculture products and maximizing the market acceptance. Generally, pollution-free aquaculture products have much higher prices and larger market demands than traditional products. (4) The aquaculture effluent could be treated by advanced microalgae biotechnology at low cost.

Thus, the wastewater treatment cost that was paid by the aquaculture industry to sewage treatment plant in the past could be avoided, reducing the financial burden of industry to some extent. (5) As the harvested biomass is used to partly replace the traditional aquaculture feed, the cost of fish rearing could be controlled.

Therefore, considering the aforementioned advantages, it is expected that the aquaculture industry could be upgraded with the assistance of microalgae, bringing great benefits to factory, consumer and society. In a real-world application, to truly use microalgae for aquaculture wastewater remediation, some important issues should be considered. First, cost-saving cultivation systems for efficient biomass production are needed to grow microalgae for large-scale treatment of aquaculture wastewater. Second, microalgal strains with value-added components should be selected for both wastewater remediation and aquaculture feed production. Third, advanced harvesting technologies are needed to simplify the harvesting process and reduce the biomass cost. Fourth, effects of microalgae on the growth of aquatic animals and relevant mechanisms should be fully understood. Recently, researchers developed a lot of efforts in aforementioned issues and had a lot of important findings that merit attention from the fields of environmental protection and aquaculture.

3.2. Microalgae-Based Wastewater Remediation

3.2.1. Mechanisms of Wastes Assimilation

Nitrogen Assimilation

Nitrogen is one of the compositions in wastes secreted by aquatic animals and a high concentration of ammonium in a water body is unfavorable or even toxic to the aquatic animals. Common forms of nitrogen in wastewater include ammonium (NH_4^+–N), nitrate (NO_3^-–N), and nitrite (NO_2^-–N) [26,33]. Ammonium could be absorbed by microalgae cells through active transport and directly utilized for amino acids synthesis while nitrate and nitrite absorbed by microalgae through active transport have to be converted to ammonium by nitrate reductase and nitrite reductase before the further assimilation process [33]. In microalgal cells, amino acids are synthesized from ammonium through glutamine synthetase-glutamine oxoglutarate aminotransferase (GS-GOGAT) pathway and glutamate dehydrogenase (GDH) pathway. Since α-ketoglutarate, a metabolic intermediate of the Krebs cycle, is an essential substrate in both the GS-GOGAT pathway and GDH pathway for nitrogen assimilation, carbon metabolism and nitrogen metabolism are closely connected [34]. In microalgae cultivation, parameters of the C/N ratio, light intensity and quality, and carbon forms could be adjusted to enhance carbon assimilation, further promoting the nitrogen assimilation.

Carbon Assimilation

Carbon sources in aquaculture wastewater for microalgae growth include inorganic carbon (CO_2 and HCO_3^-) and organic carbon (saccharides and volatile fatty acids). As the assimilation of CO_2 or HCO_3^- is driven by photosynthesis, it is a feasible way to promote the fixation of inorganic carbon by creating favorable conditions, particularly light and temperature, for photosynthesis [35]. With the fixation of CO_2 and the release of O_2, content of dissolved oxygen (DO) in a water body will be increased, constructing an oxygen-rich environment for aquatic animals. Compared with inorganic carbon assimilation, organic carbon assimilation might be more complicated and time-consuming in some cases since some forms of organic carbon could be utilized efficiently by microalgal cells [36]. For example, owning to the large size, some insoluble solids rich in carbon could not be absorbed by microalgal cells directly [14].

To promote the carbon assimilation in aquaculture wastewater, the construction of algal-bacterial consortia has been widely studied [37,38]. In consortia, CO_2 released by bacteria could be utilized by microalgae for photosynthesis, which generates O_2 for heterotrophic metabolisms in bacterial cells. As described by Hernández et al. (2013), in wastewater remediation, bacteria convert indigestible carbon forms to digestible carbon forms, such as volatile fatty acids, amino acids, and glucose, by secreting extracellular enzymes. Meanwhile, digestible carbon forms could be assimilated by microalgae in an efficient way. Therefore, compared with pure microalgae system, algal-bacterial consortia have a much better performance in nutrients recovery.

3.2.2. Properties of Aquaculture Wastewater

Boyd (1985) reported that the production of one kilogram of live catfish releases about 51 g of nitrogen, 7.2 g of phosphorus and 1100 g of COD into the water body as organic wastes, which accumulates in the form of soluble nutrients or insoluble sludge [4]. According to the data in Table 1, aquaculture wastewater should be a good medium for microalgae cultivation and value-added biomass production. First, aquaculture wastewater is rich in essential macro-elements, such as nitrogen, phosphorus, and carbon, for microalgae growth. Since nutrients of aquaculture wastes are impacted by stocking density, water replacement frequency, feed addition, and some other factors, different nutrients profiles were observed (Table 1). In most cases, supernatant of aquaculture wastewater contains lower concentrations of nutrients than sludge obtained from the bottom of aquaculture system. Second, different from industrial effluent, municipal waste stream and mining wastewater, aquaculture wastewater contain much less toxic components, such as heavy metals [39]. On one hand, the stream without toxic components may create a good environment for microalgae growth. On the other hand, harvested microalgae without contamination by toxic components have the potential to be used for aquaculture feed production. Third, concentrations of NH_3–N and COD in aquaculture wastewater are not too high to threaten the survival of microalgae. As shown in Table 1, the highest concentration of NH_3–N was about 100 mg/L while ammonium toxicity for most Chlorophyceae occurs when the concentration of NH_3-N reaches 300 mg/L [40].

Table 1. Nutrients profiles of aquaculture wastewater.

Animal Type	TN (mg/L)	NH_3-N (mg/L)	TP (mg/L)	COD (mg/L)	Total Solids (g/L)	Reference
Shrimp	361	90	*NA*	1321	*NA*	[41]
NA [a]	1023.84	28.08	239.76	904.2	21.6	
NA	777.87	50.25	383.91	348.8	20.1	[28]
NA	533.42	23.84	458.92	2494	14.9	
Shrimp	>365	83.7	*NA*	1593	*NA*	[42]
NA	110.8	0.07	*NA*	19.7	*NA*	[43]
Shrimp	>395	101.7	*NA*	1201	13.1	[27]
Rainbow trout	1.18	0.27	0.19	17.6	0.01	[44]
Crucian carp	6	0.9	>0.7	*NA*	*NA*	[45]
Water eel	12.4	4.6	5.2	48	*NA*	Our lab [b]
Crucian carp	47.6	72.0	*NA*	368	1.02	Our lab [c]

[a] "NA" is short for "Not Available". [b] Aquaculture wastewater collected from water eel rearing factory. [c] Aquaculture wastewater collected from crucian carp rearing factory.

3.2.3. Microalgae Cultivation Systems

Although various microalgae cultivation systems have been developed for different purposes by previous studies, not all of them are suitable for microalgae-based aquaculture wastewater remediation. Generally, cultivation systems with high land utilization efficiency, a low investment cost, and high photosynthesis rate, have the potential to be used in aquaculture. Two systems, including a raceway pond and revolving algal biofilm (RAB), which meet the criteria mentioned above, have more advantages in the aquaculture industry.

Raceway Pond System

As shown in Figure 1, a raceway pond system consists of a closed circulation channel with a depth of 0.2–1.0 m and one or two paddle wheels drive the circulation of water body [41,46]. As the raceway pond system has been intensively studied in both lab research and an industrial application, a couple of problems have been identified, including: (1) Contamination risks of microalgae biomass by bacteria and other microbes are high; and (2) microalgae grown in a raceway pond system might be limited by unfavorable conditions, such as temperature fluctuation and light deficiency. However, compared with closed glass photo-bioreactors, a raceway pond system has a much lower investment cost but higher volume, making it more suitable for the treatment of aquaculture wastewater [47]. In addition, a raceway pond system has lower annual operating expense ($42.65 M) and yields lipids at a lower cost ($13/gal) than a photo-bioractor ($62.80 M and $33/gal) [48]. A life cycle analysis (LCA) conducted by Sfez et al. (2015) indicated that it is a sustainable way to use microalgae-bacterial flocs in a raceway pond system for aquaculture wastewater remediation and recycle biomass for aquaculture feed. As a lot of efforts have been devoted into the fundamental research and parameters optimization, recent studies are heading towards the demonstration scale of microalgae cultivation for either wastewater remediation or value-added biomass production.

Considering the turbidity and bacteria co-growth in aquaculture wastewater, in the demonstration scale of microalgae cultivation in the raceway pond system for aquaculture wastewater remediation, two critical factors should be considered. First, location and pond depth should be selected or designed reasonably to improve the light transmittance and photosynthesis rate. Second, the relationship between microalgae and bacteria in the raceway pond system should be fully understood. Van Den Hende et al. (2014) reported that microalgal bacterial flocs contributed to the removal of 28% COD, 53% BOD_5, 31% TN, and 64% TP in aquaculture wastewater (12 m^3 raceway pond), suggesting that with the establishment of beneficial cooperation in microbial community, threat of bacteria to microalgae growth in raceway pond system could be reduced to a low level. A similar phenomenon was reported by the studies on the interaction between microalgae and bacteria or fungi in other wastewater sources [49,50]. Therefore, in the application of a raceway pond for a microalgae-based aquaculture wastewater treatment, research interests are gradually moving from the bacterial contamination control to the establishment of cooperation between microalgae and bacteria for nutrients recovery.

The use of a raceway pond system for an aquaculture wastewater treatment has a low investment cost and low operation cost, but this system is more likely to be influenced by the external environment. In addition, after algae cultivation in a raceway pond system, the harvesting process of biomass is time-consuming and energy-intensive.

Revolving Algal Biofilm (RAB) System

The RAB system, which was developed to grow microalgae on a film, was considered as a potential technology to improve land utilization efficiency and simplify the harvesting process [51,52]. As shown in Figure 1, revolving algal biofilm is a system consisting of a microalgal biofilm, drive unit, and open pond with wastewater. Since the biofilm is established vertically on the open pond, theoretically, the RAB system has higher land utilization efficiency and biomass productivity than the standard raceway pond system [51]. Besides, compared with conventional harvesting methods, such as centrifugation and chemical-floculation, it is more cost-saving and eco-friendly to harvest biomass attached on film by using a scraper [53]. In a real-world application, to construct biofilm with a high biomass density, previous studies compared a couple of film materials and found that cotton is a good material for biofilm construction, yielding a biomass at 16.20 $g \cdot m^{-2}$ [52]. In addition to the research in the lab, a pilot-scale RAB system performed well in a wastewater treatment and biomass yield. According to the studies of Gross and Wen (2014) and Christenson and Sims (2012), in the pilot-scale RAB system, nutrients removal rates were 2.1 and 14.1 $g \cdot m^{-2} \cdot day^{-1}$ for total dissolved phosphorus (TDP) and total dissolved nitrogen (TDN) and biomass productivity could reach 31 $g \cdot m^{-2} \cdot day^{-1}$ [51,53]. Another advantage of the RAB system is that the harvesting process does not reply on any chemicals, thus, the harvested biomass has a high safety level for aquaculture use. Therefore, from the perspectives of nutrients recovery and biomass reuse, the RAB system is a promising technology for the wastewater remediation and resource recycle in aquaculture.

As the RAB system is exposed to the atmosphere during operation, bacteria or fungi would grow together with microalgae on the biofilm. In spite of the competition on nutrients, the cooperation between microalgae and other microbes in the biofilm formation and nutrients uptake merits more attention. It was reported that extracellular polymeric substances (EPS) released by the bacteria function as "glue" to promote the formation of biofilm and extracellular enzymes produced by bacteria convert high-molecular-weight organics to low-molecular-weight organics, which could be assimilated by microalgae more efficiently [54–56] (Figure 1). However, to our knowledge, until now, mechanisms related with biofilm formation, nutrients conversion and assimilation, and algal-bacterial interaction in the RAB system have not been fully understood [57]. In the coming future, with the wide use of RAB system for aquaculture wastewater remediation, those scientific questions and technical problems currently bothering researchers will be addressed.

The most important advantages of the RAB system is that this system integrates algae cultivation with biomass harvesting. Besides, compared with the raceway pond system, the RAB system has better performance in land utilization and harvesting cost control. However, the RAB system has a much higher investment cost than the raceway pond system. In a real-world application, the appropriate microalgae cultivation system should be selected according to the actual conditions.

3.3. Technologies for Biomass Production

To improve the biomass productivity and increase the economic performance, previous studies devoted a lot of effects into the technologies for biomass production in wastewater, particularly wastewater pretreatment and construction of algal-bacterial consortia, which have been proven to play an important role in aquaculture wastewater remediation. The existence of solid organics and the unbalanced nutrients profile are two main problems that should be addressed by wastewater pretreatment.

3.3.1. Pretreatment of Wastewater

Solid Organics

Aquaculture wastewater contains some solid organics, which are regarded as sludge in some cases [27,28,55]. Without appropriate treatment, solid organics with a large size could not be assimilated by microalgae directly, and even hinder the photosynthesis of microalgae by increasing the turbidity of wastewater. The core principle of pretreating solid organics is to convert indigestible nutrients to digestible nutrients, such as volatile fatty acids, sugar, and carbon dioxide. Common pretreatment methods include anaerobic digestion and aerobic digestion. Mirzoyan et al. (2010) that summarized the results of employing anaerobic digestion to treat aquaculture sludge revealed that the removal efficiency of a total solid (TS) could reach 80%–100%. By controlling relevant parameters of anaerobic digestion, volatile fatty acids, which are good carbon sources for microalgae metabolisms, could be produced [58]. In the treatment of aquaculture wastewater, which is not suitable to be anaerobically digested due to lower concentrations of nutrients, aerobic digestion is commonly applied to convert solid organics to carbon dioxide. Dissolved carbon dioxide could be further fixed by microalgae through photosynthesis. Thus, by appropriate pretreatment, conversion efficiency of solid organics to microalgae biomass is highly improved.

Unbalanced Nutrients Profile

According to the redfield ratio for phytoplankton, the ratio of C/N in culture medium or wastewater should be controlled around 6.1:1, which is much higher than the ratio of C/N in aquaculture wastewater [59]. A comparison between aquaculture wastewater and a commonly used mixotrophic medium also showed that carbon deficiency might be a barrier to the microalgae growth in aquaculture wastewater. For some ammonia-sensitive microalgal strains, a high concentration of ammonia could cause toxicity or even lead to the failure of microalgae growth [40]. Similar problems have been reported in the application of microalgae to treat other sources of wastewater, such as food processing wastewater and livestock industry effluent [36,60].

To address the aforementioned problems in aquaculture wastewater, some methods used in other wastewater may be useful. First, the most straightforward method to balance the nutrients profile is adding certain nutrients in wastewater. It was verified that with the addition of glucose and acetic acids, nitrogen assimilation ability of microalgae cells was improved since carbon metabolism and nitrogen metabolism are closely connected through the TCA cycle and GS-GOGAT pathway [31]. In a real-world application, Lu et al. (2016) found that mixed food processing wastewater from different sources have a more balanced nutrients profile and become suitable to microalgae growth. Thus, not only the biomass yield, but also nutrients recovery efficiency is dramatically improved by appropriate mixing. For example, the biomass yield of algae grown in mixed wastewater ranged between 1.32 g/L and 2.68 g/L while in non-mixed wastewater the biomass yields were less than 1.16 g/L. Second, Wang et al. (2015) found that microalgae cells (*Chlorella* sp.) pretreated by nitrogen starvation performed amazingly in ammonia removal, assimilating NH_3–N at 19.1 mg/L/d in wastewater with 160 mg/L NH_3–N. Therefore, it is a possible way to quickly remove ammonia in aquaculture wastewater by appropriate nitrogen starvation of microalgae.

3.3.2. Algal-Bacterial Cooperation

As mentioned above, to control the total cost, open systems are commonly used for microalgae-based wastewater remediation. In some cases, bacteria and microalgae in wastewater may form synergistic consortia, which perform much better in nutrients recovery than individual microbial system [54,56,61]. First, some substances secreted from microalgae and bacteria contribute to the formation of a synergistic relationship. Croft et al. (2005) reported that vitamins released by bacteria have beneficial effects on microalgae growth. Besides, some intermediate metabolites of microalgae could be partly released to the extracellular environment, thus providing organic carbon for bacteria growth. Second, bacterial metabolisms may accelerate the breakdown of solid organics in aquaculture wastewater and provide more digestible nutrients for microalgae growth [54,55]. It was discovered that extracellular enzymes, such as lipase and protease, released by microbes are critical to the yield of digestible nutrients [62]. Third, gas exchange between microalgae (O_2 producer) and bacteria (CO_2 producer) is beneficial to both biomass production and wastewater treatment. Such a cooperation mechanism has been fully documented by previous studies that used microalgae to treat wastewater rich in suspended solids [50,54]. With the accumulation of dissolved oxygen in wastewater by microalgal photosynthesis, an aerobic environment is created in the aquaculture wastewater, promoting the bloom of beneficial bacteria, such as nitrifying bacteria, *Bacillus subtilis*, and yeast. Thus, after microalgae-based treatment, waste, containing beneficial microorganisms, reused for aquaculture may have positive effects on the health of aquatic animals [63,64].

To establish the interspecies cooperation, competition between microalgae and bacteria in nutrients utilization should be mitigated. The study of Ma et al. (2014) studied the effects of inoculation concentration and inoculation ratio on microalgae growth in wastewater, revealing that the maximum biomass yield was obtained when inoculation concentration was set as 0.1 g/L. In addition, physical or chemical sterilization methods can be used to control bacteria in wastewater and adjust inoculation ratio. In the coming future, cost-saving technologies for bacteria control in aquaculture practice will be developed and widely used for microalgae-based aquaculture wastewater remediation.

3.4. Technologies for Biomass Harvesting

3.4.1. Criteria for Harvesting Technology Selection

To use microalgae biomass in various industries, previous studies have developed many harvesting technologies, such as centrifugation, filtration, gravity-driven sedimentation, flocculation by positively charged ions, harvesting by edible fungi, and flotation [65,66]. In a real-world application, however, not all of these technologies are suitable to the biomass harvesting for aquaculture practice. To our knowledge, in the aquaculture industry, the biomass harvesting technologies should meet three specific requirements. First, the harvesting process should be performed at a low cost to improve the competitiveness of microalgae feed over traditional feed. Second, no toxic or unhealthy chemicals could be used in the harvesting process. Otherwise, contaminated biomass may negatively impact the safety of aquaculture products or even cause the failure of the aquaculture. For example, the accumulation of aluminum ions and polyacrylamide, which have been widely used for microalgae biomass harvesting at full-scale, in food chain may cause serious safety problems. Third, as the harvesting step could directly impact the hydraulic retention time (HRT) of the wastewater treatment system and water circulation frequency of aquaculture system, the harvesting process should be efficient and time-saving. According to the criteria mentioned above, two advanced technologies, fungi-assisted harvesting and flotation, may be applicable in microalgae-assisted aquaculture (Table 2).

Table 2. Comparison of harvesting technologies for aquaculture.

	Harvesting Cost	Safety Level	Time Consumption
Centrifugation	High (Energy-intensive centrifugation equipment)	High	Short
Filtration	High (Frequent replacement of filter blocked by algal cells)	High	Short
Gravity-driven sedimentation	Low	High	Long (Repulsive force among negatively charged algal cells)
Flocculation by chemicals	Low	Low (Addition of toxic or unhealthy chemicals)	Short
Harvesting by edible fungi	Low	High	Short
Flotation	Low	High	Short

3.4.2. Fungi-Assisted Harvesting

Fungi-assisted microalgae harvesting refers to the addition of filamentous fungi, including either fungal pellets or fungal spores, into medium with microalgae. The study of Zhou et al. (2013) found that that microalgal cells could attach to or be entrapped in the fungal pellets, which could be harvested by simple filtration. It was discovered that the use of fungal pellets could harvest over 95% of microalgae biomass in 1.5 h, revealing the great performance of fungi in microalgae harvesting [67]. In practice, however, the production of fungal pellets has a strict requirement on equipment and fermentation conditions, which increase the total cost of harvesting.

Recently, to further simplify the harvesting procedure, the co-cultivation of fungal spores with microalgae in wastewater or medium has been intensively studied. In some cases, the co-cultivation will not only harvest microalgae biomass, but also promote the nutrients recovery from aqueous phase, which has been fully confirmed by the study of Gultom et al. (2014) that co-cultivated *Aspergillus* sp. with *Chlorella* sp. in molasses wastewater. According to previous studies, the parameters that impact the fungal-algal pellet formation and determine the pellet size include inoculation ratio of fungi/algae, pH value, carbon content, cultivation temperature, and so on [67–69]. Table 3, which lists some examples of fungi-assisted microalgae harvesting, confirms that it is an efficient way to collect biomass from culture medium or wastewater by employing fungal spores or fungal pellets. For aquaculture practice, fungi used for microalgae harvesting should have a high safety level, meaning that they do not contain or secrete toxic components. Otherwise, harvested biomass used as aquaculture feed may threaten the survival of aquatic animals or even lead to the failure of aquaculture. Some fungal strains, such as *Aspergillus oryzae* and *Monascus purpureus*, isolated from food production have a high safety level and contain value-added compounds [69,70]. However, beneficial effects of these fungi on aquatic animals have not attracted attention from researchers yet.

Table 3. Fungi-assisted microalgae harvesting.

Microalgae	Fungi	Harvesting Efficiency	Conditions	Reference
Chlorella sp.	*Penicillium* sp.	98.2%	Fungal pellets; 30–34 °C; pH: 4.0–5.0; Agitation speed: 120–160 rpm	[67]
Chlorella sp.	*Penicillium* sp.	99.3%	Fungal spores; 40 °C; pH: 7.0; Agitation speed: 160 rpm	
Chlorella vulgaris	*Aspergillus oryzae*	93%	Fungal spores; Heterotrophic culture; 25 °C; Agitation speed: 150 rpm; 3-day	[68]
Chlorella vulgaris	*Aspergillus* sp.	Almost 100%	25 °C; pH: 5.0–6.0; Agitation speed: 100 rpm; 2-day	[71]
Chlorella vulgaris	*Aspergillus niger*	>60%	Fungal spores; 27 °C; pH: 5.0; Agitation speed: 150 rpm; 3-day	[72]
Chlorella vulgaris	*Aspergillus fumigatus*	>90%	Fungal pellets; 28 °C; Agitation speed: 150 rpm; 2-day	[73]
Scenedesmus quadricauda	*Aspergillus fumigatus*	>90%		
Pyrocystis lunula	*Aspergillus fumigatus*	Around 30%		

In addition to the simplification of the harvesting process, co-cultivation of fungi with microalgae could promote nutrients recovery from aquaculture wastewater with high contents of solid organics. Previous work, which presented the metabolic mechanisms of fungi and microalgae, showed that fungal cells could convert high-molecular-weight organics to low-molecular-weight organics easily utilized by microalgal cells in the co-cultivation system [69]. At the same time, carbon dioxide released by fungi through heterotrophic metabolism could be assimilated by microalgae through photosynthesis, preventing the greenhouse gas emission and improving the carbon utilization efficiency. Such a synergistic relationship between microalgae and fungi has been proven by the study of Gultom et al. (2014). Therefore, the co-cultivation of fungi and microalgae in aquaculture wastewater for simple biomass harvesting and efficient nutrients recovery merits more attention from academia and industry.

3.4.3. Flotation and Modified Flotation

Flotation has been assessed as one of the most economic technologies for microalgae harvesting [74]. The main process of flotation-based harvesting is generating fine air bubbles continuously in wastewater or a culture medium with microalgae. As the air bubbles attach on suspended microalgae cells, microalgae cells will rise to the surface of aqueous phase. Flotation can be considered as an inverted sedimentation, having a small footprint, low detention period, and high overflow rate [74].

A negative repulsive charge on the surface of microalgae cells is the main reason for suspension of microalgae cells. In a real-world application, to improve the harvesting rate, flotation can be combined with flocculation, which partly neutralizes the negative charge on microalgae cells [75]. Previous studies have explored various types of flocculating agents, such as metal ion and polymer, for microalgae flocculation [66,76,77]. Sirin et al. (2012) optimized the addition content of aluminum ion in microalgae flocculation process and found that the harvesting efficiency could reach 82%. For aquaculture practice, to recycle the harvested microalgae as feed, biomass safety should be strictly controlled. Owing to the serious threats caused by metal accumulation in food chain, metal-ions based flocculation can not be considered as a safe and eco-friendly technology. Recently, some studies reported the coagulation effects of natural polymers, such as protein and polysaccharide, secreted by microorganisms [78,79]. Generally, by natural-polymer based flocculation, the harvesting efficiency of microalgae could reach 90% [78,80]. Therefore, it is suggested that in aquaculture, to harvest microalgae by flotation-flocculation, natural polymers can be used for safety purpose.

3.5. Microalgae-Based Aquaculture Feed

3.5.1. Algal Species with Commercial Potential

In aquaculture practice, microalgae are important nutrition sources of fish or shrimp either by direct consumption or as indirectly prepared feed. Advantages of microalgae feed over traditional feed in aquaculture include the abundance of nutrients and the maintenance of water quality. Microalgae are rich in proteins, lipids, and carbohydrates, which are essential nutrients to aquatic animals. In addition, determined by the regulatory gene and growth condition, microalgae could intensively synthesize a variety of value-added components, such as antioxidants and pigments [81,82]. For the purpose of aquaculture use, microalgae biomass with value-added components is highly needed. Therefore, in addition to screening robust algal strains for wastewater remediation, we need to obtain value-added algal strains with commercial potential in aquaculture. Table 4 listed some algal strains with great potential to be used for wastewater remediation and value-added biomass production. Generally, for aquaculture practice, the value-added components in microalgae could be classified into three categories. First, microalgae rich in protein and carbohydrate can be used to partly replace traditional feed, reducing the aquaculture cost. Second, antioxidants in microalgae could be exploited to enhance the immunity of aquatic animals, overcoming the problems of antibiotics abuse. Third, some components play important roles in the growth of special fish. For example, astaxanthin, which determines the skin and flesh color of some fish, is an essential pigment in salmon production industry.

Table 4. Nutrition profile of microalgae biomass.

Strain	Culture Medium	Protein (%)	Lipid (%)	Carbo Hydrate (%)	Value-Added Compound	Reference
Thraustochytrium sp.	Medium with glycerol	NA	38.95	NA	EPA and DHA (37.88% of total lipid)	[83]
Chlorella zofingiensis	Cane molasses	NA	30–50	NA	Polyunsaturated fatty acids (36.89–49.16% of fatty acid profile)	[84]
Scenedesmus sp.	Soybean oil extraction effluent	53.3	33.4	NA [a]	EPA (15.89% of fatty acid profile)	[15]
Galdieria sulphuraria	Modified Allen Medium	26.5	1.14	69.1	Dietary fiber (54.1% of carbohydrate)	[85]
Galdieria sulphuraria	Modified Allen Medium	32.5	1.77	62.9	Astaxanthin (575 mg/kg)	
Chlorella zofingiensis	Cane molasses	NA	NA	NA	Astaxnathin (56.1 mg/L)	[86]
Chlorella zofingiensis	Cane molasses	NA	30-50	NA	Astaxnathin (13.6 mg/L)	[84]
Haematococcus pluvialis	OHM medium	NA	NA	NA	Astaxnathin (>15 mg/L)	[87]
Haematococcus pluvialis	Primary-treated wastewater	NA	NA	NA	Astaxnathin (80 mg/L)	[88]
Botryococcus braunii	NA	39.9	34.4	18.5	Essential amino acids (54.4 g/100 g protein)	[89]
Tetraselmis chuii	NA	46.5	12.3	25.0	Essential amino acids (45.5 g/100 g protein)	
Phaeodactylum tricornutum	NA	39.6	18.2	25.2	Essential amino acids (45.2 g/100 g protein)	
Porphyridium aerugineum	NA	31.6	13.7	45.8	Essential amino acids (63.9 g/100 g protein)	

[a] "NA" is short for "Not Available".

3.5.2. Microalgae Feed for Aquaculture

Protein

Protein synthesis in microalgae is impacted by both growth conditions and nutrients supply in culture medium. Table 4 indicated that protein content in dry microalgae biomass ranged between 26.5% and 53.3%. Particularly, compared with soybean protein, microalgae protein has much higher productivity, making it a feasible protein source for aquaculture. According to previous studies that used microalgae for aquaculture practice, feed conversion ratio (FCR) of fish fed by microalgae is higher than that of fish fed by traditional feed [81]. However, in some cases, due to palatability problems, microalgae feed may not perform well in aquaculture. For example, in the culture of Atlantic cod, with the increase of microalgae content (0%–30%) in fish-meal, most growth parameters, including final body weight, absolute feed intake, and specific growth rate, decreased but mortality increased [90]. Hence, in a real-world application, to ensure the sustainable operation of microalgae-assisted aquaculture, palatability of microalgae feed should be comprehensively evaluated.

Polyunsaturated Fatty Acids

Polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA), arachidonic acid (AA), docosahexaenoic acid (DHA), and α-linoleic acid (ALA), have been proven to be essential for the growth of larvae. For example, EPA, functioning as a precursor for eicosanoids synthesis that partly regulates the developmental and regulatory physiology, plays a pivotal role in the growth of aquatic animals. Compared with traditional aquaculture feed, microalgae biomass contains much higher concentrations of PUFAs. As shown in Table 4, percentages of PUFAs in microalgae could reach almost 50% under some special conditions, such as low temperature. Compared with soybean and peanut, which are usually exploited as feedstock for traditional fishmeal production, microalgae with higher contents of PUFAs have much greater potential to be used for aquaculture. Therefore, microalgae can be considered as an affordable and productive source of PUFAs for aquaculture.

Special Pigments

The natural pigments, such as astaxanthin, chlorophyll, and carotene, in microalgae are important to the growth of some fish species. First, biochemical characteristics of fish are partly determined by the intake of microalgae pigments. Choubert et al. (2006) found that rainbow trout *Oncorhynchus mykiss* fed by *Haematococcus pluvialis* contained higher concentration of astaxanthin (around 20 mcg/g dry weight). In most cases, rainbow trout with more astaxanthin and a better flesh color is preferred by consumers in the market. Second, some pigments play a pivotal role in the immunity of aquatic animals. It was discovered that lysozyme activity, an indicating factor of fish immunity, of large yellow croaker *Pseudosciaena crocea*, increased with the increase of astaxanthin and *Haematococcus pluvialis* levels, suggesting that the fish immunity has a positive relationship with the intake of microalgae pigment (astaxanthin) [81]. Traditionally, for specific purposes, feed with pigments, of which the production, extraction, purification, and preservation cost are high, are added into aquaculture [91]. In microalgae-assisted aquaculture, biomass rich in natural pigments is directly added to feed aquatic animals, thus reducing the total cost and prevent the pigments degradation.

Other Applications

Besides directly using microalgae as feed, researchers also studied the microalgae-based artificial ecological system, in which herbivorous fish relying on microalgae feed is preyed on by carnivorous fish. Thus, microalgae biomass can be indirectly used to feed carnivorous fish. Considering the poor performance of microalgae in the remediation of a water body with low concentrations of nutrients, recently, some studies proposed a novel concept of integrating microalgae culture with a hydroponics system, through which microalgae and leafy plants cooperate simultaneously for nutrients recovery from aquaculture.

4. Problems and Prospects

4.1. Potential Problems

Although the concept of microalgae-assisted aquaculture has emerged into the limelight owing to aforementioned advantages, further developments are required before the industrial implementation is feasible. This novel concept itself has a couple of potential problems, which may hinder its wide application.

Firstly, the safety level of biomass recycled from wastewater as aquaculture feed has not been assessed comprehensively. As the biomass production in wastewater is coupled with bacteria growth, harvested biomass consists of both microalgal biomass and bacterial biomass. To our knowledge, some bacterial species may contain toxic components or release bio-toxin, thus, the survival of aquatic animals will be seriously threatened if the biomass contaminated by bacterial toxins is used as aquaculture feed. Hence, strict control of toxic bacteria in wastewater treatment is pivotal to the successful application of microalgae-based aquaculture.

Secondly, flotation and fungi-assisted harvesting have a couple of problems related with economic feasibility and biomass safety. For example, flotation may bring solid wastes in aquaculture wastewater to the surface of the aqueous phase with microalgae biomass together, thus reducing the safety level of harvested staff in aquaculture practice. In addition, the cost of fungi-pellets production in an artificial medium is not low enough to support the commercial application of fungi-assisted harvesting. Hence, without addressing the problems associated with economic feasibility and biomass safety, harvesting technologies are not mature enough to support the industrialization of microalgae-assisted aquaculture.

Thirdly, the lack of knowledge on economic assessment and life cycle analysis is a barrier to the industrialization of microalgae-based aquaculture. Based on previous studies on technologies, the use of microalgae in aquaculture industry, including feed production, water quality control, and nutrients recovery could promote nutrients recycle, mitigate greenhouse gas emission, and reduce aquaculture cost [92]. However, the economic performance of microalgae-based aquaculture has never been fully studied. Besides, the life cycle analysis has not been conducted yet to evaluate the effects of microalgae-based aquaculture on natural environment. Therefore, aiming at controlling potential threats of microalgae-based aquaculture, it is necessary to comprehensively evaluate its economic performance and environmental impacts.

4.2. Prospects

As the eco-friendly aquaculture and the nutrients recovery from wastewater are becoming more important to the global sustainability, microalgae-assisted aquaculture bringing great beneficial benefits to natural environment merits more attention from both academia and industry. With the solution of aforementioned problems, microalgae-assisted aquaculture will move forward from lab research to industrial application. Generally, the great advancement brought to aquaculture by the wide use of microalgae biotechnology can be classified into environmental and economic aspects.

Firstly, the threats of aquaculture to the environmental sustainability can be controlled to some extent. Owing to the great performance of microalgae in carbon dioxide fixation and wastewater remediation, pollution caused by aquaculture can be alleviated. In addition, since microalgae feed has beneficial effects on the health of aquatic animals, the abuse of antibiotics or medicines could be prohibited in microalgae-assisted aquaculture. Thus, the antibiotics-resistance, which is becoming more serious in aquaculture, may be controlled. Therefore, with the wide application of microalgae-assisted aquaculture, its positive effects on the environmental safety and sustainability will contribute to the global sustainability.

Secondly, the cost of aquaculture can be reduced by the nutrients recovery and biomass production. The cost of feed accounts for a large portion of the total aquaculture cost in a real-world application, so the financial burden is a barrier to the development of aquaculture. By microalgae-assisted aquaculture, nutrients in wastewater are converted to value-added biomass, which can be further exploited to produce aquaculture feed. Thus, the nutrients recovery in aquaculture by microalgae biotechnology can reduce material input and the total cost of the whole system.

Considering the advancement in environmental and economic aspects, with the wide application of microalgae-assisted aquaculture, it will make great contribution to the aquaculture sustainability and global sustainability.

5. Conclusions

To overcome the problems in traditional aquaculture, herein, the use of microalgae for sustainable aquaculture is introduced by this work. Based on the principles of microalgae-assisted aquaculture, this novel system could convert wastes to value-added biomass as aquaculture feed, alleviate water deterioration, and reduce energy consumption for aeration. In recent years, technologies have been widely developed to promote the application of microalgae-assisted aquaculture, key technologies or theories include microalgae-based nutrients assimilation, design of the raceway pond system and RAB system, wastewater pretreatment methods, establishment of algal-bacterial cooperation, fungi-assisted harvesting and flotation, composition analysis of microalgae biomass, and beneficial effects of microalgae on aquatic animals.

In spite of the great progress in the aforementioned fields, microalgae-assisted aquaculture still has some problems related with the biomass safety level, economic feasibility, and the lack of knowledge on economic assessment and life cycle analysis, which hinder its industrialization or commercialization in a real-world application. In the near future, with the solution of these problems, microalgae will play a more pivotal role in aquaculture for sustainable development.

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Biofloc technology as a promising tool to improve aquaculture production

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Abstract

Given that the expansion of the aquaculture industry is associated with increased environmental impact and its strong dependence on fishmeal in the diet, the use of biofloc technology can reduce these problems. This technology absorbs inorganic nitrogen from aquaculture wastewater and improves water quality as well as produces microbial protein that is directly used as a suitable feed supplement for aquatic animals. Furthermore, this technology reduces the feed conversion ratio and subsequent production cost. Biofloc is available to aquatic animals throughout the day and provides the necessary nutrients, fatty acids and minerals. Biofloc along with formulated diets provides a complete food chain for aquatic animals' growth and thus improves growth performance. Since feed constitutes a major part of the aquaculture cost, having accurate information about biofloc system in aquatic animals' nutrition can be beneficial and helpful. Compared to traditional aquaculture techniques, biofloc technology provides a more sustainable approach with minimal water exchange along with reduced feed intake and transforms it into a low-cost sustainable technology for sustainable aquaculture development. Overall, this study highlights the significance of developing biofloc technology to improve aquaculture production and could be an alternative system for cultivation of important commercial species in aquaculture.

Key words: aquatic species, biofloc, carbon substrate, microbial interactions, nutrition, water quality management.

Introduction

Due to the rapid growth of population worldwide, the food production industries such as aquaculture industry needs to be well developed. The growth of the aquaculture industry must take into account environmental, economic and social conditions and somehow consider the factors of sustainable development in the expansion of this industry. The intensity of aquaculture activities, especially in coastal areas, increases the load of organic matter in the water and will have environmental risks in the long-term period (Piedrahita 2003; Sharifinia *et al*. 2018, 2019). In recent decades, recirculating systems have been developed that are in the direction of sustainable aquaculture development and including an appropriate approach to control aquaculture wastewater (Gutierrez-Wing & Malone 2006). In this system, 10% of the total volume of water is replaced daily (Twarowska *et al*. 1997), but due to the high operating and maintenance costs, adoption of the recirculating system among farmers, especially in developing countries, is low (Badiola *et al*. 2012; Ahmad *et al*. 2017; Karimanzira *et al*. 2017). Therefore, the need for a low-cost, sustainable and environmentally friendly technology that is acceptable to farmers and can be used on a large scale is evident and noteworthy.

Biofloc system, also called as biofloc technology (BFT), has recently attracted great attention as a cost-effective, sustainable and environmentally friendly (as it is basically zero water exchange and artificial feeding ratio are reduced) way that improves water quality as well as produces microbial protein for aquatic species (Avnimelech & Kochba 2009; Ekasari & Maryam 2012; Sgnaulin *et al*. 2018; Dinda *et al*. 2019; Gao *et al*. 2019). Biofloc system due to maintain water quality, improving feed conversion ratio (FCR), application of low-protein diets, reducing costs of production and replacing conventional high-cost feeds with alternative protein sources (Wasielesky *et al.* 2006; Ballester *et al.* 2010; Emerenciano *et al.* 2013a; Ahmad *et al.* 2017; Sgnaulin *et al.* 2018; Khanjani *et al.* 2019a) have received great attention in recent decades. The use of this system in shrimp farming and to some extent in finfish culture farming has been extensively studied (Emerenciano *et al.* 2011, 2012a, 2013a; Ekasari & Maryam 2012; Xu & Pan 2012; Monroy-Dosta *et al.* 2013; Caldini *et al.* 2015; Khanjani *et al.* 2016, 2017, 2019a; Najdegerami *et al.* 2016; Abbaszadeh *et al.* 2019a,b; Ebrahimi *et al.* 2020).

Production in biofloc system in the large scale aquaculture can have environmental benefits in marine and coastal ecosystems, and aquaculture wastewater and its environmental effects can be controlled by replacing soybean or fish meal with floc compounds in aquatic nutrition. Brito *et al.* (2016) and Bossier and Ekasari (2017) reported that culturing in a biofloc system can contribute to removal of total ammonia nitrogen (TAN) and nitrite, reducing water utilization and waste generation, decrease in Vibrio density, improve feed utilization efficiency and increased body-bound crude protein. Furthermore, studies by Khatoon *et al.* (2016) and Kamilya *et al.* (2017) have suggested that enhance of growth performance in a biofloc system could be attributed to presence of microbial floc and maintaining water quality by this system. Ekasari (2014) reported that biofloc systems can improve net productivity by 8–43% in comparison with non-biofloc systems such as conventional and recirculating aquaculture system. More importantly, to minimize environmental issues in aquaculture industry the biofloc nutrient-rich waste can be used as a feed in BFT. Therefore, the aim of this article is to (i) review the studies that have been done on the development and application of biofloc technology in aquaculture industry; (ii) intends to make this information available for an international audience and give an overview of the current status on biofloc technology; (iii) intends to highlight the significance of developing biofloc technology for improve aquaculture production as an alternative system for cultivation of important commercial species in aquaculture; and (iv) make recommendations for management of biofloc system in aquaculture.

Biofloc technology

Biofloc technology is a technique of enhancing water quality by adding extra external carbon sources in accordance with high level of aeration to produce high levels of microbial bacterial floc in aquaculture system (Crab *et al.* 2012; Ahmad *et al.* 2017). Maintaining a carbon-to-nitrogen ratio above 10 is essential in this system by adding carbon-containing organic materials such as molasses, wheat flour, starch or reducing the protein level of the feed to increase the activity of heterotrophic bacteria (Crab *et al.* 2012; Khanjani *et al.* 2017, 2019a). Under such circumstances, the production of microbial proteins takes place and thereby improves water quality as well as serve as sources of dietary protein for fish and shrimp (Crab *et al.* 2012). The uptake rate of inorganic nitrogen compounds by heterotrophic bacteria is higher than denitrifying bacteria; therefore, the growth rate and production of microbial biomass per unit substrate in heterotrophic bacteria is 10 times higher (Hargreaves 2006). Therefore, if there are sufficient organic carbon sources, immobilization of ammonia by heterotrophic bacteria usually takes place rapidly in bioflocs during hours or days (Hargreaves 2006).

This technology works based on the principle of flocculation or co-culture of heterotrophic bacteria and algae within the system (Avnimelech 2006; Ahmad *et al.* 2017). Emerenciano *et al.* (2017) stated that biological interaction can takes place between some group of microorganisms in biofloc system such as bacteria-microalgae. Some strains of bacteria can have a positive effect on growth of pelagic and benthic microalgae species (Fukami *et al.* 1997). The extracellular carbohydrates of benthic microalgae (diatoms) may be used as carbon source by heterotrophic microorganisms (Bruckner *et al.* 2008). Biofloc technology because of economic, environmental and social benefits has been successfully applied in aquaculture, especially shrimp farms. Compared to traditional aquaculture techniques, BFT technology provides a more sustainable approach with minimal water exchange along with reduced feed intake and transforms it into a low-cost sustainable technology for sustainable aquaculture development (De Schryver *et al.* 2008; Avnimelech & Kochba 2009). Taking advantage of this system can reduce water exchange and increase density and biosecurity. For sustainable intensive aquaculture, farm biosecurity and biofloc technology need to be considered as two major factors. BFT provides higher degree of biosecurity by limited water exchange, higher environmental control, biological and physical (indoors) barriers against pathogens and enhances immune system. Ju *et al.* (2008) biofloc has bioactive compounds that contribute for a healthy status of cultured prawns. Also, expressions of certain hemocytes enzymes related to immune system (Jang *et al.* 2011) and antioxidants status (Xu & Pan 2013) enhanced in *Litopenaeus vannamei* reared in biofloc system. Furthermore, BFT showed positive impacts on the immune response to higher resistance against infectious myonecrosis virus (IMNV) challenge (Ekasari 2014) and *Vibrio* (Liu *et al.* 2017). Therefore, with respect to the present issues it appears that biofloc technology to be the solution.

Biofloc formation

To create biofloc, the tanks were firstly filled with water then a certain amount of nitrogenous material (aquatic feed and urea fertilizer) was added in order to supply the nitrogen then carbonate organic materials (such as molasses, wheat flour, starch) about 0.7 of diet were distributed on the surface of the tanks in order to provide carbon. Clay is also added to the microbial reservoirs to help forming the microbial mass after softening and passing through the sieve (53-μm-sized particles or less pass with mesh number of 270). Adding clay at the beginning and during biofloc formation is suitable for further mass continuity. In addition, the use of farm wastewater containing nitrogenous wastes is helpful as an inoculum. 20 g of clay, 10 mg of ammonium sulphate and 200 mg of carbonaceous organic matter such as molasses stimulate biofloc formation in one litre of water. Various studies have shown that the use of clay and water rich in a biofloc production cycle as the primary inoculum improves microbial mass formation in the new culture system (Gaona *et al.* 2011; Zemor *et al.* 2019).

After providing the necessary elements in the system, in order to activate the activity of bacteria in water the aeration was conducted. The presence of carbonated organic matter has made heterotrophic bacteria more active than other bacteria, which these bacteria remove nitrogen and carbon from water by absorption process, and produce microbial biomass, as well as, other organisms in the water attach to them to feed on the microbial biomass and form biofloc (Khanjani *et al.* 2017, 2019a). During the biofloc formation period, algae first develop and then foam form, and eventually, the development of a brown state indicates the presence and activity of heterotrophic bacteria. During the experimental period, when there are aquatic animals in the tanks, the physicochemical parameters (temperature, oxygen, pH, alkalinity, total nitrogen, ammonium, nitrite and nitrate) should be measured and subsequently appropriate responses as outlined below should be adopted quickly (Avnimelech 2009; Khanjani *et al.* 2015):

- If ammonia levels were high: adding carbohydrates to the tank and reducing protein in the feed
- If the nitrite level is high: checking for low-oxygen areas, sludge collection, putting aerators and adding carbon
- If the microbial biomass is low: adding carbohydrates
- If the volume of biofloc is too high: excretion of waste material and some of the bioflocs

The amount of settled solids in the BFT culture tanks (using Imhoff cone; measured in 15–20 min to be well deposited), and total suspended solid (TSS) must also be measured to better management of the system (Avnimelech & Kochba 2009).

Factors such as the amount of salinity and type of carbon affect the rate and duration of biofloc formation (Khanjani *et al.* 2017, 2019a). Maicá *et al.* (2012) found that increasing salinity increases the density of biofloc formation, and the type of carbon source also affects the quality of the flocs, so that saltier waters promote better formation of stable bioflocs. Furthermore, addition of simple carbonaceous organic matter such as molasses to the aquaculture system without water exchange leads to improved water quality and faster growth of heterotrophic bacteria compared to complex carbohydrates like wheat flour. Figure 1a,b shows the formed and condensed of wet and dry biofloc, respectively, that can be used by aquatic animals, as well as Figure 1c shows how the heterotrophic bacteria are interconnected in a chained and porous manner. The open and porous structure is characteristic of biofloc micrographs, which allows water and chemicals to flow throughout the floc and is effective in supplying nutrients and eliminating the metabolites from in and out of biomass in the microbial mass (Khanjani *et al.* 2015, 2016, 2019b).

Mechanism of microbial cell bonding

The massing of microbial communities is a complex process involving the physical, chemical and biological processes in its formation (De Schryver *et al.* 2008). There are several mechanisms that influence the formation, appearance and stability of the microbial mass. Many organisms repel polymeric compounds of humic, proteins and polysaccharides that cover their outer surface; these slimy polymers act as adhesives and integrate other cells and particles to form a biofloc. Another mechanism is the balance between the forces of gravity (molecular, dipole, hydrogen bond) and electrostatic repulsion forces. Most organisms are negatively charged and cause counter electrostatic repulsion. If this repulsion reduced, then strong gravity forces can occur; this is the case when the salt concentration is high and multivalent ions are present in the environment (Avnimelech 2009). Calcium and aluminium ions stimulate the formation of stable flocs and algae, fungi or bacteria organisms can help to bonding between the components of different flocs (De Schryver *et al.* 2008; Avnimelech 2009). Another advantage of the biofloc in terms of biofloc formation is that the presence of common groups of microorganisms in BFT. Fungi, ciliate, protozoa, rotifer, copepod and nematode complement the biofloc community play an essential role in recycling of organic matter in the BFT system. Ciliates are the largest group of protozoa in nature; they eat bacteria (including cyanobacteria) and small phytoplankton (Emerenciano *et al.* 2017). High porosity of biofloc causes relatively low mass density, which keeps the bioflocs suspended in water and reduces sedimentation rate (Avnimelech 2009).

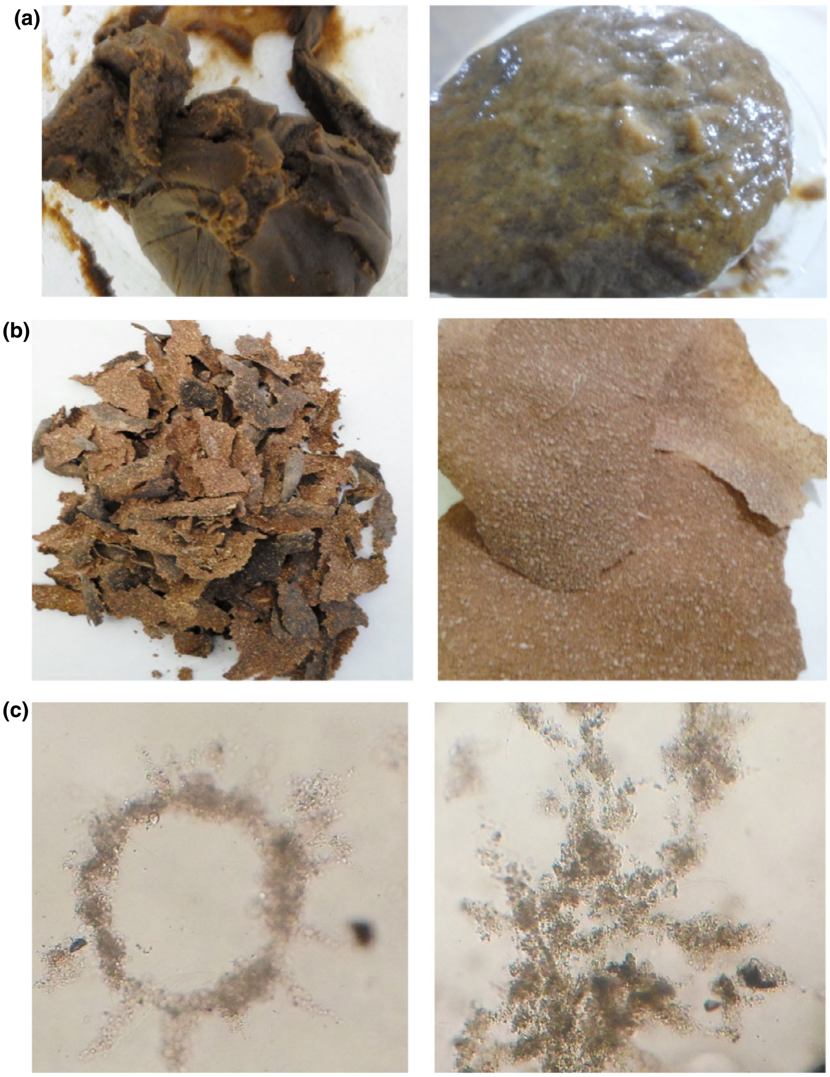

Figure 1 (a) Wet biofloc, (b) dry biofloc and (c) produced floc in rearing tanks of pacific white shrimp, bioflocs are porous and has a lot of bacteria that were connected like a chain (Khanjani 2015, 2019b).

Microbial communities in biofloc

Biofloc can be defined as a set of organic matter in a culture environment that forms at a high density as suspended particles (Cuzon *et al*. 2004; Emerenciano *et al*. 2012b, 2013a) and includes compounds of organic materials (60–70%), heterogeneous combination of microorganisms (i.e. fungi, algae, bacteria, protozoa, rotifer, nematode) and inorganic substances (30–40%) such as colloids, organic polymers, binary ions, salts and dead cells (Chu & Lee 2004). Bacterial communities are primarily responsible for maintaining water quality in the zero water exchange system by relying on heterotrophic and nitrifying chemoautotrophic bacteria (Ebeling *et al*. 2006; Hargreaves 2006). In BFT systems, the heterotrophic bacteria community are able to colonize dead organisms, moults, unconsumed food and faeces to produce bacterial biomass. This community use the organic compounds as a carbon source and can reduce accumulation of ammonia by incorporation as bacterial biomass in the water column that are a main component of biofloc (Emerenciano *et al*. 2017). During biofloc formation, the colour changes from green to brown as progress for the development of bacteria due to transition from algal to bacterial in a BFT system. Avnimelech (2007) suggested that the number of bacteria in the ponds containing biofloc can be between 10^6 and 10^9 colonies per ml, with each ml of biofloc containing 10–30 mg of dry matter. Khanjani (2015) and Khanjani *et al*. (2019a) stated that the 3.36×10^7 number of heterotrophic bacteria per ml can indicate the maturation of a biofloc system.

The different groups of organisms present in the biofloc depend on various factors such as the type of carbon source, salinity level and cultured species (Ray *et al.* 2010). Ju *et al.* (2008) reported that bioflocs collected from Pacific white shrimp tanks consist of 24.6% phytoplankton (often diatoms such as *Thalassiosira*, *Chaetoceros* and *Navicula*), 3% biomass of bacteria (two-thirds Gram-negative and one-third Gram-positive), some protozoa communities (98% flagellates, 1.5% rotifer and 0.5% amoeba), 32.2% detritus and 39.25% ash. Also, Yunos *et al.* (2017) showed that the biofloc structure contained of 29% microalgae, 35% bacteria, 24% fungi and 12% zooplankton.

Khanjani *et al.* (2016) quantified the amount of different organisms in the biofloc system based on methods applied by Thompson *et al.* (2002) and Emerenciano *et al.* (2012b) and found that biofloc compounds comprised 73.47% of flagellum and ciliated protozoa, 20.53% of microalgae, 3.33% of nematodes and 2.67% of other suspended organisms. Also, biofloc sizes of 20–437.5 μm were observed. Zhao *et al.* (2012) stated that dominant species of bacteria that exist in bioflocs include *Proteobacteria*, *Bacillus* and *Actinobacteria* and there are also some other minor species of bacteria such as the genus *Roseobacter* and *Cytophaga*.

Monroy-Dosta *et al.* (2013) reported that a great number of heterotrophic microbial communities related to bioflocs include genera such as the *Sphingomonas* (*Sphingomonas paucimobilis*), *Pseudomonas* (*Pseudomonas luteola*, *Pseudomonas mendocina*), *Bacillus*, *Micrococcus*, *Nitrospira*, *Nitrobacter* and yeast Rhodotorula sp. These microorganisms are suitable for maintaining water quality and the physiological health of organisms in culture (Monroy-Dosta *et al.* 2013). In the biofloc production system, the development of microbial groups can vary depending on the species, environmental conditions, amount of feed and especially the carbon source (Monroy-Dosta *et al.* 2013). Gutiérrez *et al.* (2016) investigated the cultivation of *Puntius conchonius* fish in a biofloc system and found that the genera *Aeromonas* and *Vibrio* were dominant biofloc-related bacteria in the first weeks of bacterial cultivation period. At week 12, the concentration of these bacteria was very low in the culture medium, but in the last two weeks, the concentration of *Bacillus subtilis* and yeast *Rhodotorulla* increased, which showed probiotic properties. Therefore, the biofloc system could be considered as a microbial ecological sequence model. Bacteria in the biofloc system consume organic matter and nitrogen compounds for growth and development. The ability to adhere to suspended particles and surfaces as well as the use of organic matter are important physiological properties of bacteria in a biofloc. Microorganisms play a major role in natural aquatic resources, and the intensity of solar energy, organic matter density and added carbon sources affect their activity. In the biofloc system, bacteria are more dependent on organic matter and strong aeration to maintained C: N ratio (Pérez-Rostro *et al.* 2014).

Nutritional value of biofloc

Bioflocs have a dynamic nutritional value (Ekasari *et al.* 2010) and can be used as a complete aquatic food source, as well as being able to supply bioactive compounds (Ahmad *et al.* 2017). Therefore, analysing the factors affecting nutritional value of biofloc such as aquatic nutrition priority, their ability to ingest and digest microbial protein, and biofloc density in water are of interest (Hargreaves 2006). The single-cell protein formed by heterotrophic bacteria through uptake of inorganic N can be used as a food source for cultured aquatic species such as shrimp, tilapia and carp (Burford *et al.* 2004; Monroy-Dosta *et al.* 2013; Khanjani *et al.* 2019a; Ebrahimi *et al.* 2020). The nutritional value of bioflocs for a particular organism depends on its particle size, digestibility and biochemical compounds. Ekasari *et al.* (2014) reported that flocs with particle size of > 100 and < 48 μm due to higher nutritional value and N recoveries are more favourable for shrimp *L. vannamei*, red tilapia *Oreochromis niloticus* and mussel *Perna viridis*. They also found that with particle size of > 100 μm contained highest levels of protein and lipid, whereas the flocs < 48 μm rich in essential amino acids.

The growth performance of Pacific white shrimp increases in the presence of biofloc (Khanjani *et al.* 2016). Improving growth performance of aquatic species in the biofloc system is related to the simultaneous presence of biofloc production with the artificial diet, which is a natural food supplemented with a formulated diet, forms a complex food chain that the aquatic animal uses. Khanjani *et al.* (2015) investigated the effects of BFT on feed conversion ratio (FCR) and recorded the FCR values 1.52 and 1.2–1.29 in clear water and BFT treatments, respectively. Khanjani (2015) reported that the presence of biofloc can lead to decrease in FCR (1.20–1.29) and increase in feed efficiency (78.61–84.26%) compared to clear water treatment (1.52 for FCR and 66.81% for feed efficiency). In terms of quality, based on the dry matter biofloc contains 38% protein, 3% lipid, 6% fibre, 12% ash and 19 kJ g^{-1} energy (Azim & Little 2008). They reported that the quality of biofloc is independent of the feed quality used for biofloc production. Ballester *et al.* (2010) found that bioflocs contained 30.4% crude protein, 4.7% crude fat, 8.3% fibre, 39.2% ash and 29.1% nitrogen-free extract based on dry matter. Wheat bran and molasses's were used as sources of carbohydrates in this study. Brown *et al.* (1996) assessed the nutritional value of seven species of yeast and reported protein content of 25–37%, carbohydrates 21–39%, lipids 4–6%, and, additionally, the presence of all essential amino acids. Yeasts are chemoorganotrophic microorganisms that reported in the biofloc and used organic compounds of carbon as a source of energy, which are quite diverse and include sugars, polyols, organic and fatty acids, aliphatic alcohols, various heterocyclic and polymeric compounds (Emerenciano *et al.* 2017). Ekasari *et al.* (2010) reported that biofloc with glycerol as carbon source contained higher total $n - 6$ PUFAs than those with glucose. This suggested that the nutritional value of bioflocs can be dynamic and microbiota is likely to affect the nutritional value of bioflocs.

Khanjani *et al.* (2017) were used different carbon sources including molasses, wheat flour, starch and their mixtures for biofloc formation and found different nutritional value results of the bioflocs grown on each carbonaceous material. They suggested that changing in carbon sources alters the nutritional composition and quality of bioflocs. Tacon *et al.* (2002) stated that biofloc enhances ingestion and digestion of feed and provides a complete food source for cultured aquatic organisms. The presence of biofloc in the diet of brood-stocks of *L. vannamei* (Emerenciano *et al.* 2013b) and *Litopenaeus stylirostris* results in improved reproductive performance and egg quality (Cardona *et al.* 2016). Emerenciano *et al.* (2013b) found that *L. vannamei* females fed with fresh food under biofloc conditions achieved better eggs production (higher number per spawn), less unfertilized spawns, spawned more quickly and showed higher levels of highly unsaturated fatty acids (HUFA) in eggs. They suggested that the enhanced reproductive performance and egg quality of brood-stocks fed with fresh food under biofloc conditions can be explained by their high HUFA content (are essential nutrients for gonad development) and the contribution of biofloc particulates as the source of essential nutrients for reproduction including antioxidant and essential lipids. Hoa *et al.* (2009) reported that a large fraction of HUFA often observed in natural fresh food items such as squid and mussels. Cardona *et al.* (2016) reported that higher spawning rate and frequency as well as higher gonado-somatic index and number of spawned eggs in shrimp brood-stocks maintained in biofloc system compared to clear water might be explained by the dietary supplement obtained from natural productivity during BFT rearing and the contribution of biofloc particulates as a source of dietary glutathione and lipids, particularly essential phospholipids and HUFAs for shrimps.

Consumption and recycling of biofloc in the culture system increase feed efficiency (Hargreaves 2006). Microorganisms and their associated microbial communities not only remove excess waste but also increase growth rate, feed efficiency and weight gain in farmed species (Burford *et al.* 2004; Wasielesky *et al.* 2006; Khanjani *et al.* 2016, 2017). The nutritional properties of biofloc and their ability to maintain water quality in the BFT system depend on the type of carbon source used for biofloc production (Khanjani *et al.* 2017). Various carbon sources influence on the carbon/nitrogen ratio, increased activity of heterotrophic bacteria, protozoa, algae as well as microbial compounds and social structures in the bioflocs (Crab *et al.* 2010, 2012). Furthermore, carbonaceous organic matters such as dextrose and molasses in the biofloc system increases the production of Pacific white shrimp (de Lorenzo *et al.* 2015). Table 1 presents the various studies conducted on different aquatic species by adding different carbon sources.

Numerous studies have reported that biofloc provides essential nutrients such as protein, lipid, essential fatty acids, minerals, vitamins, carotenoids and digestive enzymes to facilitate digestion and consequently improves nutritional status (Crab *et al.* 2012; Xu & Pan 2012; Xu *et al.* 2012; Khanjani *et al.* 2016; Abbaszadeh *et al.* 2019a;

Table 1 Studies with different species and carbon sources used in biofloc production system

Reference	Carbon sources	Species
Crab *et al*. (2010)	Acetate	*Macrobrachium rosenbergii*
Suita (2009)	Dextrose	*Litopenaeus vannamei*
Crab *et al*. (2010)	Glucose	*M. rosenbergii*
Burford *et al*. (2004)	Molasses	*L. vannamei* and *P. monodon*
Asaduzzaman *et al*. (2008)	Starch	*L. vannamei* and *M. rosenberg*
Emerenciano *et al*. (2012a,b)	Wheat flour + Molasses	*Farfantepenaeus brasiensis*, *F. duorarum*
Avnimelech (2009)	Cellulose	Tilapia
Azim and Little (2008)	Wheat flour	Tilapia (*O. niloticus*)
Emerenciano *et al*. (2011)	Wheat bran + Molasses	*F. paulensis*
Emerenciano *et al*. (2012a,b)	Wheat bran + Molasses	*F. brasiliensis*
Wang *et al*. (2016)	Wheat bran	*Litopenaeus vannamei*
Serra *et al*. (2015)	Molasses + dextrose + rice flour	*Litopenaeus vannamei*
Khanjani *et al*. (2017)	Molasses + wheat flour + Starch	*Litopenaeus vannamei*
Abbaszadeh *et al*. (2019a,b)	Molasses + palm sap	*Litopenaeus vannamei*
Mirzakhani *et al*. (2019)	Wheat flour and Molasses	Tilapia (*O. niloticus*)

Ahmad *et al*. 2019). Ahmad *et al*. (2019) investigated the effects of different organic carbon sources on haematological indices, digestive and metabolic enzyme activities of *Labeo rohita* fingerlings rearing under biofloc conditions. They reported that activities of amylase and protease enzymes in the whole intestine were significantly higher in tapioca biofloc system while lipase activity did not show significant differences in biofloc systems with different carbon sources. De *et al*. (2015) stated that nutrient digestibility is positively associated with activity of digestive enzyme and higher activities of digestive enzymes in biofloc systems can improved utilization of macromolecules (Ahmad *et al*. 2019). Ahmad *et al*. (2019) suggested that these changes may occur due to compositional characteristic of the carbon sources used. Mahanand *et al*. (2013) noted that the nutrient content of the bioflocs indicates that the bioflocs can be successfully used to feed herbivorous and omnivorous fish and achieve optimal growth. Previous studies have shown that using bioflocs in the zero water exchange system significantly contribute to the growth and production of tilapia (Avnimelech 2007; Azim & Little 2008). Furthermore, the nutritional value of bioflocs extracted from different studies is shown in Table 2.

Impact of biofloc on growth performance, immune system and activity of digestive enzymes

Biofloc and its attached microorganisms have a positive effect on the activity of digestive enzymes in aquatic species (Xu & Pan 2012; Ahmad *et al*. 2019). Inclusion of biofloc in the diet resulted in about 75% improvement in growth performance and digestive enzyme activity in common carp (Najdegerami *et al*. 2016). Also, biofloc as a dietary supplement at 4% level in feeding of tiger shrimp increases growth and activities of digestive enzyme (Anand *et al*. 2014). Bioflocs have recently been recognized as a new strategy for controlling and reducing pathogens as antibiotics and antifungals that exhibit probiotic activity (Emerenciano *et al*. 2013a). Biological compounds in biofloc are contributing factors affecting the growth and immunity of fish and shrimp (Burford *et al*. 2004; Ju *et al*. 2008). Activity of serum lysozyme (LSZ) is an important factor in the fish immune defence, which plays an important role in the antibacterial activity against Gram-positive and Gram-negative bacteria (Saurabh & Sahoo 2008). Long *et al*. (2015) reported that biofloc can enhance the immune response of tilapia to a certain extent. Ju *et al*. (2008) and Crab *et al*. (2012) noted that bioflocs can provide abundant natural microbes, immunostimulatory and bioactive compounds such as carotenoids and soluble vitamins that could stimulate the immune response in cultured fish. Furthermore, biofloc played a positive role in utilization of feed and activities of digestive enzyme in cultured fish, which may improve the assimilation of dietary bioactive substances from the feed and then exerted an immune-stimulating effect on the fish (Xu & Pan 2012; Long *et al*. 2015; Promthale *et al*. 2019).

Albumin and globulin are among the most important proteins in plasma and increasing the levels of proteins can lead to strong innate immune status (Rao *et al*. 2006). Mansour and Esteban (2017) found that the immune status of fish reared in biofloc conditions is stronger than that in clear water and also reported that the levels of these proteins, total immunoglobulin, myeloperoxidase and lysozyme noticeably increased in fish reared under biofloc conditions. Myeloperoxidase and lysozyme are immune enzymes involved in defence against bacterial infection. In fish, lysozyme is made by leucocytes and leads to bacterial cell wall lysis, therefore stimulating and triggering the complement system and phagocytosis of different pathogens (Cecchini *et al*. 2000; Mansour & Esteban 2017). Moreover, myeloperoxidase expressed and stored in neutrophils and

Table 2 Nutritional value of bioflocs obtained from different studies

Reference	Crude protein%	Carbohydrate%	Lipid%	Crude fibre%	Ash%
Wasielesky *et al*. (2006)	31.1	23.6	0.50	–	44.8
Ju *et al*. (2008)	30.4	–	1.9	12.4	38.9
Kuhn *et al*. (2009)	49	36.4	1.13	12.6	13.4
Kuhn *et al*. (2010)	38.8	25.3	0.1>	16.2	24.7
Maicá *et al*. (2012)	28.8–43.1	–	2.1–3.6	8.7–10.4	22.1–42.9
Emerenciano *et al*. (2012a,b)	30.4	29.1	0.5	0.8	39.2
Emerenciano *et al*. (2012a,b)	18.2–29.3	22.8–22.9	0.4–0.7	1.5–3.5	43.7–51.8
Emerenciano *et al*. (2012a,b)	18.4–26.3	20.2–35.7	0.3–0.7	2.1–3.4	34.5–41.54
Emerenciano *et al*. (2012a,b)	28–30.4	18.1–22.7	0.5–0.6	3.1–3.2	35.8–39.6
Khanjani *et al*. (2017)	27.43	–	0.86	–	39.83
Khanjani *et al*. (2017)	23.8	–	1.14	–	21.81
Khanjani *et al*. (2017)	30.73	–	2.18	–	29.97
Abbaszadeh *et al*. (2019a,b)	–	–	0.5–0.8	6.8–8.9	7.5–9.3

plays a role in respiratory burst through peroxide to produce hypochlorous acid (Dalmo *et al.* 1997). The effects of biofloc as immune-stimulants appeared to be carbon source-dependent and the improvement observed in the immunological factors may be due to some probiotic microorganisms such as *Bacillus* and *Lactobacillus* present in BFT system (Anand *et al.* 2014; Ahmad *et al.* 2016; Zhao *et al.* 2016). Panigrahi *et al.* (2019) suggested that biofloc can improve the shrimp immune status by presence of the microbial cell wall in the biofloc, containing of peptidoglycans, lipopolysaccharides and glucans, which were known to have a potential to activate the immune response in shrimps by triggering the major non-specific defence mechanism (Labbe & Little 2009; Rao *et al.* 2010; Panigrahi *et al.* 2018).

Bioflocs have various compounds include microbial protein (Ballester *et al.* 2010; Hargreaves 2013) organic polymer (PHB: Poly-β-hydroxybutyrate) created by bacteria (De Schryver *et al.* 2010), microalgae, protozoa, nematodes (Azim & Little 2008), copepods and rotifers (Ray *et al.* 2010). PHB is a biodegradable polymer with several benefits including helping to improve digestibility in the intestine, increase unsaturated fatty acids and improve growth in fish and shrimp (Crab *et al.* 2007; Emerenciano *et al.* 2013a). Polyhydroxybutyrate acts as a probiotic for aquatic species and can, therefore, adjust the gut microbial population, which is useful for improving aquatic health (Trainer & Charles 2006).

In the Khanjani (2015) study, feed conversion ratios of 1.51 and 0.98–1.27 were obtained in control (non-biofloc) and biofloc treatments, respectively, and it was found that biofloc could account for about 30% of daily food intake Pacific white shrimp. Megahed and Mohamed (2014) found that dietary protein levels could be reduced from 45% to 25% in the presence of biofloc without affecting the growth of *Fenneropenaeus indicus*. Biofloc biochemical compounds can provide important nutrients such as protein, fat and minerals to shrimp. Various studies have shown that biofloc can make amino acids, fatty acids and minerals accessible for shrimps (Izquierdo *et al.* 2006; Emerenciano *et al.* 2012b; Toledo *et al.* 2016). The presence of biofloc in shrimp rearing tanks improves the feed conversion ratio (Wasielesky *et al.* 2006; Abbaszadeh *et al.* 2019b), enhances feed efficiency (Xu & Pan 2012), improves growth performance (Megahed & Mohamed 2014; Khanjani *et al.* 2016), reduces feed cost (Xu & Pan 2012; Xu *et al.* 2012), increases digestive enzyme activity (Xu & Pan 2012) and impacts on biochemical composition of shrimp body (Izquierdo *et al.* 2006; Ju *et al.* 2008; Xu & Pan 2012; Khanjani *et al.* 2017). Additionally, several studies revealed that bioflocs may contribute in production of exogenous microbial enzymes like proteases (Arnold *et al.* 2009; Xu & Pan 2012; Zhang *et al.* 2016) and moreover induce the generation of endogenous digestive enzymes (Xu & Pan 2014; Najdegerami *et al.* 2016; Mirzakhani *et al.* 2019) facilitating the digestion and absorption of feed nutrients.

Essential fatty acids, carotenoids, free amino acids, chlorophylls (Ju *et al.* 2008), trace minerals (Tacon *et al.* 2002) and vitamin C (Crab *et al.* 2012) are considered as the bioactive compounds of bioflocs which improve antioxidant status, growth and reproduction in aquatic species. Khanjani (2015) reported that weight gain, length, percentage of body weight gain, growth rate and specific growth rate showed better performance in treatments with biofloc compared to control (no biofloc). Yun *et al.* (2016) stated that when juvenile Pacific white shrimp were cultured in BFT tanks, the dietary protein content could be reduced by up to 10% without affecting growth performance, body composition and hemolymph properties. In zero water exchange systems, improved growth performance is due to the presence of a suitable substrate for the growth and attachment of microorganisms, ultimately, creating a colony and biofloc, together with commercial diets, provide a complete food chain for shrimp growth. Studies have shown that the presence of biofloc improves the digestive system of shrimp, increases growth by 15% and decreases food conversion ratio by 40% (Wasielesky *et al.* 2006). The presence of biofloc-dependent microorganisms in the biofloc treatments results in improving growth performance compared to the clear water treatment (Thompson *et al.* 2002). Biofloc consumption in zero water exchange treatments decreased feed conversion ratio (1.39–1.03) and increased growth rate (0.39–1.25 g week^{-1}; Wasielesky *et al.* 2006). The findings from studies on growth performance in fish and shrimp indicated that species reared under biofloc conditions significantly improves growth parameters compared to non-biofloc systems. These improvements could be due to beneficial microbiota dominance in gut and consequent improvement of digestive function (Zhou *et al.* 2013; Wang *et al.* 2018).

Economic aspects

The main factors affecting the growth and development of the aquaculture industry are environmental protection and feed cost (Avnimelech 2009). Reducing production costs and more profitability are considered as important goals in the aquaculture industry. Growth rate and feed conversion ratio play an important role in aquaculture costs, which are improved in biofloc system compared to conventional system and profitability is also better in the biofloc treatments (Khanjani 2015). Better feed recycling, improved feed conversion ratio, increased specific growth rate and survival rate are key components of aquaculture management costs. Hanson *et al.* (2009) found that biological parameters such as survival rate are effective factors in cost return and profitability. Increasing stocking density (up to 20%) and growth rate enhances profitability by 57% and 45%, respectively (Browdy *et al.* 2001). On the other hand, a 20% reduction in feed costs has a significant impact on profitability. Specifically, investing in aquaculture bases such as eggs, larvae and feed increases survival rate, growth rate and stocking density, which has a positive effect on cost return. In the biofloc system, it is more profitable than the clean water system due to the reduction of commercial feed consumption and the use of biofloc and consequently lower food prices. Production of one kilogram of green tiger shrimp (*Penaeus semisulcatus*) and tilapia using biofloc was associated with 33% (Megahed 2010) and 10% reduction in cost (De Schryver & Verstraete 2009), which depends on the species, diet, amount of consumed biofloc and price of carbohydrates. The biofloc system eliminates the cost of organic and inorganic fertilizers and only covers the cost of carbon source. Reducing the cultivation period, increasing growth rate and survival per cent has made the biofloc system more useful than the clear water system (Sontakke & Haridas 2018). Furthermore, biofloc systems provide more economical benefits such as reducing expenses of water treatment by 30%, and additionally, the efficiency of protein utilization is twice as high in biofloc technology systems in compared with conventional water treatment technologies (De Schryver *et al.* 2008; Avnimelech 2009). Conventional technologies to manage and remove nitrogen compounds are based on either earthen treatment systems, or a combination of solids removal and nitrification reactors (Crab *et al.* 2007).

Application of BFT technology for sustainable aquaculture

In recent years, numerous studies have been carried out on the application of new technologies in aquaculture (multi-species aquaculture, hybrid aquaculture, recirculation system, aquaponics and more recently BFT technology) to increase production. Due to lack of flawless technology, it is more difficult to justify BFT technology and persuade the farmers to set it up than conventional methods. On the other hand, droughts, scarcity and expensive water for the development of aquaculture, the destructive effects of aquaculture effluents on the environment, pollutions and the spread of infectious diseases and consequently attention to the farm biosecurity have resulted that the amount of water exchange in the farms is minimized (Avnimelech 2009). The successful experiences of BFT technology as well as the economic benefits of this technology need to be taught to farmers in a practical way. One of the most important parts in launching BFT technology in aquaculture is monitoring and evaluation of ponds. Water quality monitoring including determining and stabilizing the total concentration of suspended solids, settling solids, the number of aerators, their type and location in the ponds are important (De Schryver *et al.* 2008). Future research should focus on the role and importance of BFT technology to persuade the farmer to launch this technique. Consumers also need to be encouraged to buy organic aquaculture products from BFT ponds. Recycling faeces and converting it into aquatic feed may cause consumers to refrain from buying such products. However, as the population grows, aquaculture strategies are needed to conserve wild fish stocks and control the price of edible fish (Jiang 2010). An increase in population, followed by a shortage of seafood and a pressure on fish stocks, is driving up fish prices (Péron *et al.* 2010). In contrast to the fisheries strategic plans, it is important to preserve fish stocks and reduce fish prices and increase commercial fish stocks. Therefore, BFT technology can reduce the pressure on aquatics stocks and improve social welfare by reducing the cost of fish production, which is beneficial to both the farmer and the consumer. The consumer wants the guarantee that the fish produced is not harmful to its health, respect to ethical and social considerations. BFT technology has been successful in this field. Many researchers are looking to integrate this technology with other aquaculture methods to control water quality and its effects.

Combining the BFT system with the presence of periphyton (Asaduzzaman *et al.* 2008), combining heterotrophic and autotrophic communities in the culture system to control environmental factors (Avnimelech 2009), the use of nitrification, denitrification and anaerobic oxidation of ammonia for nitrogen removal have been investigated (Kumar & Lin 2010). The results of these researchers showed that these technologies with low energy consumption have the potential to control harmful compounds in aquaculture systems. Other researchers have investigated the use of the BFT system for multi-species culture such as tilapia with vegetables, shrimp with microalgae, mussels and seaweed, which have achieved positive results (Kuhn *et al.* 2009). BFT technology can be combined with multi-species ponds to improve water quality, access to natural food, better nutrition performance, growth and production (Rahman *et al.* 2008). BFT technology shows that aquaculture is a sustainable tool that emphasizes environmental, social and economic issues as it develops. Researchers are working to further develop this method and persuading farmers to implement the system principally in new-generation aquaculture. The development of this method requires careful adjustment and implementation so that researchers, farmers and consumers need more research and information to build a platform for this system, which is the basis of sustainable aquaculture.

Management aspects

It is important to control the amount of suspended solids (TSS) in BFT tanks, which is associated with dissolved oxygen, carbon dioxide, pH and inorganic nitrogen compounds (Ray *et al.* 2010), and it also prevents gill clogging. Excessive increase in suspended solids results in reduced oxygen, increased carbon dioxide and pH, and the system loses its performance in a short time (less than one hour). As the flocs develop, the density of filamentous bacteria increases unpredictably, creating a 'filamentous bulking' state that impairs TSS control and interferes with shrimp gills and leads to death (Fig. 2). The major challenge with the biofloc system is the control of TSS. Its concentration should be around 500 mg L^{-1} may reach up to 1000 mg L^{-1} (Avnimelech 2009). Therefore, the level of turbidity increased and the visibility reduced, so FCR will rise and production will decrease. Poor growth performance and FCR as a result of high TSS concentration (more than 500 mg L^{-1}) in tilapia have been reported (Azim & Little 2008). The same result has also been stated about growth performance of shrimp (Furtado *et al.* 2011).

Vibrio bacteria accumulate in shrimp BFT systems and can cause pathogenicity by changing the system conditions (low or high concentrations of suspended solids). In most recirculated aquaculture systems, especially BFT systems, nutrients and minerals (especially metals) accumulate in the water. In shrimp ponds with limited water exchange, nitrate can accumulate up to several hundred mg L^{-1}, which reduces shrimp food intake at one level; in marine systems, maintaining a nitrate concentration of about 50 mg L^{-1} is an effective way to reduce the production of high volumes of toxic hydrogen sulphide. In this regard, water quality management, bacterial control and pollution in the BFT system are indispensable that should be taken into consideration.

Monitoring of the following parameters is important (Avnimelech 2009):

- Oxygen, reduce number of aerators if the level of dissolved oxygen is high. However, if the level of dissolved oxygen is less than 4 mg L^{-1}, some aerators should be added.
- TAN. Low level of TAN (<0.5 mg L^{-1}) means that the system works properly. If TAN increases, the amount of carbon should be added.
- NO_2. Nitrite negatively impacts tilapia. An increase in nitrite may be an indication of the presence of anaerobic areas. If the level of nitrite increases the sludge piles may exist in the pond, so the place of aerators should be changed.
- Floc volume (FV) determination using Imhoff cones is easy and cheap. FV should be in the range of 5–50 mL L^{-1}. Carbohydrates should be added if the level of FV is low and sludge should be removed if its level is more than 50.

Concluding remarks and future directions

Water resources constraint, increasing demand for seafood consumption and land resources constraint to expand aquaculture activities are identified as major problems. To respond to the growing demand for animal protein, intensive aquaculture is one of the main options. The needs for sustainable development and the development of environmentally

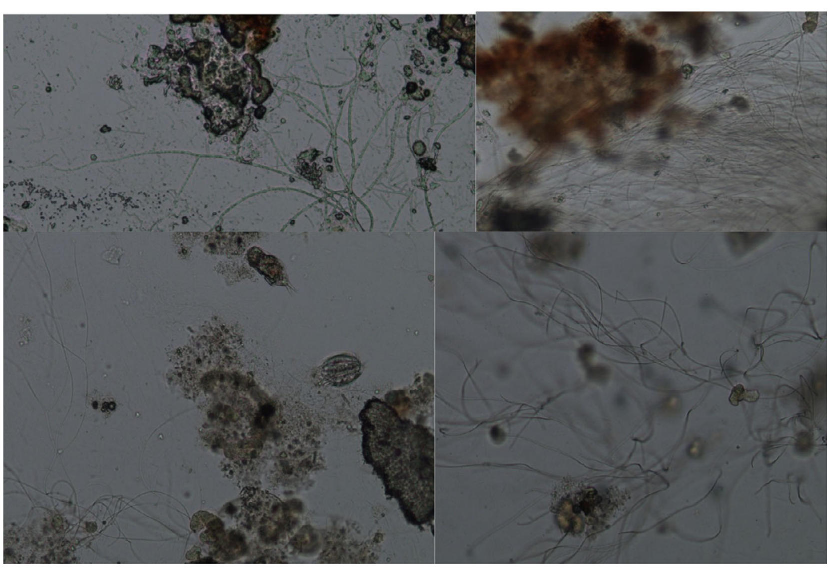

Figure 2 Filamentous bacteria in biofloc system (Khanjani 2015; 2019b).

friendly aquaculture can be achieved through the use of biofloc technology. The use of biofloc in aquatics nutrition is important as it provides aquatics supplementation needs and can be a good substitute for fish meal in the aquatics diet. Bioflocs contain microbial protein, organic polyhydroxybutyrate polymer as well as bacteria containing peptidoglycan and lipopolysaccharides in their cell walls, which, together with commercial diets, formulate a complete food chain for aquatics growth and thus improve growth performance. Studies have shown that the presence of biofloc in the zero water exchange system leads to improved water quality and better growth performance of western white shrimp, which these particles are used by shrimp. Commercial pellets may not provide all the required nutrients for growth of shrimp, some of the nutrients come from the biofloc in the rearing tank. Therefore, biofloc can be used as a food supplement for aquatic animals. Biofloc is produced as a rich food source in the culture system that is available for aquatics 24 h a day. Benefits of the biofloc production system include lower feed intake, reduced water exchange, increased biosecurity, and reduced risk of pathogens, increased growth, and survival and thus increased productivity and production. Also, the use of natural food in the same culture area has increased and the commercial diets are less used, which results in reduced environmental impacts of wastewater. Due to the nutritional value of bioflocs, applying them damply at the same culture area as well as the use of dried biofloc and its addition to the aquatic diet will effectively reduce feed costs and subsequently reduce production costs. Standardization of methods, techniques and equipment for pond construction, stocking management and harvesting in BFT aquaculture systems is required. It is necessary to conduct a detailed study for the construction of BFT ponds; circular ponds with central conical outlet and slope towards the centre are appropriate. Water flow velocity and water disruption in circular ponds are better, and the slope towards the centre causes the excess waste to move outward and discharge easily, and the conical outlet also helps to accumulate excess waste. At the specified time, open the outlet to remove the waste depending on the stocking density, rearing species, size of the fish, etc. Put two pieces at the outlet of the six-inch pipe; the first of which is lower than the surface of the pond water, and by removing the upper part of the pipe, the wastewater will be removed, which the first piece will be inserted again, after the waste has been removed.

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AQUACULTURE WASTEWATER

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Bioflocculation formation of microalgae-bacteria in enhancing microalgae harvesting and nutrient removal from wastewater effluent

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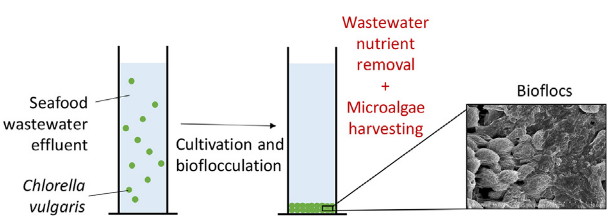
GRAPHICAL ABSTRACT



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ABSTRACT

Microalgal bacterial flocs can be a promising approach for microalgae harvesting and wastewater treatment. The present study provides an insight on the bioflocs formation to enhance harvesting of *Chlorella vulgaris* and the removal of nutrients from seafood wastewater effluent. The results showed that the untreated seafood wastewater was the optimal culture medium for the cultivation and bioflocculation of *C. vulgaris*, with the flocculating activity of 92.0 ± 6.0%, total suspended solids removal of 93.0 ± 5.5%, and nutrient removal of 88.0 ± 2.2%. The bioflocs collected under this optimal condition contained dry matter of 107.2 ± 5.6 $g{\cdot}L^{-1}$ and chlorophyll content of 25.5 ± 0.2 $mg{\cdot}L^{-1}$. The results were promising when compared to those obtained from the auto-flocculation process that induced by the addition of calcium chloride and pH adjustment. Additionally, bacteria present in the wastewater aided to promote the formation of bioflocculation process.

1. Introduction

Microalgae have appeared as promising renewable raw materials to provide a wide variety of compounds with commercial interest, such as lipids, proteins, pigments and carbohydrates (Becker, 2007; Borowitzka, 2013; Lee et al., 2017). However, large-scale production of microalgae is limited by the high-energy inputs required for the harvesting of microalgae (Ummalyma et al., 2017; Vandamme et al., 2013). Microalgae harvesting requires an intensive effort to separate a small amount of biomass from a large volume of culture broth, either from open pond reactor or photobioreactors. Besides, the small size of microalgae cells (from 2 to 20 µm) has contributed to their high colloidal stability in liquid suspension, and thus making the harvesting by simple sedimentation process is not feasible. The cost of microalgae harvesting can easily achieve 20–30% of the total cost of microalgae production and, in some circumstances, might reach 60% of the total

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cost when post-production is needed (Greenwell et al., 2009; Grima et al., 2003; Van Den Hende et al., 2011). Therefore, many studies have devoted to develop a cost-effective and economical strategy for microalgae harvesting (Grima et al., 2003; Milledge and Heaven, 2013; Quijano et al., 2017).

Various approaches for solid-liquid separation have been investigated, including adherence techniques using coagulation, flocculation and flotation, and the force applications such as centrifugation and filtration (Barros et al., 2015; Laamanen et al., 2016; Singh and Patidar, 2018; Wan et al., 2015). Amongst these methods, adherence approach is promising. However, chemical coagulation method using metal coagulants like alum and iron chloride might consume large amounts of coagulants and flocculants which lead to high operation cost and metal contamination of the harvested biomass. In view of these constraints, bioflocculation is an alternative technological approach. Bioflocculation is a flocculation process of microalgae cells assisted with microorganisms (Lee et al., 2013). During bioflocculation process, the aggregation of bacteria and microalgae cells creates large flocs and settle down by gravity, without the use of any metal and chemical flocculants, or the alteration of medium's pH (Vandamme et al., 2013). Beside of allowing speedy harvesting of microalgae, the microalgal bacterial flocs (MaB-flocs) tend to adsorb suspended compounds in surrounding medium to form co-bioflocculate and thus enhance the removal of nitrogen and phosphorous (Alcántara et al., 2015; Ummalyma et al., 2017). Therefore, the MaB-flocs technique was incorporated into conventional aerobic activated sludge technologies for wastewater treatment to enhance nutrient removal and effluent recovery (Tang et al., 2018; Van Den Hende et al., 2016; Van Den Hende et al., 2011; Yan et al., 2019). This technology was reviewed for its fundamentals and applications recently (Abinandan et al., 2018; Posadas et al., 2017). Moreover, several studies reported that the technique can be applied for the wastewater treatment coupled with the flue gas treatment or biogas upgrading simultaneously (Posadas et al., 2016; Tang et al., 2018; Van Den Hende et al., 2016; Van Den Hende et al., 2011). Despite that it is generally accepted that bacteria aids in inducing flocculation, there is still lack of the investigation on the contribution of the bacteria to the overall wastewater treatment performance (Posadas et al., 2017). Additionally, the key factor for the formation of the MaB-flocs is not clear yet. It is therefore important to provide more insights of the technique to optimize its efficiency. Moreover, the bioflocculation formation without adjusting of bioflocculants plays an important role in this investigation.

In this study, the seafood wastewater effluent (SWE) obtained from the cleaning units of a seafood production factory was used as culture medium for microalgae production. *Chlorella vulgaris* was selected as a strain of microalgae in this study owing to its fast growth rate and lipid productivity compared to others strains (Fu et al., 2012; Kang et al., 2014). The study started with the investigation of the effect of initial concentration of *C. vulgaris* on the MaB-flocs formation. After obtaining the optimal initial microalgae concentration, different culture media, as presented in Table 1, were prepared for the cultivation of microalgae, in order to examine the key factors for the formation of bioflocculation. Aiming to compare the bioflocculation process with the autoflocculation induced by pH modulation and presence of metal ions, Sueoka broth was prepared with added calcium chloride and adjusted pH, and their results were addressed. Besides, *Escherichia coli* was added as bacteria model in several media, in order to verify its effect. The results were evaluated based on the flocculating activity, total suspended solids (TSS) removal, dry matter and chlorophyll content of the flocs collected, and the wastewater nutrient removal. Furthermore, all the flocs collected from different culture media were treated and observed using compound microscope and scanning electron microscope (SEM).

Table 1
Different culture media prepared for the investigation of flocculation process.

Medium	M1	M2	M3	M4	M5	M6	M7	M8
Description	Sterilised SWE	Sterilized SWE + *E. coli*	Treated SWE with low Ca^{2+} and Mg^{2+} content	SWE	Sueoka broth + *E. coli*	Sueoka broth	Microalgae grown in M6 were harvested by centrifugation	Sueoka broth + $CaCl_2$ and pH at 10.0 ± 0.5
Bacteria content (CFU/mL)	–[a]	4.0×10^5	720.0×10^5 for aerobic bacteria, 2.1×10^5 for *Coliforms*	240.0×10^6 for aerobic bacteria 5.0×10^5 *Coliforms*	4.0×10^5	–[a]	–[a]	–[a]
Divalent metal concentration (mg/L)	107.7 Ca^{2+} + 14.1 Mg^{2+}	107.7 Ca^{2+} + 14.1 Mg^{2+}	56.2 Ca^{2+} + 2.1 Mg^{2+}	107.7 Ca^{2+} + 14.1 Mg^{2+}	6.8 Ca^{2+} + 13.8 Mg^{2+}	6.8 Ca^{2+} + 13.8 Mg^{2+}	6.8 Ca^{2+} + 13.8 Mg^{2+}	126.8 Ca^{2+} [b] + 13.8 Mg^{2+}

[a] No bacteria present in the medium.
[b] Total calcium content was derived from calcium concentration in Sueoka broth and calcium chloride added in.

2. Material and methods

2.1. Microalgal strain and wastewater

The microalgal strain used in this study was *C. vulgaris* SAG 211-19 and the cultivation process was performed using the protocols described elsewhere (Nguyen et al., 2014). The SWE was collected from the cleaning units of fish and shrimp in a seafood production factory in Vietnam. Before using for all the experiments throughout this study, the SWE was filtered to remove the suspended grease layer. The quality parameters of the SWE were measured and listed as follow ($mg{\cdot}L^{-1}$): NH_4^+, 277.5; PO_4^{3-}, 39.3; CO_3^{2-}, 405.0; Ca^{2+}, 107.7; Mg^{2+}, 14.1; Na^+, 186.5; chemical oxygen demand (COD), 362.0; biochemical oxygen demand (BOD), 215.5; and TSS, 468.5.

2.2. Cultivation and harvesting of microalgae in different media and conditions

First, the effect of initial concentration of *C. vulgaris* on the bioflocs formation was investigated. *C. vulgaris* inoculum that cultivated in Sueoka medium was obtained at 1000.0 ± 2.0 $mg{\cdot}L^{-1}$, and was mixed with 1 L of the SWE to make up the culture medium at different initial concentration of *C. vulgaris* starting at 10.0 $mg{\cdot}L^{-1}$ with a concentration interval of 5.0 $mg{\cdot}L^{-1}$. The cultivation process was performed in a continuous aeration mode for 14 days. The microalgae productivity ratio, namely the ratio of the COD concentration in the SWE (C_{COD}, $mg{\cdot}L^{-1}$) and initial microalgae concentration ($C_{microalgae}$, $mg{\cdot}L^{-1}$), was defined in Eq. (1).

$$Microalgae\ \ productivity\ \ ratio = C_{COD}/C_{microalgae} \tag{1}$$

After obtaining the optimal microalgae productivity ratio, the experimental studies were carried out to investigate the formation of bioflocs, specifically the self-settlement of microalgae cells by bacteria aggregation on the microalgae cell surface, and the removal of nutrients from the SWE. Several different cultivation media were prepared at a pH of 8.2 ± 0.5, unless otherwise stated, as presented in Table 1. The sterilization process of the SWE was run in an autoclave at 121 °C for 30 min. While for the preparation of M3, the SWE was treated using a Pyrex Squibb separatory funnel containing cation exchange resins (Indion 220Na) to reduce the content of Mg^{2+} and Ca^{2+}. The cation exchange process was conducted using 1 kg resin to treat 1L of the SWE. After the SWE passed through the resin bed, the filtered suspension was collected for the analysis. The quality parameters of the treated SWE were as follow ($mg{\cdot}L^{-1}$): NH_4^+, 168.5; PO_4^{3-}, 8.5; CO_3^{2-}, 315.2; Ca^{2+}, 56.2; Mg^{2+}, 2.1; Na^+, 144.6; COD, 217.8; BOD, 165.5; and TSS, 206.7. For the preparation of M7, the microalgae grown in M6 were harvested using centrifugation step. Whereas for the preparation of M8, Sueoka broth was prepared and 333.0 $mg{\cdot}L^{-1}$ of $CaCl_2$ was added and pH was adjusted to 10.0 ± 0.5 (Nguyen et al., 2014).

The microalgae cultivation process was performed under a light intensity of 150 $\mu mol{\cdot}m^{-2}{\cdot}s^{-1}$ and at 27 ± 2 °C until all the microalgae cells have been reduced to content. The flocs settled down in the bottom of flask were collected for the determination of the dry matter and chlorophyll contents. Besides, the culture medium was measured for the COD contents.

2.3. Flocculation activity

The supernatant of culture broth before and after the cultivation process was counted for *C. vulgaris* cells on a Malassez counting chamber using a microscope (ProWay China, PW-BK 5000). The flocculation activity was determined using Eq. (2), by measuring the optical density of the sample at 680 nm (OD_{680}) (Kim et al., 2011; Lee et al., 2013; Oh et al., 2001).

$$Flocculation\ \ activity(\%) = (1 - OD_{680,s}/OD_{680,i}) \times 100 \tag{2}$$

where $OD_{680,s}$ is the optical density of the sample at the sample collection time and $OD_{680,i}$ is the optical density of the sample before the flocculation process.

2.4. Dry matter and chlorophyll content

The flocs formed at the bottom of culture broth was collected for the measurement of their dry matter and chlorophyll contents. Prior to passing through the vacuum filtration device for dewatering the flocs, the supernatant was withdrawn to allow efficient separation of flocs from the culture broth. The flocs were then settled down in a glass graduated cylinder of 100 mL for 2 h to allow a complete separation of a thick stable flocs layer. The flocs were dewatered in a linen filter bag (150–200 µm pore size). Then, the samples were dried at 105 °C for 24 h and cooled in a desiccator for 10 min, before weighed using a reusable filter holder.

Besides, the chlorophyll content of microalgae can be used as an indicative parameter for the growth performance of microalgae in different culture media. In this study, the chlorophyll that contained in microalgae cells was extracted using methanol. 0.5 mL of culture broth was centrifuged using a centrifuge (Eppendorf, Minispin) at 13400 rpm for 5 min for the separation of biomass and supernatant. Then, the supernatant was discarded and 1.5 mL of methanol was added to extract the chlorophyll from microalgae cells. The biomass suspension was isolated from the light for 1 h and incubated at 44 °C in an oven. After that, the suspension was centrifuged at 13400 rpm for 5 min to remove the cell debris. The absorbance of the supernatant was measured using a UV–vis spectrophotometer (Lambda 2S, Perkin Elmer). The absorbance peaks of the supernatant at wavelengths of 652 nm (chlorophyll *b*), 665 nm (chlorophyll *a*) and 750 nm (turbidity of suspension) were obtained, and were used to determine the chlorophyll content ($mg{\cdot}L^{-1}$), using Eq. (3) (Ritchie, 2006).

$$Chlorophyll = [-8.0962(OD_{652} - OD_{750}) + 16.5169(OD_{665} - OD_{750})]\frac{V_2}{V_1{\cdot}l} \tag{3}$$

where V_1 and V_2 are volume of sample suspension and methanol used, and l is the optical path, which is 1 cm. For high concentration of chlorophyll that is more than 10 $mg{\cdot}L^{-1}$, the protocols can be simplified by adding 0.25 mL of sample suspension (V_1) to 1.25 mL of methanol (V_2) before the incubation process. The rest of the procedures followed the same as described.

2.5. Seafood wastewater quality parameters

The measurement of quality parameters of the SWE, including TSS, BOD, COD, NH_4^+, Ca^{2+}, Mg^{2+}, NO_3^- and PO_4^{3-}, was performed by referring to the American Public Health Association (APHA) standard methods (APHA/AWWA/WEF, 2012). The measurements were carried out before and after the flocculation process to determine the efficiency of the nutrient removal by the formation of flocs. The efficiency of nutrient removal (E_r), particularly the reduction of the COD in wastewater effluent, was expressed in Eq. (4).

$$E_r(\%) = \frac{C_i - C_f}{C_i} \times 100 \tag{4}$$

where C_i and C_f are the initial and final concentrations of COD ($mg{\cdot}L^{-1}$) in wastewater effluent.

2.6. Compound microscope and scanning electron microscope

The flocs collected after the centrifugation operation at 3000 rpm for 1 min were resuspended in distilled water and observed using a compound microscope to verify the presence of extracellular polymeric substances.

Besides, the flocs collected was immersed in 2.5% glutaraldehyde in

0.1 M cacodylate buffer at pH 7.2 for overnight. After that, the specimen was washed 3 times using 0.1 M cacodylate buffer and followed by a post-fixing treatment using 1% osmium tetroxide in 0.1 M cacodylate buffer for 20 min. Then, the specimen was washed 3 times using 0.1 M cacodylate buffer and dehydrated in 50, 70, 90, and 100% ethanol. After the dehydration process, the sample was transferred to the chamber of a critical point drying apparatus for drying. The sample was mounted onto a metal stub and sputter coated with gold by an ion-coater, before being examined using a SEM (Hitachi, FE-SEM S4800).

3. Results and discussion

3.1. Initial concentration of microalgae

In the study, the SWE served to supply nutrients for the growth of microalgae, and the nutrient utilization allow wastewater treatment (Cai et al., 2013). In a typical condition, microalgae consume wastewater mineral nutrients and CO_2 to produce biomass and release the O_2 required by bacteria. Whereas, bacteria ingest O_2 and release CO_2 during their action to degrade COD to mineral components (Muñoz and Guieysse, 2006).

The *C. vulgaris* SAG 211-19 was cultivated in the SWE for 14 days with different initial microalgae concentration from 10.0 to 50.0 mg·L^{-1}. The flocculating activity was evaluated through the observation of flocs formation at the bottom of flask and the measurement of TSS. TSS is one of the most essential parameters for water quality since both organic and inorganic particles that are larger than 2 μm, including bacteria and algae, can contribute to the concentration of TSS (Ayana et al., 2015). Table 2 shows the results of the culture media with different initial microalgae concentration. Significant built up of flocs volume was observed at initial microalgae concentration of 20.0 mg·L^{-1}, namely at microalgae productivity ratio of 18, with the TSS removal performance of 90.0 ± 5.5%. At the culture media with the microalgae productivity ratio less than 18, the SWE medium was dense without clear formation of flocs layer due to slow settlement of biomass and low microalgae density. The cell might not sufficient to grow in low-dense culture medium and thus prevent the aggregation of microalgae to form bioflocs (Grima et al., 2003). Therefore, the initial concentration of *C. vulgaris* added into the culture medium of 20.0 mg·L^{-1} was chosen for further studies.

3.2. Flocculation process in different media

The adhesion of bacteria and microalgae to form MaB-flocs is closely related to the secretion of the extracellular polymeric substances by bacteria (Salehizadeh et al., 2000). Many studies reported the close relationship between extracellular polymeric substances and TSS to form bioflocs (Dertli et al., 2015; Jimenez et al., 2007). The researchers reported that the amount of extracellular polymeric substances released by bacteria was closely related to the available space in bioflocs. The flocculating activity increases with the increase of the extracellular polymeric substances content. The suggestion was supported by the results presented in Table 3 with different types of culture media prepared. The decrease of TSS content in supernatant of culture medium altered the performance of TSS removal and flocs concentration in M4 that consisted of various aerobic bacteria that are capable to produce extracellular polymeric substances layer.

Besides, wastewater nutrient removal efficiency is important in a wastewater treatment protocol. The effects of bacteria on flocs formation and simultaneously the nutrient removal performance are one of the main focus of this work. Therefore, it is essential to evaluate the growth of microalgae in the SWE. The results showed that the growth of *C. vulgaris* in M1, M2, M3 and M4 was similar by the utilization of nutrients from the SWE to microalgae cells. This nutrient conversion can be evaluated by analyzing nutrient removal efficiency and chlorophyll content of flocs (Ji et al., 2013; Lee et al., 2013). Among the culture media composed of the SWE, M4 showed the highest nutrient removal efficiency of 88.0 ± 2.2% and relatively chlorophyll concentration of 25.5 ± 0.2 mg·L^{-1}. Therefore, it can be concluded that the formation of flocs has positive impact in both wastewater treatment and microalgal biomass production. Moreover, the chlorophyll content of flocs obtainned from the untreated SWE (M4) is relatively comparable to those obtained from Sueoka medium (M8), indicating the successful bioconversion of wastewater nutrients and CO_2 into microalgae biomass.

Compared to the TSS removal performance, flocculating activity was determined in relative values by comparing the optical density of the culture medium before and after the flocculation process. It is worth noting that the untreated SWE in M4 promoted a valuable flocculating activity, which indicating that *C. vulgaris* was capable to settle down by gravity in the culture broth without any harvesting technique applied. M4 consisted of excess nutrients that were sufficient to support the growth of both microalgae and bacteria to enhance the formation of MaB-flocs. Compared to M4, M1 with the sterilized SWE offered significant low flocculating activity. This might be due to the lack of bacteria, and insufficient metal cations content as well as pH value in performing flocs formation. Additionally, it was observed that the flocculating activity of at least 78.0 ± 6.4% was achieved for those culture media with the presence of bacteria (M2, M3 and M4). Nonetheless, it was found that the highest flocculating activity achieved at 99.0 ± 5.5 for M8 containing Sueoka broth with the addition of Ca^{2+} and adjusted pH value to 10.0 ± 0.5. Both high pH and sufficient magnesium concentration (> 0.15 mM) are essential to induce the autoflocculation process (Vandamme et al., 2012a). This autoflocculation process occurs due to the change of surface properties of microalgae cells when there are changes in nitrogen, pH, dissolved oxygen and presence of calcium and magnesium ions in the culture media (Ummalyma et al., 2017; Vandamme et al., 2013). The results reported were consistent with those reported by other researchers specifically about the impact of bacteria, divalent cation and pH in inducing flocculation formation (Han et al., 2016; Pacheco-Vega et al., 2018; Quijano et al., 2017; Van Den Hende et al., 2016; Vandamme et al., 2013).

The results showed that the microalgae harvested were able to grow well when they were co-cultivated together with bacteria as a bioflocculant. This technique might reduce the production cost of microalgae and at the same time improve wastewater treatment strategies without using chemical substances and the coercion in increment of pH value. The use of wastewater like the SWE in this study allows the cultivation and harvesting of microalgae, and simultaneously the removal of nutrients which eases the further wastewater treatment steps (Alcántara et al., 2015; Cuellar-Bermudez et al., 2017; Ummalyma et al., 2017; Wang et al., 2017).

Table 2
The effects of different initial microalgae concentrations on the occurrence of bioflocculation and the results of TSS removal.

Microalgae concentration (mg·L^{-1})	10.0 ± 1.3	15.0 ± 1.1	20.0 ± 1.2	25.0 ± 1.1	35.0 ± 1.0	40.0 ± 1.1	45.0 ± 1.1	50.0 ± 1.2
Microalgae productivity ratio	36	24	18	14.5	12	10	9	8
Flocculation occurrence	No clear formation	No clear formation	Yes	Yes	Yes	Yes	Yes	Yes
TSS removal (%)	20.1 ± 7.0	20.6 ± 10.5	90.0 ± 5.5	94.5 ± 4.2	95.0 ± 6.0	95.0 ± 7.2	94.2 ± 4.5	94.0 ± 6.8

Table 3
The results of different culture media prepared in terms of flocculating activity, TSS removal, chlorophyll and dry matter contents of flocs, and wastewater nutrient removal.

Medium	M1	M2	M3	M4	M5	M6	M7	M8
Flocculation activity (%)	8.7 ± 2.5	78.0 ± 6.4	85.0 ± 2.2	92.0 ± 6.0	75.0 ± 2.5	– [a]	– [a]	99.0 ± 5.5
TSS removal (%)	14.3 ± 3.5	80.0 ± 3.5	81.0 ± 7.0	93.0 ± 5.5	78.0 ± 8.2	– [a]	– [a]	90.0 ± 4.0
Flocs chlorophyll ($mg{\cdot}L^{-1}$)	3.5 ± 0.3	22.8 ± 2.4	29.0 ± 1.6	25.5 ± 0.2	22.4 ± 2.1	– [a]	– [a]	26.1 ± 0.8
Flocs dry matter ($g{\cdot}L^{-1}$)	8.3 ± 4.72	81.2 ± 3.5	82.2 ± 2.6	107.2 ± 5.6	91.1 ± 3.4	– [a]	– [a]	47.5 ± 7.8
Nutrient removal (%)	78.4 ± 2.3	82.3 ± 5.2	78.1 ± 2.5	88.0 ± 2.2	– [b]	– [b]	– [b]	– [b]

[a] No formation of flocculation in the respective culture medium.
[b] Sueoko broth was used instead of the SWE for M5 to M8. Therefore, no measurement was needed for wastewater nutrient removal performance.

3.3. Flocculation formation

To further understand the key factors for the formation of bioflocs, the results of different culture media presented in Table 3 were discussed. The flocculating activities in M3 and M4 were relatively high at 85.0 ± 2.2 and 92.0 ± 6.0%, respectively. The results were consistent with the removal of TSS calculated. Despite the absence of flocculation process in M6, M5 containing Sueoka broth with added *E. coli* demonstrated flocculation activity of 75.0 ± 2.5%. Likewise, M2 containing sterilized SWE with added *E. coli* promoted flocculation activity of 78.0 ± 6.4% when compared to M1 consisting of only sterilized SWE with the flocculation activity of only 8.7 ± 2.5 acquired. All these results suggested that the bacteria play a key role in the formation of flocculation. When compared to M5, M3 that consisted of diverse aerobic bacteria can lead to a more efficient adhesion of cells although the concentration of primary divalent cations present in the culture medium was not sufficient to promote the aggregations of microalgae cells. The adhesion of bacteria on microalgae cell surface created a large biofilm to enhance the attachment of microalgae cells around this layer until the aggregation size is sufficient for auto-settlement by gravity.

The use of chemical flocculants for microalgae harvesting might not appreciated owing to their high pH dependence, high cost, and large accumulation of many chemical compounds on microalgae cells (Farid et al., 2013; Wan et al., 2015). It must be noted that the presence of high pH in culture medium is a major factor for the creation of auto-flocculation (Bhola et al., 2011; Nguyen et al., 2014; Vandamme et al., 2012b; Vandamme et al., 2013). The same conclusion was also drawn in this study. There was no formation of flocs in M8 until the medium's pH was increased to 10.0 ± 0.5. At the pH of 10.0 ± 0.5, the flocs formed instantly and increased up to 99.0 ± 5.5%. Besides, the presence of high concentration Ca^{2+} and Mg^{2+} content in culture medium has aided in improving the formation of flocs when comparing the results obtained for M3 and M4. Nevertheless, there was no flocculation occurred in M8 containing added Ca^{2+} of 120.0 $mg{\cdot}L^{-1}$ at pH of 8.2 ± 0.5, although the concentration of Ca^{2+} in M8 is almost equivalent to those in M4, namely at 107.7 $mg{\cdot}L^{-1}$.

3.4. Microscopic images of the harvested biomass

The flocs formation mechanism was well formulated by the location of bacteria (Nguyen et al., 2014; Vandamme et al., 2013). The results of SEM images of the harvested microalgae from different media were presented in electronic supporting information. The converging of aerobic bacteria around *C. vulgaris* cells in M4 was remarkable compared to M5 containing *E. coli* that the adsorption of *E. coli* and microalgae was relatively sporadic. The higher flocculation activity of M4 than M5 might be due to the significant interactions of various bacteria on microalgae cell surface forming conditional films. The findings were in good agreements with several works published concerning the increment of bioflocs content in culture medium containing various bacteria (Pacheco-Vega et al., 2018; Quijano et al., 2017). The results suggested that the bacteria play important role in the adhesion of suspended microalgae in order to perform bioflocculation process.

Bacteria attachment on microalgae cells is the critical step in bioflocs formation. The attachment was performed by the extracellular polymeric substances excreted by bacteria to derive a conditional film (Bos et al., 1999). The layers of extracellular polymeric substances were found in those samples collected from M2, M3, M4 and M5 that contain bacteria in their medium, as illustrated in electronic supporting information. Whereas, the microscopic image of harvested samples from culture media such as M1, M7 and M8 demonstrated the absence of extracellular polymeric substance. The microalgae cells have the tendency to become a large bioflocs when slimy layers of extracellular polymeric substances were formed. Moreover, the aggregation of microbial population can be observed from the harvested biomass obtained from M3 and M4. For M3 consisting of *E. coli*, the adhesion of *E. coli* with planktonic microorganism might be difficult. The interference with other cells has led to a weak absorbance of these specific molecules. Compared to M3, the accumulation of diverse aerobic bacteria in M4 was developed fast, which might be attributed to the specific interactions between localized molecular groups. The cohesion occurred between bacteria species has accelerated the produce of extracellular polymeric substances that were beneficial for the attachment of planktonic microorganism. When the microorganisms have adhered to each other, they grew progressively to create an accumulation of large number of bacteria on microalgae cell surface.

In order to compare the difference of adherence of microalgae supporting by divalent cations and bacteria, the SEM images of the harvested biomass obtained from M1, M7 and M8 were observed. There was a discrete connection of microalgae cells in M1 and M8, while bacteria in other mediums, suggesting that the bacteria size was much smaller than microalgae. Moreover, the extracellular polymeric substances appeared as a messy stacking in every microalgae cells when observed under compound microscope. It was therefore concluded that the role of bacteria on aggregation of microalgae cells to form the bioflocs is indispensable because of their small size and the capacity to secrete extracellular polymeric substances. The proposed bioflocculation technique in this work is viable for microalgae harvesting and wastewater treatment in a single step. This method is associated with several advantages, such as simplicity, low cost, low energy consumption and environmentally friendly.

4. Conclusions

Bioflocculation using bacteria in wastewater effluent is an alternative approach for harvesting the microalgae biomass from the huge volume of culture broth. The study demonstrated the use of seafood wastewater in cultivation and harvesting of *C. vulgaris*. The direct use of untreated SWE as culture medium for *C. vulgaris* allowed the flocculation activity of 92.0 ± 6.0%, TTS removal of 93.0 ± 5.5%, and nutrient removal of 88.0 ± 2.2%. The MaB-flocs collected under this optimal condition contained dry matter of 107.2 ± 5.6 $g{\cdot}L^{-1}$ and chlorophyll content of 25.5 ± 0.2 $mg{\cdot}L^{-1}$.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2018.09.146.

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ARTICLE

Chicken Eggshell as an Innovative Bioflocculant in Harvesting Biofloc for Aquaculture Wastewater Treatment

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ABSTRACT

Implementation of biofloc technology (BFT) system in aquaculture industry shows high productivity, low feed conversion ratio, and an optimum culture environment. This study was divided into two phases. The first phase involved maintaining the water quality using the optimum carbon-to-nitrogen ratio by manipulating pH in culture water. The second phase examined the performance of harvesting biofloc (remaining phytoplankton and suspended solids in the system) using chicken eggshell powder (CESP). This study showed that pH 7 to 8 were the best biofloc performance with high removal percentage of ammonia (>99%) with a remaining ammonia concentration of 0.016 mg L^{-1} and 0.018 mg L^{-1}, respectively. The second phase of this study was performed to determine the optimal formulation and conditions of using CESP as a bio-flocculant in harvesting excess biofloc. The use of eggshell showed a higher harvesting efficiency of more than 80% under the following treatment conditions: 0.25 g L^{-1} of eggshell dosage; with rapid and slow mixing rates of 150 and 30 rpm, respectively; 30 min of settling time; settling velocity of 0.39 mm s^{-1} and pH of 6 to 7. Therefore, the results indicated that biofloc would be the best green technology approach for sustainable aquaculture wastewater and the CESP is an organic matrix that environmental-friendly bio-coagulant for biofloc harvesting.

KEYWORDS

Chicken eggshell; aquaculture; biofloc; coagulant; water quality; harvesting

1 Introduction

As food consumption has increased with population growth, aquaculture has become a growing industry for sustaining food supplies [1]. Nevertheless, the expansion of the aquaculture industry is constrained due to the environmental pollution resulting from the effluent rich in nutrient compounds such as phosphorus, ammonia, nitrite, nitrate and pathogens [2]. Aquaculture systems adopting Bioflocs Technology (BFT) could solve this problem by effectively removing the nutrients while generating beneficial microbial biomass of high protein content [3]. Bioflocs are defined as an agglomeration of macroaggregates diatoms, macroalgae, microalgae, fungi, faecal pellets, exoskeletons and dead organisms, bacteria, and invertebrates [4].

BFT enhances water quality by recycling nutrients and maintaining carbon and nitrogen ratio by introducing carbon sources to stimulate heterotrophic bacteria. The bacteria convert the nitrogenous waste from the uneaten feed and faeces into microbial biomass, making it possible for the protein (present in the feed and microorganism) to be eaten twice by the cultured fish and simultaneously reducing the waste generated in the aquaculture system [5]. This is due to the existence of microorganisms with ability to degrade ammonia, nitrite and chemical oxygen demand (COD) in aquaculture wastewater [6].

Although BFT shows high productivity, low feed conversion ratios, and provides a stable culture environment in the aquaculture industry, there are several challenges needed to be solved for the successful application of BFT, namely: determining the optimal C/N ratios, temperature fluctuations, low alkalinity and salinity, broad pH range, and high concentration of suspended solids. Solids concentration management in BFT systems is also a problem that should be highlighted. Excessive solids concentration has an adverse effect on the performance of BFT systems, including clogging gills of aquatic life that affect the productive system [7]. Therefore, this technology requires a comprehensive understanding of all microorganisms involved in every biological filter process as part of the BFT system [8]. In the activated sludge process, bioflocs can break up into smaller particles due to shear forces during aeration, pumping and dewatering process. This condition led to the breakage of weak bioflocs and could generate a large number of smaller particles that do not settle readily. This circumstance led to the increase of suspended solids and turbidity in the water bodies [9].

Therefore, the use of bioflocculant is needed to encounter the problems coagulation-flocculation method. In this process, flocculants are valuable agents for the agglomeration of colloids, cells and suspended particles. Flocculants can be categorized into three groups: synthetic organic flocculants, such as polyethyleneimine and polyacrylamide, inorganic flocculants, such as aluminium sulphate and poly aluminium chloride. And natural flocculants (bio-flocculants) such as chitosan [10] and various bioflocculants include chitosan, eggshell, *Moringa oleifera*, and microbial flocculants such as *Aspergillus niger* and *Scenedesmus* [11,12]. These types of flocculants have great potential for use in industrial applications. However, limitations, such as low flocculation efficiency and large dosage requirement, need to be dealt with [13].

Chicken eggshells are greatly recommended as bio-flocculant because they are non-toxic, non-corrosive and safe to handle [14]. Hence, the present study was proposed to determine the optimum condition of dosage, pH and settling time of eggshells as bio-flocculant to harvest the remaining or breakage bioflocs and maintain the alkalinity level. Besides, the reduction in alkalinity and pH occurs due to the consumption of inorganic carbon by bacteria present in the bioflocs. As the level of alkalinity becomes crucial in stabilizing the aquaculture system, the characteristics of eggshells that comprise an amorphous calcium carbonate matrix and the mineralized material contribute to the alkalinity supplementation [15]. Moreover, eggshells have a high cationic (Ca^{2+})charge density and can, thus, strongly adsorb and destabilize negative particles, such as negatively charged microbial cells, especially microalgae cells [16]. Calcium (principally precipitate) plays a significant role in the formation and growth of granules. In contrast, the location of calcium precipitates and microbial ecology of the granule appeared to have no additional effect on the shear strength [17].

The positive effect of Ca^{2+} on the aggregation of non-fed fine anaerobic granular sludge is likely to induce the formation of bigger particles and thus prevent their wash-out from anaerobic bioreactors due to Ca^{2+} addition significantly altered their limit viscosity values [18]. Besides, Ca^{2+} promotes cell-to-cell bridging formation, improving the biopolymer's aggregation and stabilization, which could enhance bioflocculation and the overall granulation process [19]. Therefore, chicken eggshell, as a polymer with high cationic charge density has the potential to strongly agglomerate bioflocs. It can retain microbial cells on its surface through charge neutralization and polymer bridging. Consequently, it is crucial to surmount these problems and improve the flocculation processes to optimize its effective utilization. The harvested bioflocs can be used in *ex-situ* feeding applications with acceptable water quality performance [20].

2 Material and Methods

2.1 Experimental Design

Bioremediation treatment utilizing BFT was carried out in batch culture with six different pH of 5, 6, 7, 8, 9 and 10 with an optimal C/N ratio of 15 based on our previous study stated that treatment of C/N 15 was determined as the optimum C/N ratio yielding the highest removal of ammonia (98.7%) [21]. Optimization of C/N ratio is specific to different types of aquaculture species selected, and it is crucial to effectively reduce the nutrient concentration in water. Biofloc was generated by adding organic carbon and continuous aeration, reducing dissolved nitrogen from 22% to 14%, sedimentary nitrogen from 49% to 5%, and increasing nitrogen accumulation in biofloc and fish biomass. Molasses acting as carbon sources were transferred to the treatment culture tank after being fermented for 24 h to enhance the breakdown process by the bacteria or microorganisms for bioflocs formation. The adjustment of C/N ratio by molasses was adopted from the experiment [21]. In order to observe the characteristics of bioflocs, 18 units of rectangular tanks were designed for the bioremediation treatment, and three other tanks served as controls (without the addition of a carbon source and pH adjustment). The procedure for determining the amount of molasses required for an effective treatment efficiency based on the amount of fish nitrogen excretion is shown in Fig. 1.

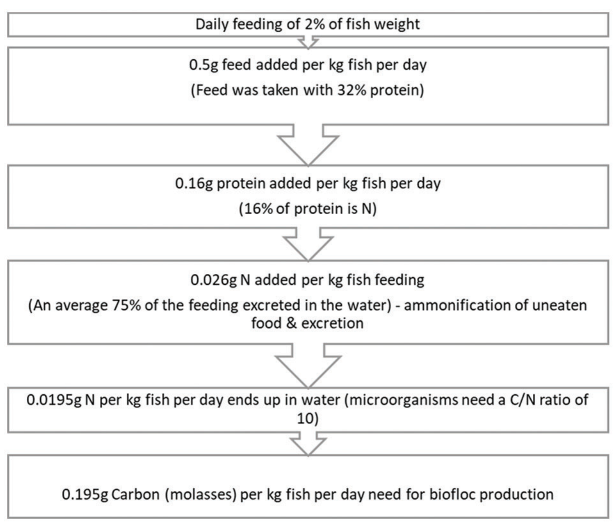

Figure 1: Carbon-Nitrogen procedure measurements

In order to ensure uniform wastewater characteristics, 20 individuals of catfish (*Clarias gariepinus*) with an average weight of 6 g/fish were maintained in each of the 8 L treatment tanks at a controlled temperature of about 28 ± 2°C. The aerator was set up in the middle section of the tank with continuous aeration. Feed (Dindings Super Keli Fish Feed 96000 with 35.43% crude protein), approximately 2% of the fish weight was given once daily. The substance of hydrochloric acid (HCL) or sodium hydroxide (NaOH) was added to the system for 30 days to maintain the pH set for the experiment. The accumulations of ammonia and nitrate concentration were observed after three days of culture period in freshwater [7]. After three days of fish rearing in each treatment tank, the water sample was collected for water quality testing to investigate the nitrogenous compound removal under the effect of pH conditions. Then, the optimal C/N ratio and pH condition for bioflocs formation was used to investigate the performance of chicken eggshells as a coagulant in harvesting the remaining bioflocs. Finally, the mechanism output was used to determine the optimal condition of the harvesting process based on the harvesting efficiency. The potential users of the harvested product produced from this research which consists of solid product (bioflocs biomass) and liquid product (settled water) were determined based on proximate analysis (AOAC Method) and water quality analysis.

2.2 Bioflocs Formation

The volume of bioflocs was produced to indicate the biofloc performance. A simple method to measure the *in-situ* bioflocs formation was to use Imhoff cone [7]. 1000 mL of water sample was collected and transferred into Imhoff cones. Then, the volume of the biofloc was allowed to settle for 15 min before the water quality analyses and harvesting process.

2.3 Water Quality Analysis

Water quality testing is an integral part of environmental monitoring in the aquaculture industry and is highlighted in this study. The water quality analyses were carried out with the collection of 50 mL of the water sample from each treatment and control tank at 3-day intervals until 30 days treatment period. The water sample was centrifuged at 6000 rpm for 10 min by Eppendorf 5702 Variable Speed Multi-Purpose Centrifuge to obtain a clear supernatant [2]. The supernatant was analyzed using a Dual-Beam UV-Vis spectrophotometer to monitor the ammonia, nitrite, and nitrate concentration. The percentage of ammonia removal was calculated using Eq. (1):

$$\text{Percentage Removal, } (\%) = \frac{\text{Control} - \text{Remaining (R)}}{\text{Control}} \times 100\ (\%) \tag{1}$$

2.4 Preparation of Chicken Eggshell Solution

The collected chicken eggshell from cafeterias around Universiti Malaysia Terengganu (UMT) was thoroughly washed using warm distilled water. Then, the eggshell was dried in a conventional oven at 104°C for 24 h. The dried eggshell was ground and sieved using a 325-mesh sieve to obtain a fine powder. The chicken eggshell solution (bio-flocculant) was prepared based on the method reported by [14]. The stock solution was prepared by dissolving 100 mg of eggshell powder in 10 mL of 0.1 mol L^{-1} HCl solution with continuous stirring at 100 rpm for 30 min until a clear solution was achieved. The stock solution was diluted with 100 mL of deionized water to obtain a final concentration of 1000 mg L^{-1}.

2.5 Harvesting of Bioflocs Using Chicken Eggshell Powder (CESP)

Excess bioflocs commonly occur in the culture ponds that use BFT. The excess bioflocs that were not settled at the bottom tank and released as waste from BFT were harvested using eggshells as a coagulant. It can be used as a feed for the aquaculture industry while minimizing environmental problems. The floc jar test was used to determine the optimal condition in terms of dosages of eggshell and mixing rate of eggshell. Various dosages of (0, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.40, 0.45 and 0.5 g L^{-1}) and pH ranges (5–10) were run at fixed rapid and slow mixing rate at 150 and 30 rpm, respectively. After determining the optimal dosage and pH, this study examined the flocculation condition by varying the slow mixing rate from 10 to 50 rpm. The optical density of the supernatant was measured at 600 nm using Dual-Beam UV-Vis Spectrophotometer (Shimadzu UV-1800, Japan) to determine the flocculation by determination of harvesting efficiency and biomass recovery. The harvesting efficiency (%) and biomass recovery (%) of nutrients were determined by Eqs. (2) and (3):

$$\text{Harvesting Efficiency } (\%) = \frac{OD_{initial} - OD_{final}}{OD_{initial}} \times 100\ (\%) \quad (2)$$

$$\text{Biomass Recovery } (\%) = \frac{OD_{final} - OD_{initial}}{OD_{final}} \times 100\ (\%) \quad (3)$$

where $OD_{initial}$ is optical density 600 nm of initial culture and OD_{final} is optical density 600 nm at clarified zone.

2.6 Statistical Analysis

Statistical analyses of this study were performed through IBM SPSS ver. 23.0. Normality and homogeneity of variances of the data were satisfied via Shapiro-Wilk test and Levene's test, respectively. The removal efficiency of ammonia (%) in different pH conditions (pH 5, pH 6, pH 7, pH 8, pH 9, and pH 10) in BFT culture system was analyzed by One-Way Analysis of Variance (ANOVA), followed by Tukey HSD test. Results were considered as statistically significant at $p < 0.05$ in this experiment.

3 Results and Discussion

3.1 Effect of pH on Biofloc Formation

The study on the effect of pH was carried out at an optimal C/N ratio of 15 for 30 days treatment period to evaluate the performance of biofloc (biofloc volume) at six different pH (5, 6, 7, 8, 9 and 10). Based on Fig. 2, the acclimatization period observed for biofloc at the beginning of treatment period for about one week. The biofloc formation was observed from the Day 0 to Day 6 onwards. The volume of biofloc at pH 7 and 8 showed a significantly high trend or pattern as compared to other pH. Whereas the biofloc volume at other pH (5, 6, 9 and 10) were relatively low. During the treatment period, pH 7 and 8 produced the highest biofloc volume of more than 90.0 mL L^{-1}.

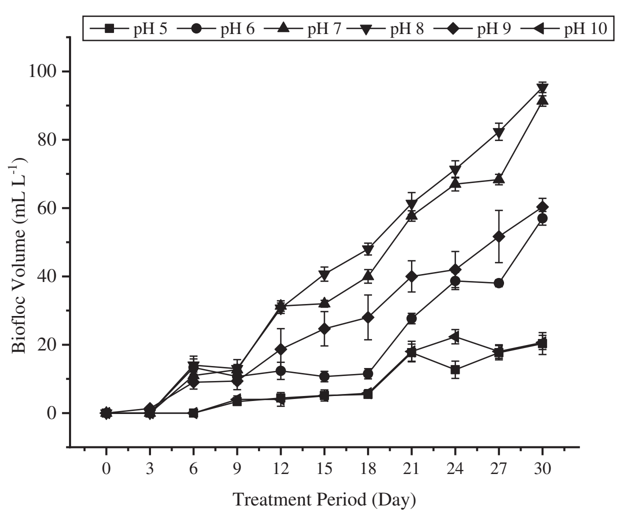

Figure 2: Volume of C/N 15 bioflocs at different pH for 30 days treatment period

This result is in accordance with Luo et al. [22], which reported that pH at neutral to weakly alkaline conditions resulted in high growth of bacteria and enhanced the formation of biofloc. An optimum pH concentration must be maintained to produce high biofloc formation since the optimum pH can increase microbial activity. This finding was supported by Martins et al. [23], who stated that the abundance of microorganisms depended on the pH of the environment (pH 6.5 to 7). Nasir et al. also reported a similar finding [24] that pH from 7 and above is suitable for the bacteria to enhance biofloc formation.

3.2 Bioflocs Performance in Reducing Nitrogenous Compound

The changing of pH might exhibit a low rate of microbial nitrogen assimilation and lead to increased inorganic nitrogen such as ammonia, nitrite, and nitrate in the culture system. This circumstance explains that the difference in pH could affect the ammonia concentration between the treatments. Table 1 shows the removal of the nitrogenous compound (ammonia) and the remaining ammonia concentration at various pH values ranging from 5 to 10. This study showed that the pH condition ranging from pH 7 to 8 showed the best performance of biofloc due to the capability of maintaining an effective nutrient reduction. This condition is due to the high biofloc volume recorded in this pH range (7 to 8) that promoted the nitrogen accumulation process by the abundance of microbial cells [4]. There are no data recorded for ammonia removal at Day 0 up to Day 6 for all pH. This is because autotrophic nitrification and heterotrophic nitrification were delayed during the lag period, so it has a long generation time to acclimate to a new environment [25]. Moreover, during this stage (Day 0 to 6), there was no biofloc formation which led to the low density of autotrophic and heterotrophic microorganisms [21]. This condition was parallel to the Navada et al. [26], which stated that the availability of nitrogen and organic carbon in water treatment resulted in a high density of autotrophic and heterotrophic microorganisms.

Table 1: The percentage removal and the remaining of ammonia in catfish farming tank under the different pH condition

Day	Control	pH 5		pH 6		pH 7		pH 8		pH 9		pH 10	
		%	*R*	%	*R*	%	*R*	%	*R*	%	*R*	%	*R*
0	0	N/A	0	N/A	0	N/A	0	N/A	0	N/A	0	N/A	0
3	0	N/A	0	N/A	0	N/A	0	N/A	0	N/A	0	N/A	0
6	1.14^{a}	N/A	2.15^{b}	N/A	2.10^{b}	N/A	2.20^{b}	N/A	2.12^{b}	N/A	2.12^{b}	N/A	2.15^{b}
9	2.78^{a}	10.07	$2.50^{a,b}$	35.30	1.80^{d}	31.29	$1.91^{c,d}$	37.05	1.75^{d}	21.22	$2.19^{b,c}$	8.27	2.55^{a}
12	2.79^{a}	12.19	2.45^{b}	37.99	1.73^{d}	53.41	1.30^{e}	56.63	1.21^{e}	17.56	2.3^{c}	22.94	2.15^{c}
15	2.39^{a}	1.67	2.35^{b}	28.03	1.72^{c}	71.55	0.68^{d}	58.16	1.00^{e}	38.08	1.48^{c}	12.97	2.08^{b}
18	2.42^{a}	10.33	2.17^{b}	29.75	1.70^{c}	84.71	0.37^{d}	69.01	0.75^{e}	58.26	1.01^{f}	34.71	1.58^{c}
21	2.31^{a}	24.24	1.75^{b}	48.05	1.20^{c}	94.37	0.13^{d}	83.55	0.38^{e}	69.26	0.71^{f}	48.05	1.2^{c}
24	2.28^{a}	38.60	1.40^{b}	56.14	1.00^{c}	94.74	0.12^{d}	89.04	0.25^{d}	70.61	0.67^{e}	35.09	1.48^{f}
27	2.3^{a}	45.65	1.25^{b}	67.39	0.75^{c}	99.22	0.018^{d}	99.17	0.019^{d}	71.74	0.65^{c}	45.65	1.25^{b}
30	2.31^{a}	50.22	1.15^{b}	69.69	0.70^{c}	99.31	0.016^{d}	99.22	0.018^{d}	74.46	0.59^{c}	44.59	1.28^{b}

Note: % = Percentage removal of ammonia; R = The remaining concentration of ammonia in mg L^{-1}; N/A = Not applicable.

At pH close to neutral (7–8), a significantly high ammonia removal efficiency of more than 90% was achieved, with remaining ammonia concentration recorded at 0.016 and 0.018 mg L^{-1}, respectively. In contrast, ammonia removal efficiency recorded at the other pH 5, 6, 9 and 10 were lower than that recorded at pH 7 and 8. This condition indicates the occurrence of incomplete decomposition processes performed by the probiotic bacteria; thus, nutrient removal, especially ammonia tended to decrease [27]. Furthermore, the ammonia concentration recorded on day 30 for both pH 7 and 8 showed compliance with the water quality standard of specified water sources in fish farms less than 0.3 mg L^{-1} and no statistically significant differences ($p > 0.05$ in HSD-Tukeys test) were observed [22]. However, at a pH of strong alkaline (pH 10), the trend for the R (remaining concentration of ammonia) presented from 9th day–30th day showed fluctuation. This circumstance is because ammonia depends on the pH of the culture, and the concentration of free ammonia increases with pH. Increased pH encourages the production of more toxic unionized forms (NH_3). At the daytime, with the source of solar energy, the photosynthesis process is in active mode, and this will contribute to the high dissolved oxygen (DO) level. The DO concentration affects the concentration of ammonia. This is because the heterotrophic and autotrophic bacteria can break up organic compounds and ammonia with an adequate DO concentration [28]. Whereby during the nighttime (very low), so DO become lower with no occurrence of the photosynthesis process. Therefore, the cycle process of day and night might influence the reading of ammonia remaining concentration in the treatment tank.

At pH 7 and 8, the concentration of ammonia, nitrite and nitrate was low from Day 21 onwards. This elucidated that the bioflocs underwent formation and breakage by utilizing the available nutrient in the treated water. Based on Fig. 2, a significant maximum bioflocs growth and surplus likely occurred on Day 21. Since the catfish was more endurance than the tilapia, this statement was in line with the research by Kanu et al. [29], which found that catfish had a significantly higher survival probability than tilapia, so the surplus bioflocs should be more than 50 mg L^{-1}. Therefore, surplus bioflocs should be harvested.

3.3 Performance of Chicken Eggshell as Bio-Flocculant in Harvesting Bioflocs

Harvesting technology consists of various methods such as centrifugation, gravity filtration, ultrafiltration, flotation and flocculation [30]. However, this study focused on flocculation using chicken eggshells as bio-flocculant for harvesting excess bioflocs. The important characteristics of eggshells are non-toxic, non-corrosive and easy or safe to handle. Thus, this bio-waste is potentially suitable for use as a coagulant [14].

3.3.1 Effect of Dosage Eggshell on Harvesting Process

The floc jar test results showed optimal flocculation process performance with the appropriate bio-flocculant dosage, pH and mixing agitation condition. The effect of different eggshell dosages (0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45 and 0.5 g L^{-1}) on the efficiency of harvesting bioflocs was monitored. Based on Fig. 3, the maximum harvesting efficiency and biomass recoveries were 81% and 80%, respectively, with the eggshell dosage recorded at 0.25 g L^{-1}. However, Choi [14] reported that the dosage of eggshell 0.08 g L^{-1} was encouraging dosage for harvesting single species, *Chlorella* sp. On the contrary, this study focused on harvesting a complex of species/microorganisms contained in bioflocs. The encouraging harvesting efficiency achieved using the same eggshell bio-flocculant found that the dosage of eggshell 0.08 g L^{-1} was used for harvesting only one species (*Chlorella* sp.) [14]. However, this study focused on harvesting a complex of species/microorganisms contained in bioflocs.

3.3.2 Effect of Mixing Rate on Harvesting Process

High efficiency of biomass recovery of up to 80% was achieved at a slow mixing rate between 30 and 50 rpm, as shown in Fig. 3. 30 rpm was found to show the best harvesting efficiency and biomass recovery of 81% and 80%, respectively (Fig. 4). This slow flocculation mixing rate was appropriate for bioflocs cells to be agglomerated with the eggshells. The results indicated that the mixing rate had a significant impact on flocculation efficiency, including harvesting efficiency. This is in line with the finding from Bakar et al. [21] and Li et al. [31], which stated that the mixing regime or mixing rate becomes a critical factor that controls the flocculation process towards the perspective of harvesting efficiency and energy saving.

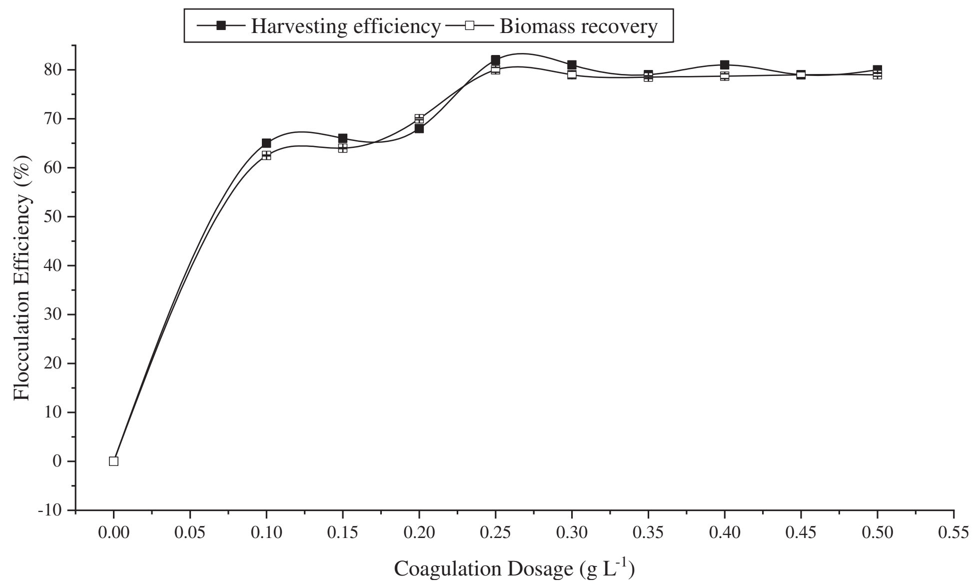

Figure 3: Effect of eggshell dosage on harvesting efficiency and biomass recovery

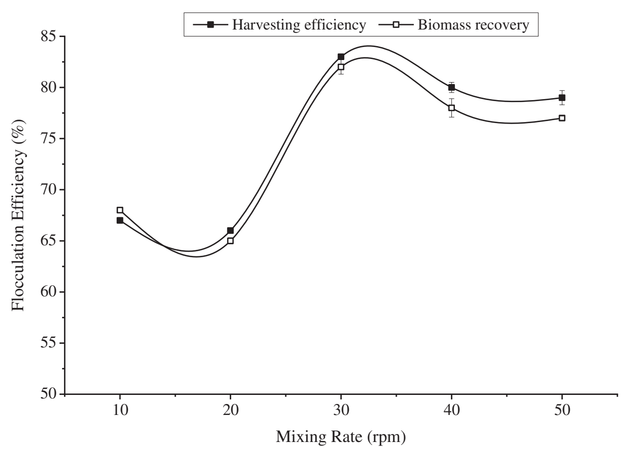

Figure 4: Effect of mixing rate on harvesting and biomass recovery

3.3.3 Effect of pH on Harvesting Bioflocs

The optimum growth pH refers to the most favorable pH for the growth of an organism. The lowest pH value an organism can tolerate is called the minimum growth pH, and the highest is called the maximum growth pH [32]. The selected optimal dosage 0.25 gL^{-1} and mixing rate 30 rpm that produced the highest harvesting efficiency of more than 80% was used to study the effect of pH (Fig. 5). The results showed that pH at 6–7 was appropriate since it produced the highest harvesting efficiency of more than 80%. A similar group of harvesting efficiency and removal efficiency was discovered between pH 6 and pH 7 (v/v) via Post hoc Tukey's HSD test ($p > 0.05$). However, a slight decrease in the harvesting efficiency was observed (65%) when the pH was increased to 8–10. The effect of pH could be explained by the physical properties of eggshells and physicochemical interactions between eggshells and microorganisms in bioflocs cells [33]. At neutral pH, coagulants retained a coil-like structure that fascinated the other microorganisms in bioflocs to bind and form a large molecule, resulting in high harvesting efficiency.

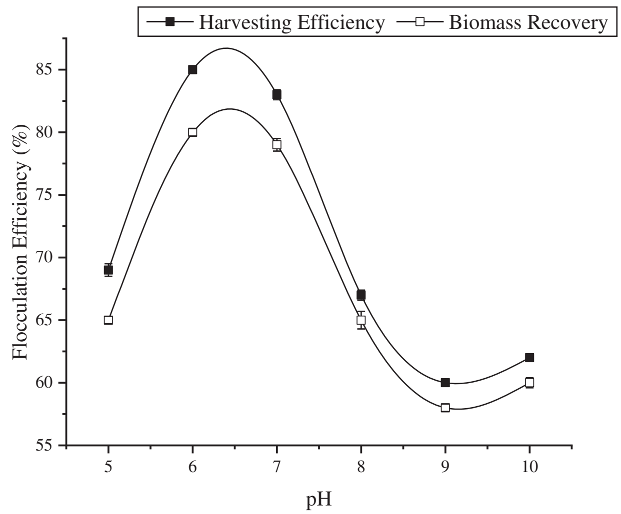

Figure 5: Effect of eggshell pH on harvesting efficiency and biomass recovery

Besides, the divalent metal ion, such as ion calcium (Ca^{2+}) that was present in bio-coagulant (eggshell), could influence the harvesting efficiency by coagulating the negatively charged microorganisms in bioflocs to form a large and robust molecule [34]. At low pH, the hydrogen ion concentration was high. Thus, ion Ca^{2+} had to compete with the ion hydrogen for binding sites on microorganisms in bioflocs, and this led to reduced harvesting efficiency [35]. On the contrary, at pH 6–7 with a low concentration of hydrogen ion, the presence of Ca^{2+} promotes the binding process with microorganisms in bioflocs that produce higher harvesting efficiency and contribute to higher biomass recovery. For example, the optimal pH between 6–7 produced a higher biomass recovery of >70%. In addition, the promotion of Ca^{2+} through eggshells could maintain water quality in BFT by the maintenance of alkalinity levels in order to minimize the daily fluctuation of pH [36].

4 Conclusion

The results obtained from this study demonstrated the best condition of effective bioflocs formation at pH close to neutral with recorded the encouraging of ammonia removal at day-30 of more than 98% percentage removal efficiency. However, a little bit high concentration of ammonia (0.75 mg L^{-1}) was recorded on day-18 due to the optimum growth of bacteria at this stage. In addition, the utilization of eggshell as an organic matrix showing potential to be used as a coagulant to control the nutrient in aquaculture wastewater with more than 80% harvesting efficiency under condition of 0.25 g L^{-1} of eggshell dosage; rapid and slow mixing rate of 150 rpm and 30 rpm; and pH of 6 to 7. The use of eggshells indicated good performance in harvesting bioflocs and polishing aquaculture wastewater and is considered a sustainable practice for the aquaculture industry towards an environmentally friendly approach.

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formatting, conduct on final revision of manuscript & submission of manuscript, Nurfarahana Mohd Nasir: comments on the critical manuscript writing, Fareza Hanis Mohd Yunos: comments on the critical manuscript writing, Sofiah Hamzah: comments on the critical manuscript writing, Ahmad Jusoh: supervise of the research project and comments on the critical manuscript writing.

Availability of Data and Materials: Data are available upon reasonable request from the corresponding author.

Ethics Approval: All applicable international, national, and/or institutional guidelines for the care and use of animals or chemicals handling were fully utilized and followed.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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Distribution, combined pollution and risk assessment of antibiotics in typical marine aquaculture farms surrounding the Yellow Sea, North China

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ABSTRACT

This study focused on the distribution, combined pollution, potential source and risk assessment of 17 antibiotics in an aquaculture ecosystem surrounding the Yellow sea, North China. Antibiotics were detected in various matrices (seawater, sediment/biofilm, organism and feed) in different aquaculture modes (greenhouse and outdoor aquaculture) during the wet and dry seasons in coastal areas of Shandong province. The innovation points of the study were as follows: (1) To the best of our knowledge, this study was one of the few to investigate the occurrence and distribution of antibiotics in mariculture environments along the Yellow Sea coast; (2) Biofilms, a focus of the study, might act as a sink for antibiotics in the aquaculture ecosystem; and (3) The correlation of heavy metals and antibiotic concentrations was proved, which could correspondingly be used as an indicator for antibiotic concentrations in the studied area.

The levels of antibiotics in water were observed to be relatively low, at the ng/L level. Trimethoprim was the most prevalent antibiotic, and was detected in all water samples. Oxytetracycline was detected at high concentrations in biofilms (up to 1478.29 ng/g). Moreover, biofilms exhibited a higher antibiotic accumulation capacity compared to sediments. Concentrations of oxytetracycline and doxycycline were high in feed, while other antibiotics were almost undetected. Tetracycline was widely detected and the concentration of enrofloxacin was highest in organisms.

Correlation analysis demonstrated that environmental parameters and other coexisting contaminants (e.g. heavy metals) significantly affected antibiotic concentrations. In addition, the concentration of Zn was significantly correlated with the total antibiotic concentration and was proportional to several antibiotics in water and sediment (biofilm) samples ($p < 0.01$). High Mn concentrations were closely related to total and individual (e.g. sulfadiazine, sulfamethazine and enrofloxacin) antibiotic levels, which may result in the combined contamination of the environment. Antibiotics in estuaries and groundwater generally originated from aquaculture wastewater and untreated/treated domestic sewage. Most of the detected antibiotics posed no risk to the environment. Ciprofloxacin and enrofloxacin found in water may present high ecological and resistance risks, while the two antibiotics observed to accumulate in fish may pose a considerable risk to human health through diet consumption. All antibiotics detected in seafood were lower than the respective maximum residue limits. This study can act as a reference for the government for the determination of antibiotic discharge standards in aquaculture wastewater and the establishment of a standardized antibiotic monitoring and management system.

1. Introduction

In recent years, antibiotics have attracted attention across the globe due to their large-scale use and potential harmful effects on both the ecosystem and human health. Antibiotics have been widely used as an antimicrobial agent in order to prevent and treat bacterial diseases in humans and animals, and have been added in feed as growth promoters in husbandry and aquaculture. In particular, animal husbandry and aquaculture wastewater usually contain high concentrations of antibiotics, which may pose potential risks to ecosystems once discharged into the environment (Yang et al., 2018). Moreover, antibiotics may induce a series of genetic effects in human, such as allergic reactions. Long-term exposure to antibiotics can also result in chronic toxic effects on organisms (van den Bogaard and Stobberingh, 2000).

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Mariculture is a booming global industry, however the lack of restrictions on antibiotics usage during the culturing process has detrimental effects of the sustainable development of the industry. At present, research focusing on antibiotic residues in marine aquaculture environments is limited. To the best of our knowledge, the earliest study investigating mariculture antibiotic residues focused on aquaculture environments in Italy (Lalumera et al., 2004). In recent years, research on pharmaceuticals and antibiotics in aquaculture environments has generally focused on the southern sea of South Korea, Bangladesh and China (Hossain et al., 2017; Kim et al., 2017). China has become the largest producer of aquaculture products, compromising 71% of the global supply (Sapkota et al., 2008). China leads the world in the production and consumption of antibiotics, and antibiotic residue has become a serious problem in the Chinese aquaculture industry (Xu et al., 2007b; Zhou et al., 2013). However, the use of antibiotics differs regionally. The Pearl River Delta (PRD) is an important marine aquaculture area for China. In this area, fluoroquinolone residues in marine aquaculture environments (water, sediment and fish) have been detected to be relatively higher than other aquaculture environments (He et al., 2012). Although the levels of pharmaceutical and personal care products (PPCPs) in water and sediments are relatively low, higher concentrations are observed in feed and organisms in the marine aquaculture areas of the PRD (Xie et al., 2019). Antibiotic concentrations in marine aquaculture farms surrounding Hailing Island and the Beibu Gulf in South China have also been the subject of recent research (Chen et al., 2015a; Zhang et al., 2018). Table S1 compares antibiotic levels in global aquaculture environments. It can be seen that studies on antibiotics found in marine aquaculture in China mainly focus on the South China Sea. The Yellow Sea coastal area is also an important marine aquaculture zone located in northern China, yet comprehensive studies on this location are still lacking. Our study may partially fill in the data gap of antibiotic residues in marine aquaculture in northern China.

Previous studies on the detection of antibiotics generally focus on open sea mariculture and pond farming, while little information is available on antibiotic residues in greenhouse farming (Chen et al., 2015a; He et al., 2012; Zhang et al., 2018). Biofilm refers to the extracellular polymeric substances deposited on the surface of the inside wall or discharging tube in greenhouse ponds. Research on antibiotics in biofilms is limited. Our study fills this gap by provided information on the distribution and accumulation of antibiotic residues in biofilms.

The distribution and level of antibiotics in water can be affected by a variety of environmental variables, including water temperature (T), dissolved oxygen (DO), nitrate nitrogen (NO_3^-–N) and chemical oxygen demand (COD) (Wang et al., 2017). Coexisting contaminants (e.g. heavy metals) may also affect the occurrence of antibiotics. Previous studies concentrated on the correlation between antibiotic resistance genes (ARGs) and heavy metals, while few focused on the combined contamination of antibiotics with heavy metals. It has been reported that the relative abundance of ARGs is positively correlated with heavy metals (e.g. As, Cd, Cu, and Zn) (Graham et al., 2010; Guo et al., 2018; Jiang et al., 2013). A significant positive correlation has also been found between some ARGs and Cu, Zn, Pb, Co and Hg (Graham et al., 2010; Jiang et al., 2013). In fact, the combined contamination of heavy metals with antibiotics is a current serious problem. Thus, our study investigates the coexisting mechanism and analyzes the interaction in detail.

Principal component analysis–multiple linear regression (PCA–MLR) has been applied in previous research in order to analyze the potential source contributions of polycyclic aromatic hydrocarbons, chemicals of emerging concern, pharmaceutically active compounds, PPCPs, antibiotics and their metabolites (Dai et al., 2015; Jia et al., 2011; Jiang et al., 2016; Lin et al., 2018; Liu et al., 2018; Zhang et al., 2012). However, results are usually based on other literature or insufficient experimental data prevents the representation of all possible sources, thus causing uncertainties. The field experiments in our study allowed for the collection of a large amount of data on pollution sources, strengthening the accuracy of the results.

Furthermore, the resistance risk assessments are limited. Previous studies have focused on the ecological risks of antibiotics at three trophic levels, yet few have investigated the resistance risks of antibiotics to bacteria. Thus, special attention must be paid on resistance selection risks due to their long-term effects on the ecosystem.

The objectives of the present study are as follows: (1) to investigate the occurrence of antibiotics in water, sediments (biofilms), organisms and feed in the marine aquaculture areas surrounding the Yellow Sea; (2) to compare the spatial and temporal distribution of antibiotics in two types of aquaculture modes (greenhouse mariculture and outdoor breeding) during important aquaculture periods (wet and dry seasons); (3) to reveal the correlation between antibiotics and environmental parameters or other coexisting contaminants; (4) to analyze the potential sources of antibiotics in receiving water bodies and environment matrices in mariculture ponds; and (5) to assess the ecological, resistance and health risks of antibiotics to the environment and to humans. This study is based on the following assumptions. When analyzing potential sources of antibiotics in receiving water bodies, it is assumed that the water samples collected from estuaries and groundwater represent receiving water bodies in the area. In terms of health risk assessments, we assume that the daily seafood consumption amounts in the region are consistent with other studies.

2. Materials and methods

2.1. Chemicals and standards

The 17 antibiotics detected in this study are as follows: sulfonamides (SAs) consisting of sulfadiazine (SDZ), trimethoprim (TMP), sulfamethazine (SMT), sulfamethoxazole (SMX), sulfaquinoxaline (SQ) and sulfamonomethoxine (SMM); fluoroquinolones (FQs) consisting of norfloxacin (NOR), ciprofloxacin (CIP), ofloxacin (OFL), enrofloxacin (ENR) and sarafloxacin (SAR); tetracyclines (TCs) consisting of doxycycline (DOC), tetracycline (TC), and oxytetracycline (OTC); and macrolides (MLs) consisting of erythromycin–H_2O (ETM), roxithromycin (RTM) and clarithromycin (CTM). The studied antibiotics are widely used in human activity, animal husbandry and the aquaculture industry, and are commonly detected at high concentrations in other mariculture areas within China (Chen et al., 2017; Chen et al., 2015a, 2015b; He et al., 2012; Na et al., 2013; Xie et al., 2019). Detailed information on the target antibiotics and reagents are reported in Text S1 and Table S2. Furthermore, nine metals were analyzed: Cr, Mn, Co, Ni, Cu, Zn, As, Cd, and Pb.

2.2. Sample collection

The sampling sites located in Haiyang, southeast of Shandong Peninsula along the Yellow Sea (Fig. 1). Haiyang has a warm temperate East Asian monsoon continental climate, which is suitable for the marine aquaculture of various organism species. In addition, Haiyanǵs proximity to the Yellow Sea provides water sources for the aquaculture industry. Therefore, Haiyang was selected as a typical mariculture area in northern China. The Dongcun and Mahegang Rivers both flow into the Yellow Sea, with mariculture ponds located at the lower reaches of the two rivers by the coast. A sewage treatment plant (STP) is located within the upper reaches of the Dongcun River. Water and sediments (biofilms) from mariculture ponds and estuaries were collected during August (wet season) and November (dry season) in 2018, while organism samples were only collected in November of the same year. Water, sediment, and organism samples in S3, S9 and S10 in November were not collected due to the end of aquaculture activities in this winter season. Also, only crabs were collected in S8 (shrimp-crab polyculture pond), as the shrimp was totally fished out of the pond during the sampling period. Similarly, only sea cucumbers were collected in S11

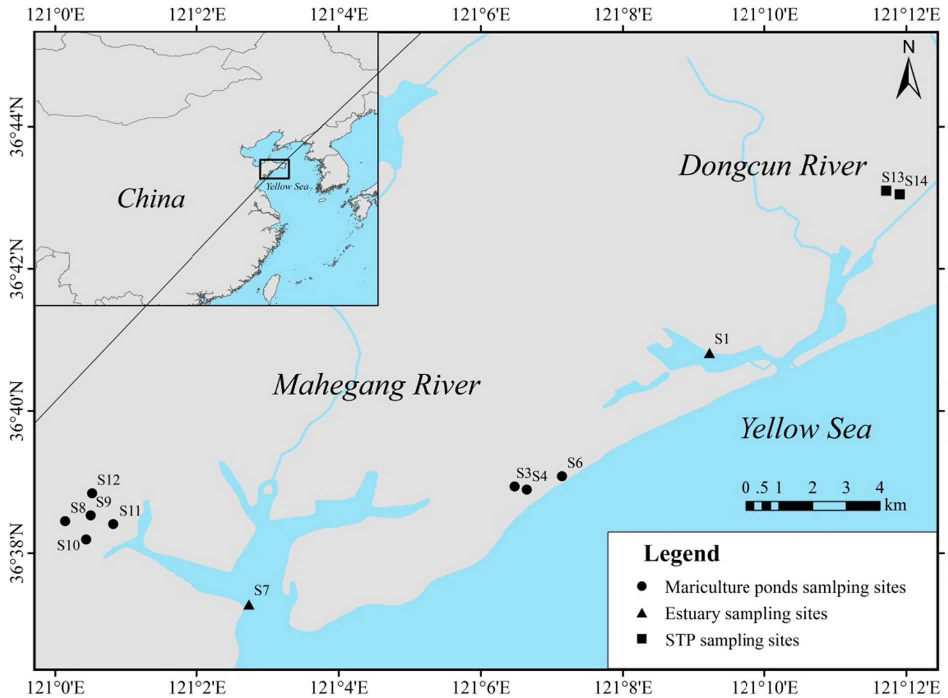

Fig. 1. Locations of the sampling sites around the Yellow Sea. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(shrimp-sea cucumber polyculture pond). Further details on the sample collection are reported in Text S2, and Tables S3 and S4.

2.3. Analysis of antibiotics, heavy metals, TOC and water quality parameters

Antibiotics detected in the samples were extracted using the solid phase extraction method and concentrations were analyzed via high–-performance liquid chromatography–electrospray ionization tandem mass spectrometry (HPLC–ESI–MS/MS) using the multiple reaction monitoring (MRM) model. Heavy metals were measured using atomic fluorescence spectrophotometry (AFS) and an inductively coupled plasma–mass spectrometer (ICP–MS). Further details on the treatment procedures and instrumental analysis are reported in Texts S2 and S3. The TOC contents in the water and sediment samples were determined using a Total Organic Carbon Analyzer coupled with a SSM–5000A Solid Sample Module (TOC–LCPH–SSM500A, Shimadzu Corporation, Japan). The water temperature (T), salinity (SAL), dissolved oxygen (DO) and pH of the water samples were measured in situ using a portable multi–parameter water quality analyzer (INESA DZB–712, Shanghai, China). Total nitrogen (TN), total phosphorus (TP), ammonia nitrogen (NH_3–N), chemical oxygen demand (COD), turbidity (Tur), and chlorophyll *a* (Chla) were determined in the laboratory according to the Chinese State Environmental Protection Agency (SEPA) Standard Methods (Chinese SEPA, 2002).

2.4. Quality assurance and quality control (QA/QC)

The experimental process followed strict quality assurance and quality control procedures. Procedure blanks were set up in each batch of experiments. Parallel samples were set up for the extraction and analysis in order to avoid accidental errors. The instrument blank analysis was conducted every 8 samples to monitor the background value of the instrument. Internal standard curves (5, 10, 20, 50, 100, and 200 ug/L) were used for quantitative analysis ($R^2 > 0.99$). The recovery, regression equation and correlation coefficients for the target antibiotics are reported in Table S7. For the water, sediment/biofilm and organism samples, the detection limit ranges (LOD) were 0.09–0.60 ng/L, 0.04–0.30 ng/g and 0.04–0.30 ng/g respectively. Moreover, the quantification limits (LOQ) were 0.30–2.00 ng/L, 0.15–1.00 ng/g and 0.30–2.00 ng/g respectively. The recovery rates for the 17 types of antibiotics in the water, sediment and organism samples were 55–141%, 49–169% and 43–187%, respectively, while recoveries of heavy metals were 96.37–101.30%, 94.12–119.33% and 93.40–114.43%, respectively. More details on the recovery are provided in Tables S7 and S8.

2.5. Assessment methods

The risk quotient (RQ) is used to assess the ecological and resistance risks of antibiotics to aquatic organisms, and is classified into three levels: Low risk (RQ < 0.1), medium risk (0.1 < RQ < 1), and high risk (RQ < 1). RQ is derived as follows:

$$RQ = \frac{MEC}{PNEC} \tag{1}$$

The health risk (HQ) of antibiotics to humans is generally determined by calculating the quotient of the estimated daily intake (EDI; ng/(d·person)) and the acceptable daily intake (ADI):

$$HQ = \frac{EDI}{ADI \times W_{people}} \tag{2}$$

According to previous studies, HQ ≤ 1% indicates a negligible risk, 1% < HQ < 5% indicates a considerable risk, and HQ > 5% denotes a distinct risk for humans (Vragović et al., 2011).

The bioconcentration factor (BCF) is commonly used to assess the bioaccumulation of different aquatic species, and is calculated according to the following equation:

$$BCF = \frac{C_{organism}}{C_w} \times 1000 \tag{3}$$

In aquatic organisms, chemicals are defined as “bioaccumulative” if the BCF is greater than 5000 L kg^{-1}, and as “potentially bioaccumulative” if the BCF lies within 2000–5000 L kg^{-1} (Wu et al., 2010).

Table 1
Concentrations of antibiotics in the surface water (ng/L) and sediments (ng/g dry weight) from mariculture ponds during the wet and dry season.

Water	Wet Season (n = 10)					Dry Season (n = 6)				
	Min	Max	Mean	Median	Freq.	Min	Max	Mean	Median	Freq.
SDZ	n.d.	n.d.	n.d.	n.d.	0	n.d.	0.51	0.21	0.13	50%
TMP	0.31	10.69	2.57	0.96	100%	1.69	9.57	5.99	4.23	100%
SMT	n.d.	0.68	0.16	n.d.	40%	n.d.	0.64	0.19	0.16	50%
SMM	n.d.	n.d.	n.d.	n.d.	0	n.d.	n.d.	n.d.	n.d.	0
SMX	n.d.	0.88	0.21	0.09	50%	1.41	5.17	2.77	2.46	100%
SQ	n.d.	0.48	0.05	n.d.	10%	n.d.	0.4	0.14	0.11	50%
OTC	n.d.	42.63	7.29	0.44	50%	n.d.	41.03	13.12	1.18	83.33%
TC	n.d.	n.d.	n.d.	n.d.	0	n.d.	0.9	0.42	0.36	50%
DOC	n.d.	6.53	1.28	0.46	60%	0.32	3.73	1.2	0.75	100%
OFL	n.d.	34.68	6.01	n.d.	30%	0.18	3.25	0.86	0.89	100%
NOR	n.d.	22.51	3.5	n.d.	30%	0.82	1.53	1.21	1.28	100%
CIP	n.d.	103.08	21.06	n.d.	30%	n.d.	48.26	14.94	1.67	83.33%
ENR	n.d.	995.02	135	0.48	50%	0.56	125.96	26.74	1.17	100%
SAR	n.d.	1.00	0.34	0.34	80%	0.58	1.14	0.9	0.93	100%
ETM	n.d.	n.d.	n.d.	n.d.	0	n.d.	0.83	0.56	0.65	83.33%
RTM	n.d.	n.d.	n.d.	n.d.	0	n.d.	1.26	0.42	0.29	50%
CTM	n.d.	n.d.	n.d.	n.d.	0	n.d.	n.d.	n.d.	n.d.	0
Sediment	Wet Season (n = 8)					Dry Season (n = 5)				
	Min	Max	Mean	Median	Freq.	Min	Max	Mean	Median	Freq.
SDZ	n.d.	0.44	0.08	n.d.	25%	n.d.	0.29	0.06	n.d.	20%
TMP	n.d.	0.46	0.55	n.d.	25%	n.d.	6.58	1.4	0.07	80%
SMT	n.d.	0.08	n.d.	n.d.	12.50%	n.d.	0.08	0.02	n.d.	20%
SMM	n.d.	n.d.	n.d.	n.d.	0	n.d.	n.d.	n.d.	n.d.	0
SMX	n.d.	0.09	0.02	n.d.	25%	n.d.	2.55	0.38	0.26	50%
SQ	n.d.	n.d.	n.d.	n.d.	0	n.d.	0.16	0.03	n.d.	20%
OTC	n.d.	1478.29	186.10	n.d.	37.50%	n.d.	59.78	26.92	15.37	60%
TC	n.d.	7.43	0.93	n.d.	12.50%	n.d.	0.51	0.1	n.d.	20%
DOC	n.d.	0.26	0.05	n.d.	25%	n.d.	0.24	0.05	n.d.	20%
OFL	n.d.	0.47.	0.08.	n.d.	25%	n.d.	0.43	0.1	n.d.	40%
NOR	n.d.	0.46	0.12	n.d.	25%	n.d.	0.84	0.3	0.22	60%
CIP	n.d.	0.4	0.05	n.d.	37.50%	n.d.	5.75	1.3	0.22	60%
ENR	0.1	869.04	111.97	0.59	100%	n.d.	895.32	184.12	0.47	100%
SAR	n.d.	0.44	0.10	n.d.	25%	n.d.	0.76	0.46	0.43	100%
ETM	n.d.	n.d.	n.d.	n.d.	0	n.d.	n.d.	n.d.	n.d.	0
RTM	n.d.	n.d.	n.d.	n.d.	0	n.d.	n.d.	n.d.	n.d.	0
CTM	n.d.	n.d.	n.d.	n.d.	0	n.d.	n.d.	n.d.	n.d.	0

Further details of the calculation and underlying explanations of the parameters in Formulas (1)– (3) can be found in Text S4.

2.6. Statistical analysis

Multivariate analysis was used to assess the correlation between antibiotic concentrations and environmental variables using Canoco for Windows (V.4.5). In addition, Pearson's correlation analysis was applied to evaluate the determined correlations with SPSS (V.20.0). Source apportionment analysis was conducted using PCA–MLR via SPSS. Text S5 presents more details on the statistical analysis.

3. Result and discussion

3.1. Occurrence and spatiotemporal distribution of antibiotics

3.1.1. Occurrence of antibiotics in mariculture environments

As reported in Table 1, the concentrations of all detected antibiotics were observed at the ng/L level. All raw data are reported in Tables S9–S12. A total of 11 out of the 17 antibiotics were detected (at detection frequencies between 10 and 100%) within mariculture pond surface waters during the wet season. The concentrations of 6 antibiotics were lower than the LODs due to low usage or instability in water. The concentrations of antibiotics ranged from n.d. to 995.02 ng/L, indicating large variations in antibiotic concentrations in mariculture ponds. TMP, which is often used as a synergist of sulfonamides in aquaculture (Gräslund and Bengtsson, 2001), was the most frequently detected antibiotic in all mariculture water samples. This suggests the universal presence of TMP in mariculture wastewater within this area. During the wet season, CIP and ENR were the predominant antibiotics in all mariculture water samples, with concentrations ranging from n.d. to 103.08 ng/L and n.d. to 995.02 ng/L, respectively.

The average concentrations of the 17 antibiotics ranged from n.d. to 26.74 ng/L during the dry season. All antibiotics were detected during the dry season, with the exception for SMM and CTM. Detection frequencies ranged between 50 and 100%, indicating that the detected antibiotics were widely distributed in mariculture wastewater. The detection frequencies of TMP, SMX, DOC, OFL, NOR, ENR and SAR reached 100%. In the dry season, TMP and ENR concentrations were generally higher among these high-detection-frequency antibiotics in all mariculture water samples. In addition, the maximum and mean concentrations of CIP reached up to 48.26 and 14.94 ng/L, respectively, indicating that CIP was also a notable antibiotic in this area. ENR was detected as a predominant antibiotic, with observed concentrations between 0.56 and 125.96 ng/L. The water and sediments of estuaries, influent and effluent of STP were analyzed for antibiotics, as shown in Text S6.

SMM, ETM, RTM and CTM were not detected in the mariculture sediment samples of the two seasons. Moreover, SDZ, SMT, SQ, DOC, OFL and NOR were detected during the wet and/or dry seasons at very low concentrations. OTC and ENR exhibited relatively medium-to-high concentrations during the two seasons. In particular, ENR exhibited maximum and average concentrations of 895.32 and 184.12 ng/g respectively, and was detected in all sediment samples during the dry season. Moreover, ENR was observed as the predominant antibiotic in mariculture sediments. FQs were predominant antibiotics in water and sediment samples in the dry season, while TCs were predominant in sediment samples in the wet season – yet TCs concentrations in water were low. This may be attributed to the physicochemical characteristic of TCs. More specifically, TCs tend to adsorb on sediments (Zhang et al., 2011) and are generally not completely catabolized by organisms, thus entering the environment through excrement. This may explain the large difference in TCs concentrations between water and sediments. In previous studies, similar concentrations of TCs were found in sediments across several sites. For example, TC and OTC concentrations in sediments from Nanming river reached 312 and 335 ng/g respectively, while TC (OTC) concentrations in sediments from the Hai river (Liao river) of China were observed as 422 ng/g (653 ng/g) (Liu et al., 2009; Zhou et al., 2011).

A total of 15 antibiotics were detected in the organism samples, with detection frequencies within 17.65–52.94%. Concentrations ranged from 0.09 to 24.75 ng/g ww in mariculture organisms surrounding the Yellow Sea. This is similar to that of shrimps from mariculture farms in the Beibu Gulf, China (1.80–10.6 ng/g ww) (Zhang et al., 2018). However, our detected concentrations were far lower than those of mollusks from Hailing Island in South China, with concentrations of 0.8–15,090 ng/g ww (Chen et al., 2015a). Turbots (S4) accumulated the highest total concentration of antibiotics among all organisms (Fig. 2), reaching up to 41.53 ng/g ww. In addition, ENR and CIP exhibited high concentrations in turbots, at 24.75 and 9.62 ng/g respectively. Note that TC levels were significantly higher than other antibiotics detected in sea cucumbers (S6, 5.18 ng/g), crabs (S8, 12.08 ng/g) and shrimps (S12, 9.65 ng/g). In summary, TCs and FQs exhibited the highest concentrations among mariculture organisms, indicating that these antibiotics may be used as common veterinary drugs or are easily accumulated in organisms.

Feed was collected from three sites: S4, S6 and S8. Total antibiotic concentrations within different feed varied greatly, ranging from 4.23 to 208.14 ng/g. High concentrations of TCs were detected in F1, F2 and F3 (from S4), particularly OTC and DOC (Fig. S1). This may indicate the accumulation of TCs in organisms from these feeds. Antibiotic concentrations in F4, F5 (from S6) and F6 (from S8) were very low, implying that these feed samples were not key antibiotic sources.

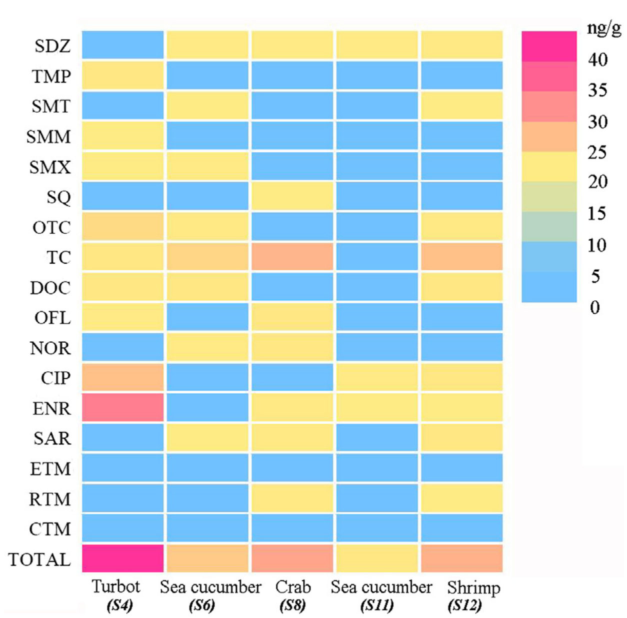

Fig. 2. Concentrations of antibiotics in aquatic organisms across mariculture sites.

3.1.2. Spatiotemporal distribution of antibiotics in mariculture water and sediments.

In general, antibiotic concentrations were higher in the wet season compared to the dry season. FQs were predominant antibiotics in mariculture water, which may be related to the frequent use of FQs (e.g. ENR and CIP) as veterinary drugs in aquaculture. That also showed that aquaculture drugs might be an important factor affecting the occurrence of antibiotics in aquaculture water.

Antibiotic concentrations in greenhouse mariculture pond water samples (S3–S4) were significantly higher during the wet season compared to the dry season (Fig. 3a). However, the opposite was observed for outdoor breeding ponds (S5–S12). This difference in antibiotic distribution characteristics may be explained by cultivation modes and feeding behavior. First, the greenhouse mariculture pond was a relatively closed system with a poor water exchange ability, thus facilitating the accumulation of antibiotics. In contrast, outdoor breeding ponds are connected to the sea, and water is exchanged during the rise and fall of the tides. This may dilute the concentration of antibiotics in the outdoor pond. Moreover, the greenhouse pond was in the dark, while the outdoor breeding pond was exposed to sunlight. Photolysis effects of antibiotics in outdoor culture systems may reduce the concentration of antibiotics. Therefore, antibiotics were observed at higher concentrations in greenhouse mariculture compared to outdoor breeding. Second, high summer temperatures can induce disease, thus farmers tend to spray antibiotics into ponds. This may explain the temporal trend of higher observed antibiotic concentrations during summer. S4 is an exemplary for the spatial and temporal distribution characteristics of the studied area. The total antibiotic concentration of S4 (greenhouse mariculture) during the wet season was higher than that in the dry season, which contrasts to observations of other sites (outdoor breeding). In addition, the influent of greenhouse mariculture ponds exhibited high antibiotic concentrations in both seasons. This can be attributed to the contaminated underground water.

The antibiotic concentrations of biofilm in S4 were much higher than those of other sediments in both seasons (Fig. 3b), indicating that biofilms may have a greater ability to accumulate antibiotics. The adsorption or release of antibiotics by biofilm may affect the concentration levels of antibiotics in ponds. Therefore, biofilms may be key for the control of antibiotic concentrations in greenhouse ponds. In addition, the total concentrations of antibiotics in sediments from S6 and S8 were higher in the dry season than in the wet season, suggesting that long-term aquaculture may cause the accumulation of antibiotics in sediments.

3.2. Correlation analysis

3.2.1. Correlation between antibiotic concentrations and water quality

Redundancy analysis was used to assess the correlation between the antibiotic concentrations and environmental variables. Raw data on water quality, heavy metals and TOC in the water, sediment, organism and feed samples are reported in Tables S13–S17. In addition, the model selection is presented in Table S18. Results demonstrate that water quality influenced the antibiotic distribution in both seasons (Fig. S2). TP and TN were positively correlated with OFL over the two seasons, which might indicate that nutrients and OFL have similar sources, such as domestic sewage and aquaculture wastewater (Chen et al., 2015b). DO was negatively correlated with almost all antibiotics in the wet and dry seasons. This was consistent with the correlation between antibiotics and environmental parameters in Lake Honghu (Wang et al., 2017). DO may affect antibiotic concentrations in the following ways. First, DO directly affects microbial activity such as biodegradation. Previous studies have shown that the biodegradation rate of antibiotics under aerobic conditions is higher than that under anaerobic conditions (Ingerslev et al., 2001). Aerobic microorganisms may multiply and degrade antibiotics in the environment with high concentrations of DO, causing a decrease in the concentrations detected in water. This can

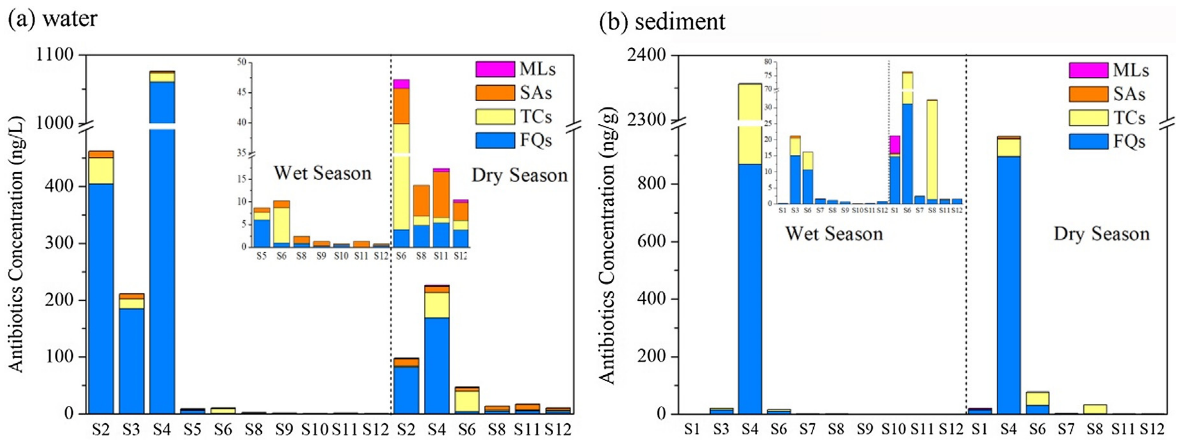

Fig. 3. Distribution of antibiotics in water (a) and sediments (b) from mariculture sites during the wet and dry seasons.

explain the negative correlation between antibiotic concentrations and DO. Second, DO may indirectly influence the antibiotic usages of mariculture. Adequate DO in water may increase the tolerance of cultured organisms to adverse environmental conditions (such as ammonia nitrogen and nitrite) and enhance the resistance to environmental stress. Organisms that grow in low-oxygen environments over a long time may be more susceptible to disease. When organisms are infected by disease, farmers tend to use large amounts of antibiotics, which may also result in a negative correlation between DO and antibiotics. In addition, the water samples collected from Mahegang River (S7-12) clustered together in the wet season, indicating that the correlation between antibiotic concentration and water quality in aquaculture ponds was similar to that in estuaries in this watershed. In the dry season, the influent and effluent samples of the STP (S13 and S14) were separated from the mariculture and estuary water, showing that the correlations between antibiotic concentration and water quality of the two kinds of water were obviously different. It might be related to the mixing of multiple sewage in STP, which resulted in a more complex relationship between antibiotic concentration and water quality.

3.2.2. Correlation between antibiotics and TOC

There was no correlation between antibiotic concentrations and TOC in water and sediments (Fig. S3). However, TOC concentrations in sediments exhibited a significant positive correlation with log total antibiotic concentrations in sediments/biofilms ($r = 0.803$, $P < 0.01$) (Fig. S4). It may be possible to estimate total antibiotic concentrations in sediments using TOC concentrations, however since the data in this study was limited, more data is needed to verify this hypothesis.

3.2.3. Antibiotic and metal combined contamination

Redundancy analysis and Pearsońs correlation analysis were used to assess the correlation between antibiotic and heavy metal concentrations. Zn concentrations were significantly correlated with the total antibiotic concentration and were positively correlated with several antibiotics, such as OTC ($p < 0.01$) (Fig. S5). This may be attributed to the following reasons: First, Zn can bind several antibiotics, including quinolones, tetracycline and macrolides (Uivarosi, 2013), which may lead to the correlation between Zn concentrations and several antibiotics. Second, Zn has been reported to promote the development of antibiotic resistance in environmental bacteria (Wales and Davies, 2015). Third, Zn is often used as feed additive to promote animal growth, indicating that feed may be the common source of Zn and antibiotics. In general, the concentration of antibiotics was observed to be directly proportional to the concentration of Zn. Thus, Zn can potentially be applied as an indicator for the level of antibiotics in the studied area.

The combined contamination of heavy metals and antibiotics may cause complex effects on organisms in the environment. First, Zn may affect the potency of antibiotics to organisms. For example, Zn can bind to tetracycline, making antibiotics less effective (Wei et al., 2011), while for most quinolones, Zn can enhance efficacy (Uivarosi, 2013). Reports confirm the synergistic effect of Zn and quinolones on *P. aeruginosa* biofilms, as well as the enhanced efficacy of CIP against *MDR Acinetobacter baumannii* caused by Zn (Elkhatib and Noreddin, 2014; Ghasemi and Jalal, 2016). Second, antibiotics and Zn have a synergistic selective effect on bacteria within the environment. Antibiotics in the environment provide common selective pressure for bacteria, and high heavy metal concentrations (e.g. Zn and Cu) may kill bacteria (Lemire et al., 2013). In general, it is necessary to comprehensively evaluate the ecological effects of the complex interactions between antibiotics and metals, and also improve existing environmental risk assessment systems.

There was a significant positive correlation between CIP concentrations and those of Mn, Co and Ni in water samples ($p < 0.01$). CIP is able to complex with metal cations and has been combined with Mn, Co and Ni to synthesize complexes in previous studies (Patel et al., 2012; Psomas, 2008). Yet the relationship between them in water environments remains to be reported. The correlation between metals and antibiotics in sediments was observed to be highly significant in this study. There was a significant positive correlation between Mn, Ni, Zn, Cd and Cu levels with the concentrations of total antibiotics and numerous individual antibiotics. In addition, concentrations of Mn, Ni, Zn, Cd, and Cu in biofilms were all higher than those in sediments in both seasons, indicating the accumulation characteristics of biofilms. The concentration of Mn was observed to be extremely high level; one to two orders higher than that of other metals. In particular, for biofilms, concentrations of Mn reached 34,866 and 31,730 mg/kg in the wet and dry season, respectively. High Mn concentration is characteristical to the mariculture in the studied area.

As shown in Fig. 4(b), the sediment samples collected from estuaries (S1 and S7) and some mariculture ponds (S11 and S12) clustered together in both seasons, indicating that the correlations between antibiotic concentrations and heavy metals in sediment samples from these sites were less affected by seasons. It might be related with the properties of sediment. Sediment is a very complex system that contains clay minerals, metal oxides and many other components, which have good adsorption capacity for antibiotics (Allen, 1993). The types and concentrations of antibiotics and heavy metals in the sediments of these sites might be relatively stable and would not change easily with time. Therefore, the correlations were similar.

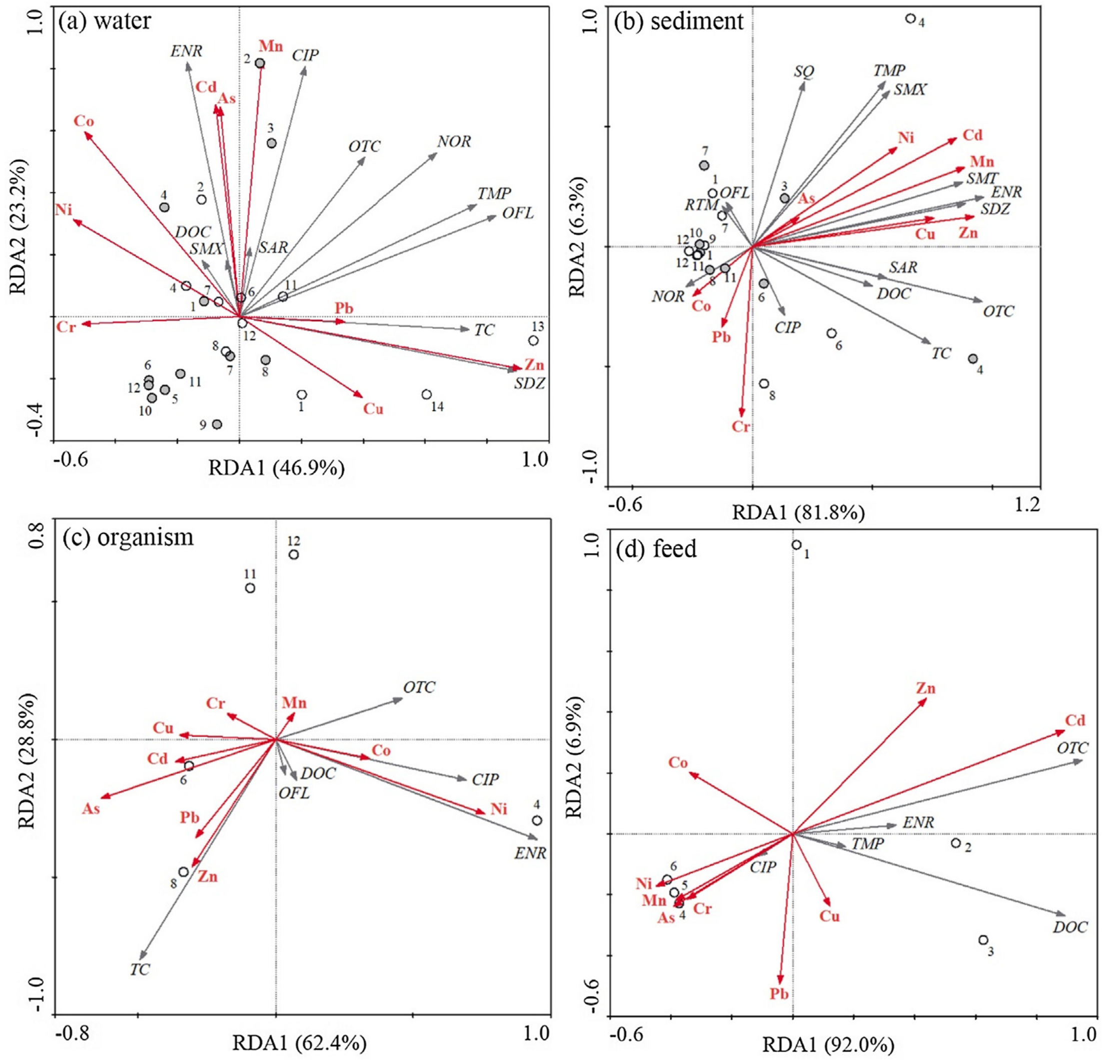

Fig. 4. Redundancy analysis of antibiotic and metal concentrations in water (a), sediment (b), organism (c) and feed (d) samples. Solid circle symbols represent sampling sites in wet season, and empty circle symbols represent sampling sites in dry season. The numbers represent number of sampling points.

3.3. Antibiotic source analysis

3.3.1. Potential sources of antibiotics in receiving water bodies

Antibiotic-contaminated water flows to marine environments through estuaries. Marine environment is a sink for antibiotic-contaminated water, and groundwater can be denoted as a tributary of offshore seawater. Therefore, both groundwater and estuary water are classified as receiving water bodies. The correlation between the principal components and antibiotics in receiving water bodies was demonstrated using the factor loadings. Two principal components (PC1 and PC2) accounted for 77 and 23% of the variance, respectively (Table 2). PC1 was related to the most frequently detected antibiotics, which were reported to have high detection frequencies and concentrations in typical STPs in previous studies (Xu et al., 2007a). PC1 accounted for the largest proportion of total antibiotic concentrations in STP influent and effluent in this study (Fig. S6), indicating that PC1 may represent domestic sewage. PC2 was predominantly associated with quinolone antibiotics of NOR, CIP and ENR, which are widely used as key veterinary drugs in aquaculture activity. In particular, ENR was developed exclusively for animal usage. In addition, total concentrations of NOR, CIP, and ENR accounted for 57% of the total antibiotic concentrations, implying that PC2 represented a source of aquaculture wastewater.

Source apportionment analysis was conducted using PCA–MLR in

Table 2
Varimax-rotated component matrix following principal component analysis (PCA) of receiving water bodies samples.

Variable	Rotated component number	
	1	2
SDZ	**0.869**[a]	0.495
TMP	**−0.981**	0.195
SMT	**0.844**	0.536
SMM	**0.843**	0.538
SMX	**−0.996**	−0.090
SQ	0.728	0.685
OTC	**−0.809**	0.588
TC	**0.949**	−0.316
DOC	**0.885**	0.466
OFL	**0.943**	0.332
NOR	0.187	**0.982**
CIP	−0.087	**−0.996**
ENR	−0.115	**−0.993**
SAR	**0.864**	0.503
ETM	**−0.841**	−0.541
RTM	**0.908**	0.419
CTM	**0.908**	0.419

[a] Bold val ues denote PCA loadings higher than 0.8.

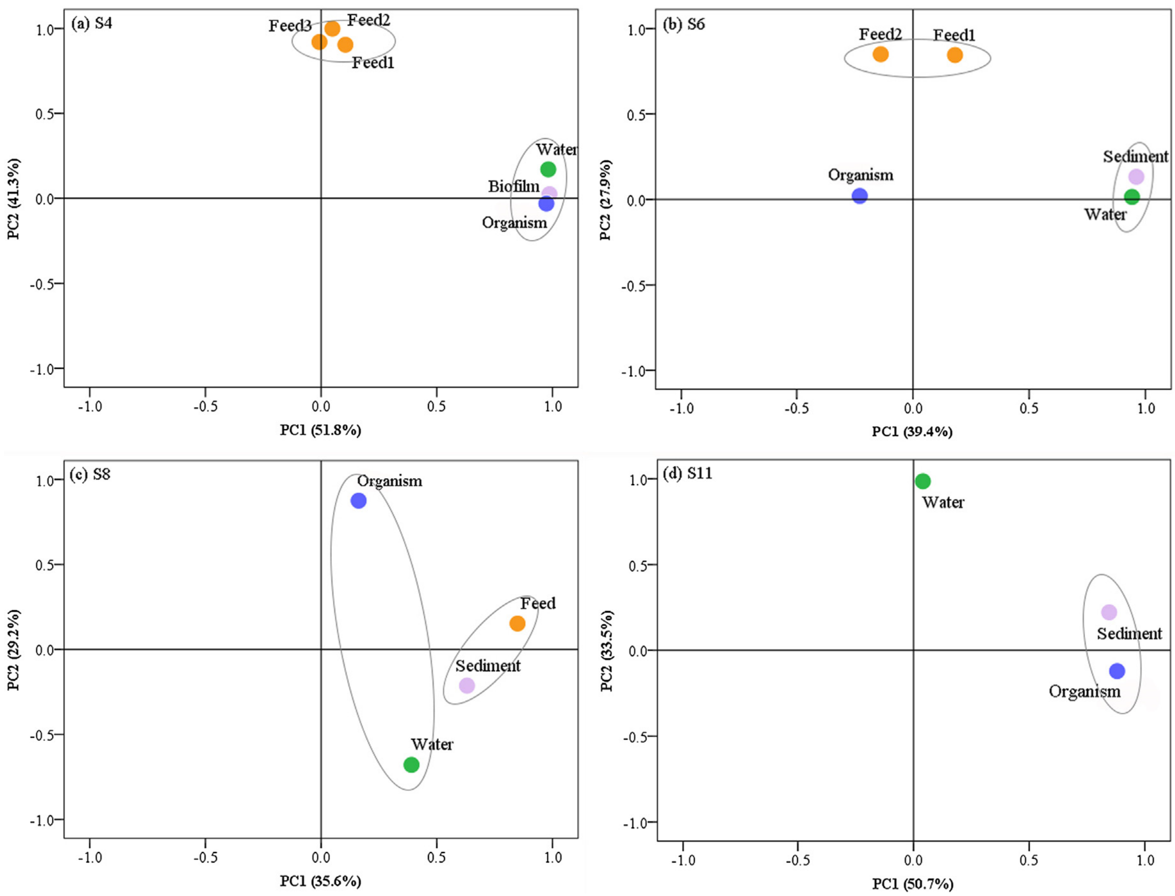

Fig. 5. PCA based on antibiotic concentrations of different matrices at each mariculture pond.

order to determine the mass apportionment of each source to total concentrations. Details on the calculation and analyses are provided in Text S7.

3.3.2. Potential sources and distribution of antibiotics in various matrices in mariculture ponds

PCA was used to trace antibiotics in the mariculture ponds and for the distribution analysis of various matrices at each mariculture pond (Fig. 5). All variables were reduced to two principal components (PC1 and PC2) using the maximum variance rotation method (Table S20–S23). The Spearman correlation analysis is reported in Table S24–S27.

Feed was grouped together, and water, biofilm and organism samples were closely grouped in S4 (Fig. 5a). This suggested that feed may not be the main source of several high concentration antibiotics (such as TMP, CIP and ENR) in water, biofilms and organisms. The close connection of antibiotics among water, sediments, and organisms may be attributed to the transformation activity of antibiotics in these three matrices in mariculture systems. Water and biofilms continuously experience adsorption and desorption processes. Antibiotics dissolved in the water can be ingested and accumulated in fish, while undecomposed antibiotics are excreted via faeces and deposited in the biofilms. This leads to the acumination of antibiotics in this mariculture system, particularly in the biofilms. Spearman correlation analysis (Tables S24) demonstrated the significant correlations among the concentrations of antibiotics in water, biofilms and organisms ($p < 0.01$). Sea cucumbers generally feed on dosed clean sediment and industrial processed feed in studied area. Feed was classified as one group, while water and sediment were grouped together (Fig. 5b), indicating that feed may not be the main source of antibiotics in water, sediments or organisms. Spearman correlation analysis (Tables S25) also showed the significant correlations between antibiotic concentrations in water and sediments ($p < 0.01$). Furthermore, sediments and organisms were closely grouped in S11 (Fig. 5d), indicating that the antibiotics detected in organisms may be derived from those attached to the sediments. Sea cucumbers were cultured in the ponds located at S6 and S11. The adsorption and desorption of antibiotics in water and sediments may be the principle migration activity affecting the distribution of antibiotics in S6. Sea cucumbers feed on the sediments and faeces deposited on the sediments, which may result in the migration of antibiotics in S11.

In general, the distribution of antibiotics in the studied mariculture system matrices were influenced by the adsorption and desorption of antibiotics in water and sediments, the feeding behavior of organisms on water and sediments, and the deposition of feed onto sediments. Each mariculture pond was a relatively closed system. More specifically, with the flow of water, the input and output of antibiotics in mariculture ponds formed a cycle through the solid phase deposition, the accumulation in organisms, and the gas phase release. Research on the sources and distribution of antibiotics in multi-matrix systems may aid in the analysis and control of total antibiotic amounts.

3.4. Preliminary risk assessment

3.4.1. Ecological risks

In order to assess the possible risks in worst-case scenarios, this study focused on the toxicity of the selected antibiotics to the most sensitive species. Toxicity data and PNECs values for the detected antibiotics are presented in Table S28 and Table S29. PNEC values were calculated using the species sensitivity distribution (SSD) for OTC and ENR due to the availability of enough toxicity data. The risks of other detected antibiotics were determined using the assessment factor

Fig. 6. RQs of selected antibiotics to the most sensitive species across sampling sites.

method due to insufficient toxicity data.

OFL, CIP and ENR posed medium-to-high ecological risks during the wet season in S3 and S4 (Fig. 6a), indicating that mariculture wastewater directly discharged into the sea may increase ecological risks to marine ecosystems. In addition, the antibiotics in the inflowing water (S2) of greenhouse ponds may pose direct toxicity effects to organisms contained in the ponds (S3 and S4). In summary, FQs demonstrated relatively high RQ values for organisms for all detected antibiotics. Moreover, CIP was the most influential antibiotic, posing medium-to-high risks to aquatic organisms in almost all dry season samples (Fig. 6b). Antibiotics were more likely to pose high ecological risks in S1, suggesting that the organisms found in this pond may be subject to the toxic effects of antibiotics. Several antibiotics in the influent and effluent of STP (S13 and S14) still posed medium-to-high risks. Results demonstrated that STP wastewater treatment processes largely reduced the potential ecological risks caused by antibiotics, while high ecological risks to the environment were still possible with OFL, RTM and CTM.

In general, most antibiotics found in mariculture water in the Mahegang River basins may not pose ecological risks to this mariculture ecosystem. The ecological risks associated with mariculture water in ponds surrounding the Dongcun River basins were higher than those of the Mahegang River basins. The mixing of antibiotics in aquaculture wastewater and the dilution of seawater can affect the ecological risk of marine ecosystems, with further evaluation required. CIP posed the highest ecological risk, indicating that CIP usage must be controlled in order to reduce its associated negative effects and ecological risks.

3.4.2. Resistance risks

Antibiotic residues in the environment may pose long-term selective pressure to bacterial communities, thus promoting the antibiotic resistance of bacteria and inducing the generation of ARGs. In addition, ARGs in aquatic bacteria are able to transfer to terrestrial bacteria via the horizontal gene transfer, possibly inducing sever negative effects within the biosphere (Cabello et al., 2013; Sharma et al., 2016). The derived resistance selection PNEC values of the antibiotics are reported in Table S30.

ENR and CIP demonstrated medium-to-high risks in greenhouse pond samples for both seasons (Fig. 6c and d). Results indicate that the bacterial community in the greenhouse pond may be more resistant to CIP and ENR. This is consistent with the aquaculture production of *Fenneropenaeus chinensis* in China, in which the RQ of ENR in mariculture wastewater was observed as high, possibly inducing severe resistance risks (Sun et al., 2016). Antibiotics of outdoor breeding ponds demonstrated low resistance risks for both seasons. This is in agreement with the surface water of finfish and shellfish aquaculture in Bangladesh, where RQs were observed to be lower than 1 for all detected antibiotics (Hossain et al., 2017). In addition, multiple antibiotics in the influent and effluent of STP may also induce medium-to-high resistance risks, indicating the potential of aquaculture wastewater, untreated domestic sewage and STP effluent as ARGs sources in the environment. The resistance risks were lower than the ecological risks, indicating that higher concentrations of antibiotics were required to induce resistance risks. Moreover, CIP and ENR can possibly pose medium-to-high ecological and resistance risks. In summary, antibiotic concentrations must be monitored and their risks should be evaluated in order to reduce the associated environmental impacts.

3.4.3. Bioconcentration factors (BCFs)

Antibiotics in the environment can accumulate and synthesize toxic compounds in aquatic organisms, which may pose potential risks to human and animal health (Klosterhaus et al., 2013). In the present study, the BCFs of detected antibiotics ranged from 27 to 2317 L kg^{-1} in shrimp, 0 to 2261 L kg^{-1} in crab, 0 to 306 L kg^{-1} in fish, and 0 to 1561 L kg^{-1} in sea cucumber (Table S31). Results indicate the potential bioconcentration of OTC in shrimps, and OFL in crabs (Fig. S8). Other antibiotics in organisms exhibited BCF values lower than 2000 L kg^{-1}, indicating low bioaccumulative characteristics.

The BCF range in this study (0–2317 L kg^{-1}) was lower than those detected in organisms in the Baiyangdian Lake (0–16,700 L kg^{-1}) (Li et al., 2012). Moreover, SDZ was observed to accumulate in crabs in our work, with the BCF value determined at 1554 L kg^{-1}, which was lower than that of crabs (*Scylla paramamosain*) (9691–14,452 L kg^{-1}) from the Beibu Gulf, China (Zhang et al., 2018). The BCFs of SDZ in fish (306 L kg^{-1}) and shrimps (552 L kg^{-1}) in our study were lower than those in fish (*Trachinotus ovatus*) (781 L kg^{-1}) and shrimps (*Fenneropenaeus penicillatus*) (1392 L kg^{-1}) from the Hailing Island, South China Sea (Chen et al., 2015a). Due to species-specific characteristics, significant differences among the BCF values of SDZ were observed across organisms. In general, the bioconcentration may be related to the habitat environment, reproductive status and growth phase of organisms (Liu et al., 2011), and may also be affected by the environmental behavior of compounds (e.g. adsorption, photolysis and microbial degradation) (Zhang et al., 2018).

3.4.4. Health risks quotients

As shown in Table 3, the EDIs of SAs, TCs, FQs and MLs in seafood were determined as 1.25–65.23, 28.99–335.64, 6.89–2060.68 and 0–1.32 ng/(d·person), respectively. Moreover, the EDIs of FQs and TCs were the highest in the four categories. Studies have shown that FQs and TCs are widely used in aquaculture. In particular, TCs, which accumulate in seafood and are subsequently consumed by humans, can cause tooth damage in children (Kummerer, 2009). The only antibiotics with observed high HQ values in organisms were ENR and its metabolite CIP in fish (Fig. 7). HQ values were well above 1%, indicating that the high concentration residue of these antibiotics in fish may pose a considerable health risk to human consumers. Exception of these two antibiotics, the residual concentrations of all other antibiotics across organisms were unlikely to pose risks to human health through their consumption.

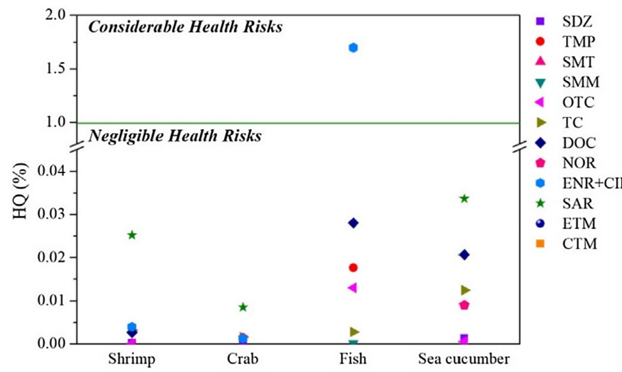

Fig. 7. HQs of selected antibiotics in different organisms.

ADIs and MRLs are commonly used together in order to assess the health risks (Zhang et al., 2018). In our study, all the antibiotics detected in seafood were lower than the respective MRLs. Note that although ENR and CIP may pose considerable health risks to human, the total concentrations of these two antibiotics in organisms were lower than that of MRLs. This implies that even if the amount of antibiotics in the seafood consumed by humans each day is lower than MRLs, it may still pose a health risk to humans. Thus, it is necessary to establish a unified standard in order to assess human health risks associated with diet consumption and to strengthen the supervision of seafood quality to avoid direct detrimental effects.

4. Conclusions

A total of 17 antibiotics in the aquaculture environment surrounding the Yellow Sea were analyzed during the wet and dry seasons of 2018. Compared with other regions in the world, the levels of

Table 3
Estimated daily intake (EDI), acceptable daily intake (ADI) and maximum residue limits (MRLs) of antibiotics.

Antibiotics	EDI ng/(d·person)				ADI ug/(kg bw·d·person)[b]	ADI × 60 ug/(d·person)	MRLs (ng/g)
	Shrimp [a]	Crab	Fish	Sea Cucumber			
Seafood consumption (g/d)	**6.05**	**2.4**	**59.3**	**43.26**			
SDZ	2.78	0.96	0	16.01	20	1200	
TMP	0	0	44.48	0	4.2	252	50
SMT	0.54	0	0	4.33	50	3000	
SMM	0	0	11.27	0	130	7800	
SMX	0	0	9.49	17.30			
SQ	0	0.29	0	0			
Sum of SAs	**3.33**	**1.25**	**65.23**	**37.64**			**100**
OTC	2.90	0	234.24	10.38	30	1800	100
TC	58.38	28.99	51.00	224.09	30	1800	100
DOC	4.84	0	50.41	37.20	3	180	100
Sum of TCs	**66.13**	**28.99**	**335.64**	**271.67**			
OFL	0	1.99	22.53	0			
NOR	0	1.78	0	10.82	2	120	50
ENR + CIP	4.78	1.58	2038.14	0	2	120	100
SAR	4.54	1.54	0	6.06	0.30	18	30
Sum of FQs	**9.32**	**6.89**	**2060.68**	**16.87**			
ETM	0	0	0	0	5	300	200
RTM	0.73	1.32	0	0			
CTM	0	0	0	0	0.2	12	
Sum of MLs	**0.73**	**1.32**	**0**	**0**			
Sum of antibiotics	**79.50**	**38.45**	**2461.54**	**326.18**			

[a] The data was based on the consumption of seafood (shrimps, crabs and fish), obtained via a questionnaire-based dietary survey in Guangdong Province, South China (Guo et al., 2010). The daily seafood consumption amounts of sea cucumber were obtained from a previous study, based on the assumption that one sea cucumber is consumed per person per day (Zhu et al., 2018).

[b] The data was obtained from the guidelines of the Ministry of Agriculture of the People's Republic of China (2002), the European Medicines Agency, the U.S. Food and Drug Administration and the Joint FAO/WHO Expert Committee on Food Additives.

antibiotics in water and sediments were relatively low within the studied aquaculture area. 11 antibiotics were detected (at detection frequencies between 10 and 100%) within mariculture pond surface waters, and the concentrations ranged from n.d. to 995.02 ng/L. OTC and ENR exhibited relatively high concentrations in sediments, with the maximum concentrations of 1478.29 and 895.32 ng/g respectively. The culture mode played an important role in the antibiotic levels of the aquaculture environment in studied area. Antibiotic concentrations in greenhouse ponds were observed to be higher during the wet season than the dry season, while the opposite was true for outdoor breeding ponds. Turbots cultured in greenhouse ponds accumulated the highest concentration of antibiotics among all organisms, which may be related to culture modes.

Biofilms could be regard as a sink for antibiotics in aquaculture environment. Antibiotic concentrations in biofilms were far higher than those for sediments, due to the strong accumulation capacity of the former. In addition, concentrations of Mn, Ni, Zn, Cd, and Cu in biofilms were all higher than those in sediments, indicating the accumulation characteristics of biofilms. The adsorption or release of contaminants by biofilm may affect the concentration levels of antibiotics in ponds. Therefore, biofilms may be key for the control of antibiotic concentrations in greenhouse ponds.

The correlation of antibiotics and heavy metals was proved, which could reflect the general trend of antibiotic concentrations in the studied area. High Mn concentrations were proportional to several antibiotic levels, which to some extent caused the combined contamination to the environment. Zn was closely related to the total antibiotic concentration ($p < 0.01$), which can subsequently be used as an indicator for antibiotic concentrations in the studied area. TOC exhibited a significant positive correlation to the log total antibiotic concentrations in sediments ($r = 0.803$, $P < 0.01$), which may be used possible to estimate total antibiotic concentrations.

Our results could be helpful to understand the levels of antibiotics, combined contamination and risks in mariculture environment. Although most antibiotics posed low risks to aquatic organisms, bacteria and humans, effective monitoring is still required to enhance the environmental and seafood safety in China.

Declaration of Competing Interest

The authors declared that they have no conflicts of interest to this work.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.105551.

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ARTICLES FOR FACULTY MEMBERS

AQUACULTURE WASTEWATER

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Electrochemical removal of ammonium nitrogen in high efficiency and N_2 selectivity using non-noble single-atomic iron catalyst

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Ammonia nitrogen (NH_4^+-N) is a ubiquitous environmental pollutant, especially in offshore aquaculture systems. Electrochemical oxidation is very promising to remove NH_4^+-N, but suffers from the use of precious metals anodes. In this work, a robust and cheap electrocatalyst, iron single-atoms distributed in nitrogen-doped carbon (Fe-SAs/N-C), was developed for electrochemical removal of NH_4^+-N from in wastewater containing chloride. The Fe-SAs/N-C catalyst exhibited superior activity than that of iron nanoparticles loaded carbon (Fe-NPs/N-C), unmodified carbon and conventional Ti/IrO_2-TiO_2-RuO_2 electrodes. And high removal efficiency (> 99%) could be achieved as well as high N_2 selectivity (99.5%) at low current density. Further experiments and density functional theory (DFT) calculations demonstrated the indispensable role of single-atom iron in the promoted generation of chloride derived species for efficient removal of NH_4^+-N. This study provides promising inexpensive catalysts for NH_4^+-N removal in aquaculture wastewater.

Introduction

With rapid economic development and continuous progress of human society, people's demand for aquatic products has increased greatly, and the offshore aquaculture industry has developed rapidly (Sun et al., 2021). In feed-based aquaculture, more than half of nitrogen in the protein of feeds is finally converted to ammonia nitrogen (NH_4^+-N) and enters the water (Wang et al., 2021; Zhou and Boyd, 2015). Due to the intensive breeding environment and excessive use of feeds, the NH_4^+-N in aquaculture water tends to accumulate and damages the growth of aquatic organisms and the aquatic ecological environment (Romano et al., 2020; Sun et al., 2021). To sustain the normal growth of aquatic creatures, the concentration of NH_4^+-N in the aquaculture water should be controlled below 1 mg/L. Therefore, it is important to develop suitable method to remove NH_4^+-N from aquaculture water.

Up to now, the treatment methods of NH_4^+-N wastewater mainly include zeolite adsorption (Ren et al., 2021; Xue et al.,

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2018), chemical precipitation (Huang et al., 2017; Tansel et al., 2018), chemical oxidation (Chen et al., 2018; Xiang et al., 2020), and biological nitrification (Chang et al., 2013; Du et al., 2017; Li et al., 2018, 2021; Miao et al., 2019). However, these methods are not ideal for small-scale and dispersed offshore aquaculture wastewater (Dong et al., 2019; Mook et al., 2012). For example, biological nitrification is effective in treatment of mass-scale waste water, whereas it occupies large area and is difficult to operate and maintain. Ideal method for remove NH_4^+-N from offshore aquaculture wastewater should have the following characteristics like low-cost, no chemical addition, small device volume, easy operation and high efficiency.

Recently, electrochemical oxidation method has been attempted in the removal of NH_4^+-N, and exhibits some advantage in the good-controllability, mild reaction conditions, economy, and little environmental impact (He et al., 2015; Li et al., 2011; Yao et al., 2019). In this process, NH_4^+-N could be removed through direct or indirect processes, depending on the reactive species. For the direct process, hydroxyl radicals (·OH) are generated at the anode and react with NH_4^+-N via the following reaction ($NH_4^+ + 3{\cdot}OH \rightarrow 1/2\ N_2 + 3H_2O + H^+$) (Ding et al., 2013). Indirect process takes place in the solution containing chloride ions, and hypochlorite (HClO or ClO^-) generated from chlorine evolution reaction (CER) and hydrolysis contributes to the removal of NH_4^+-N (Ghimire et al., 2019). Take neutral environment as an example, HClO (pKa = 7.53) reacts with NH_4^+-N to produce N_2 via the following reaction ($2NH_4^+ + 3HClO \rightarrow N_2 + 3H_2O + 5H^+ + 3Cl^-$). As is known to all, HClO can react with NH_4^+-N to form N_2 with high selectivity, and electrochemical oxidation of chloride electrolyte could generate hypochlorous acid efficiently. This provides a potential method to convert NH_4^+-N to N_2 through electrochemical oxidation method. Since there are enough chloride ions already in the wastewater of offshore aquaculture, additional chloride salts are not required. In addition, there is plenty of sunlight in the offshore area to support the generation of electricity, and reduce the cost of wastewater treatment. Up to now, active anode catalysts for electrochemical generation of hypochlorous acid are mostly composed of precious metals (Li et al., 2009). However, the high price of precious metals is still an obstacle for the wide application of electrochemical treatment of NH_4^+-N. In addition, corrosion of anode materials is also a great challenge in this field. Therefore, it is urgent to develop cheap and stable anodic materials for the selective oxidation of NH_4^+ to N_2.

Since the concept of "single atomic catalysis" was first proposed, the research on single atomic catalysis has been extended to the field of electrocatalysis. With the aid of single-atom strategy, some non-noble metals exhibited catalytic performance equivalent to that of precious metals in electrochemical reactions like hydrogen evolution, oxygen reduction and nitrate reduction (Fan et al., 2016; Wu et al., 2018, 2021; Zhao et al., 2021; Zhu et al., 2017). Perhaps single-atom catalysts can serve as an alternative of noble metal catalysts for electrochemical removal of NH_4^+-N.

Herein, a single-atom iron distributed in nitrogen-doped carbon (Fe-SAs/N-C) was synthesized as non-noble catalyst, and firstly attempted for electrochemical removal of NH_4^+-N. This Fe-SAs/N-C catalyst was utilized as anode material, and possessed superior stability and activity in the simulated wastewater and practical aquaculture wastewater. Further density functional theory calculations were conducted to understand the reaction mechanism and the contribution of single atom. This work may offer a promising way aiming at efficient removal of NH_4^+-N in aquaculture wastewater

1. Materials and methods

1.1. Chemicals and electrodes

2-methyl imidazole (Alfa Aesar), Zinc nitrate hexahydrate ($Zn(NO_3)_2.6H_2O$, 98%, Alfa Aesar), $Fe(acac)_3$(99%, Alfa Aesar), methanol (CH_3OH, Merck), Sodium chloride (NaCl, Aladdin), Nafion D-521 dispersion (5% W/W in water and 1-propanol) (Alfa Aesar), Sodium hydroxide (NaOH, Aladdin), Sodium hydrogen phosphate (Na_2HPO_4, Aladdin), Ethylenediaminetetraacetic acid disodium salt (Na_2-EDTA, Aladdin), Potassium hydrogen phosphate (K_2HPO_4, Aladdin), Potassium iodide (KI, Aladdin), N,N-diethyl-p-phenylenediamine (Alfa Aesar), Mercury iodide (HgI_2, 99.5%, Aladdin). All reagents used in the experiment were analytically pure without further purification. Commercial Ti/IrO_2-TiO_2-RuO_2 electrodes were purchased from Schulte Industrial Technology (Suzhou, China).

1.2. Synthesis of Fe-SAs/N-C and Fe-NPs/N-C samples

The Fe-SAs/N-C and Fe-NPs/N-C samples were synthesized by high temperature calcination. Firstly, zeolitic imidazolate frameworks (ZIF-8) precursor was synthesized by constant temperature water bath. ZIF-8 precursor was then loaded with $Fe(acac)_3$ by impregnation. Finally, Fe-SAs/N-C and Fe-NPs/N-C samples were obtained by calcination of the red powders at high temperature. Detailed synthesis process can be found in supporting information.

1.3. Electrochemical measurements

All electrochemical removal of NH_4^+-N was performed in a single-chamber cell with two electrodes. A glassy carbon electrode (exposure area of 4 cm^2, Appendix A Fig. S1) with catalysts loading was used as anode, and a graphite plate (exposure area of 4 cm^2) was used as cathode. Constant current was applied to the electrodes from a direct-current power supply. Because the reaction rate was directly proportional to the current, same current to applied to the anode to compare the activities of catalysts. The current density was calculated by the current divided by the geometric area (e.g. 0.75 mA/cm^2 means 3 mA applied to the anode). The catalyst loading method is detailed in the support information.

2. Results and discussion

The synthesized catalysts were firstly analyzed to determine its morphology and structure. The Fe-SAs/N-C catalyst is composed of uniform rhombododecahedral particles with an average diameter of ~ 200 nm (Fig. 1a and Appendix A Fig. S2a). High-angle annular dark-field scanning transmission electron microscopy (HAADF-STEM) shows that nitrogen and iron are

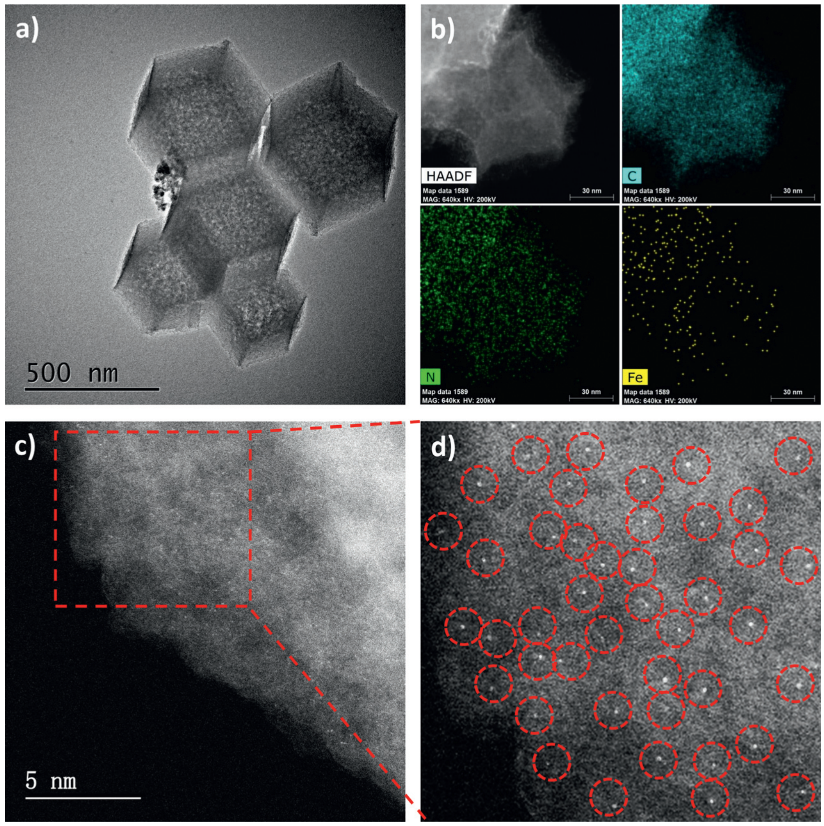

Fig. 1 – (a) TEM image of Fe-SAs/N-C; (b) HAADF-STEM image and corresponding elemental mapping images of Fe-SAs/N-C: C (blue), N (green), and Fe (yellow); (c) HAADF-STEM image and (d) enlarged view of Fe-SAs/N-C. Single Fe atoms highlighted by red circles.

evenly distributed in the carbon skeleton (Fig. 1b). Enlarged HAADF-STEM images illustrate that the iron single atoms are isolated in the carbon matrix (Fig. 1c and d). The X-ray diffraction (XRD) pattern of Fe-SAs/N-C has no characteristic peaks of iron (Appendix A Fig. S2b), but only characteristic broad peaks of carbon (Quan et al., 2019). In contrast, nanoparticles (5∼20 nm) are formed in the Fe-NPs/N-C catalyst (Appendix A Fig. S2c) and obvious characteristic peaks of iron oxides and iron clusters could be observed in the XRD patterns (Appendix A Fig. S2d). X-ray photoelectron spectroscopy (XPS) was used to probe the surface region elemental compositions and valence states of catalysts. In the C 1s spectrum of Fe-SAs/N-C (Appendix A Fig. S3a), the three characteristic peaks are attributed to C=C (283.6 eV), C=N (284.5 eV) and C-N (287.7 eV) (Afsahi and Kaliaguine, 2014; Yin et al., 2016), respectively. The N 1s spectrum of Fe-SAs/N-C is composed of three obvious peaks, which are identified as graphitic-N (402.4 eV), pyrrolic-N (399.8 eV) and pyridinic-N (397.4 eV) (Yin et al., 2016), respectively (Appendix A Fig. S3b). There is no obvious difference in the XPS results between Fe-NPs/N-C (Appendix A Fig. S3c and d) and Fe-SAs/N-C. The coupled plasma optical emission spectrometry (ICP-OES) analysis confirmed that the Fe content of Fe-SAs/N-C and Fe NPs/N-C was about 1.26 wt% and 11.7 wt%, respectively.

Atomic structural information of catalysts was further analyzed by X-ray absorption fine structure (XAFS) measurements. The position of the X-ray absorption near-edge structure (XANES) curves of Fe-SAs/N-C is located between those of Fe_2O_3 sample and Fe foil (Fig. 2a), indicating that the oxidation state of single-atomic iron is between Fe^0 and Fe^{III}. Compared with Fe-SAs/N-C, the XANES spectrum of Fe-NPs/N-C was close to that of Fe foil with valence state of Fe^0. The Fourier transform extended X-ray absorption fine structure (EXAFS) of Fe-SAs/N-C exhibited similar Fe-N(O) coordination with a main peak at approximately 1.50 Å (Chen et al., 2017) (Fig. 2b).There was no characteristic peak of Fe-Fe at 2.2 Å, suggesting that iron existed as isolated atoms in Fe-SAs/N-C. Conversely, the Fourier transform curves for Fe-NPs/N-C demonstrated the first shell of Fe-Fe paths at 2.2 Å, which is consistent with Fe-Fe in Fe foils. Furthermore, the least-squares EXAFS fitting results indicated that single Fe atoms in Fe-SAs/N-C coordinates with four nitrogen atoms (Fig. 2c, Appendix A Table S1), and its DFT calculation structure model is shown in Fig. 2c. In addition, the WT plots of Fe-SAs/N-C exhibited only one maximum at 5.20 $Å^{-1}$, which could be assigned to the $Fe-N_4(O)$ bonding (Chen et al., 2017; Zhang et al., 2017) (Fig. 2d). The WT maximum at 8.20 $Å^{-1}$ for Fe-NPs/N-C, Fe foil and Fe_2O_3 could be assigned to the Fe−Fe bonding (Appendix A Fig. S4a−c). The difference in WT results of these samples further indicates that no Fe-Fe bond exists in the Fe-SAs/N-C sample, and the Fe atoms in Fe-SAs/N-C are isolated.

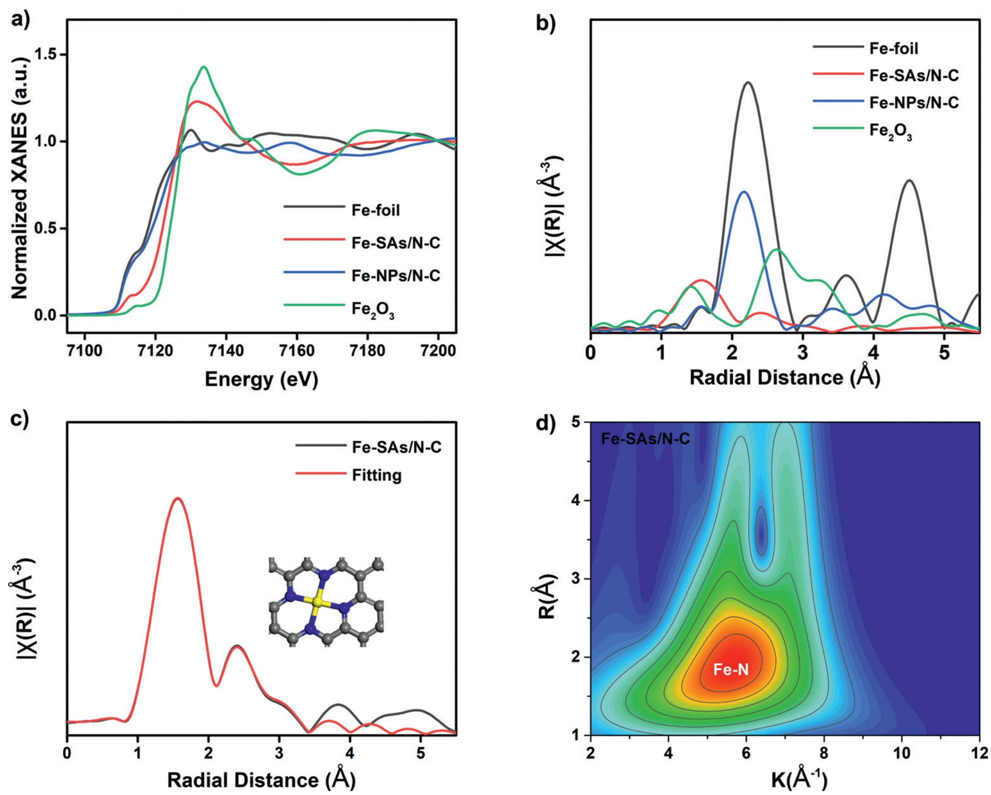

Fig. 2 – (a) XANES spectra at the Fe K-edge of Fe-SAs/N-C, Fe-NPs/N-C, Fe_2O_3 sample, and Fe foil; (b) Fourier transform (FT) at the Fe K-edge of Fe-SAs/N-C, Fe-NPs/N-C, Fe_2O_3 sample, and Fe foil; (c) the corresponding EXAFS fitting curves for Fe-SAs/N-C (Inset: DFT calculation structure model of Fe-SAs/N-C); (d) wavelet transform (WT) of Fe-SAs/N-C.

Electrochemical activity of Fe-SAs/N-C toward NH_4^+-N removal was then evaluated in a sealed electrolytic cell. Simulated NH_4^+-N wastewater was consisted of 20 mg/L NH_4^+-N and 0.1 mol/L NaCl with initial pH of 7. Firstly, the effect of current densities on removal efficiency of NH_4^+-N was investigated. 85% of NH_4^+-N was removed in 2 hr at 0.5 mA/cm^2 (Fig. 3a). With the increase of current density to 0.75 mA/cm^2, 99.3% of NH_4^+-N could be removed in the same time. If increasing the current density further to 1.0 mA/cm^2, it still took 2 h electrolysis to realize more than 99% removal of NH_4^+-N. From the perspective of energy saving, 0.75 mA/cm^2 was then selected in the following experiments. During the reaction, the change of NO_3^--N and NO_2^--N concentration were also monitored (Fig. 3b). Little amount of NH_4^+-N was converted to NO_3^--N (< 0.5 mg/L), and no NO_2^--N was detected in the reaction process. This indicates that more than 99% of NH_4^+-N is selectively converted to N_2. The control experiment in the absence of chlorine ions showed that little NH_4^+-N (< 5%) was removed (Appendix A Fig. S5), indicating that and chloride derived species contributed dominantly to the removal of NH_4^+-N. Considering that residual hypochlorite might affect the growth of aquatic products (Kim et al., 2008), we monitored the variation of hypochlorite concentration, which is as low as 1.3 mg/L in the treated water (Appendix A Fig. S6). This indicates that majority of generated hypochlorite has reacted with NH_4^+-N. Little residual hypochlorous acid existed because the amount of NH_4^+-N (< 0.2 mg/L) in the treated water was not enough to consume the hypochlorite. To avoid potential effect of hypochlorite on aquatic creatures, above treated water could be mixed with untreated aquaculture wastewater and let the hypochlorite be consumed by NH_4^+-N.

Beside the experiments in simulated wastewater, this electrochemical system was also attempted in the treatment of practical aquaculture water (Fig. 3c). The difference is that the practical aquaculture water is treated with electricity supplied by a solar cell (2.0 V). Similar removal efficiency was also achieved and the NH_4^+-N concentration decreased from initial 15 mg/L to less than 0.5 mg/L in 100 min (Fig. 3d). This demonstrates that this electrochemical system could be used for practical treatment of aquaculture water, and the utilization of solar energy would greatly reduce the processing cost. In addition, Fe-SAs/N-C catalyst exhibited satisfied activity in acidic or alkaline condition as that in neutral condition (Appendix A Fig. S7), indicating the wide pH adaptability of this catalyst. Acidic environment is relatively beneficial to the removal of NH_4^+-N due to the inhibition of oxygen evolution (Exner, 2020a) and promotion of hypochlorite generation (Appendix A Fig. S8).

As is known to all, Ti/IrO_2-TiO_2-RuO_2 electrode is commercially used as anode for hypochlorite generation and removal of NH_4^+-N wastewater. In this work, a Ti/IrO_2-TiO_2-RuO_2 electrode was evaluated in the same electrochemical system, and exhibited negligible activity toward NH_4^+-N removal at the current density of 0.75 mA/cm^2 in 2 hr (Fig. 3e). As depicted in Appendix A Fig. S9, only ~50% of NH_4^+-N could be removed under high current density of 7 mA/cm^2. By contrast, the Fe-

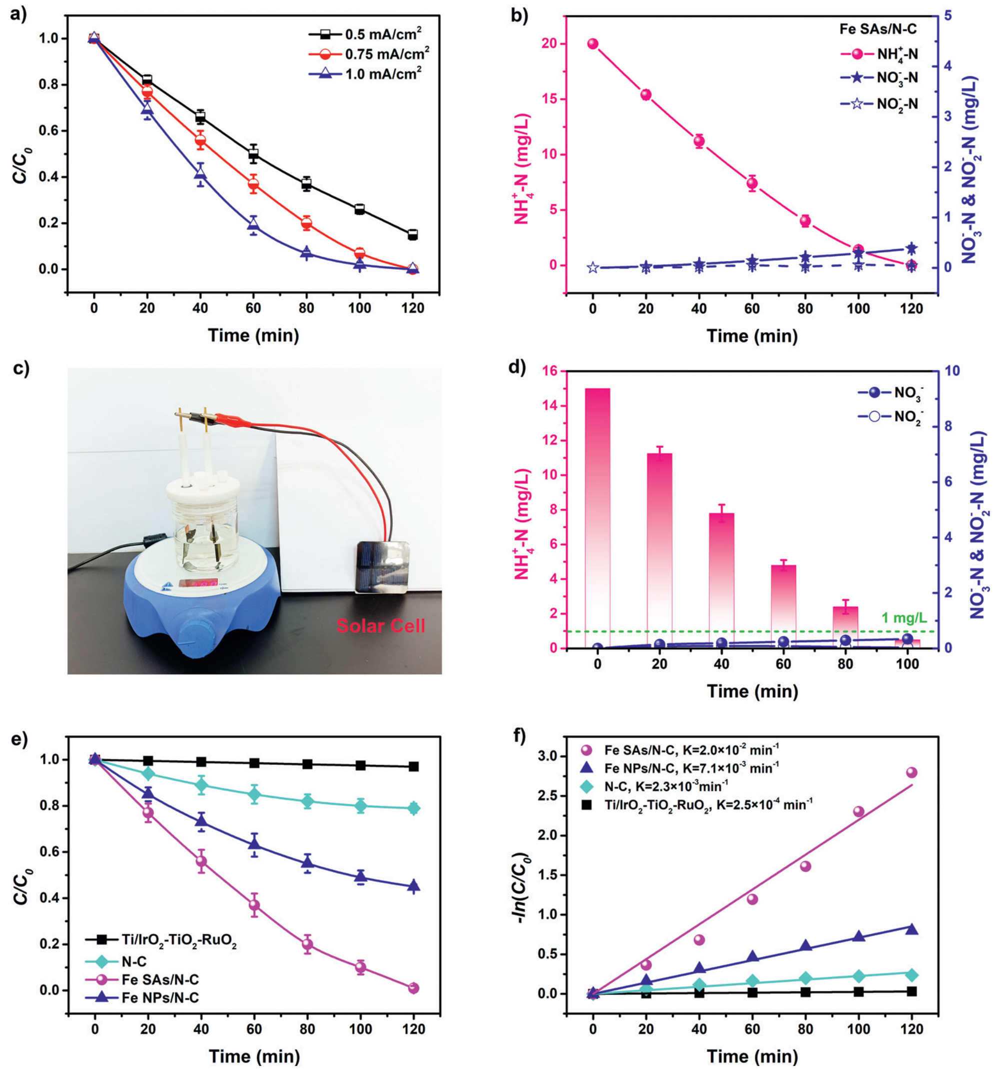

Fig. 3 – (a) Effect of current density on electrochemical removal of NH_4^+-N by Fe-SAs/N-C electrode (pH = 7.0, initial C_{NH4+-N} = 20 mg/L); (b) The change of NH_4^+-N, NO_3^--N and NO_2^--N concentration during the electrochemical removal of NH_4^+-N by Fe-SAs/N-C electrode at 0.75 mA/cm^2; (c) Photo images of the electrocatalytic cell for NH_4^+-N removal in practical aquaculture wastewater powered by a solar cell; (d) Electrochemical removal efficiency of NH_4^+-N from practical aquaculture wastewater by Fe-SAs/N-C (initial C_{NH4+-N} = 15 mg/L, pH = 7.0, 0.75 mA/cm^2, practical aquaculture wastewater is taken from Shanghai Aquaculture Company); Electrochemical removal efficiency (e) and rate constants (f) of NH_4^+-N by different electrode materials at a current density of 0.75 mA/cm^2.

SAs/N-C electrode could realize 99.3% removal of NH_4^+-N at 0.75 mA/cm^2. This indicates that less electric energy is consumed on Fe-SAs/N-C electrode to realize efficient removal of NH_4^+-N than that on Ti/IrO_2-TiO_2-RuO_2 electrode.

To determine whether the superior activity of Fe-SAs/N-C is related with single-atom iron, some control experiments were carried out. Results in Fig. 3e demonstrate that 55% and 20% of NH_4^+-N are removed when using Fe-NPs/N-C and N-C as anode catalysts, respectively. Further kinetic analysis shows that the removal processes conform to the quasi-first-order kinetic model (Fig. 3f). The rate constant achieved on Fe-SAs/N-C (0.02 min^{-1}) is 2.9 times that of Fe-NPs/N-C and 9.0 times that of N-C. The sharp contrast indicates that the unique role of Fe-SAs/N-C structure contributes greatly to its activity. As mentioned previously, the NH_4^+-N removal here was through indirect process relying on the existence of hypochlorite. Among these catalysts, Fe-SAs/N-C possessed the highest activity towards the production of hypochlorite (Appendix A Fig. S10), which was in accordance with its superior performance in the removal of NH_4^+-N. During the electrolytic process, other active species like ClO·, ·OH and Cl· might participated into the removal of NH_4^+-N. To check this, tert-butyl alcohol (TBA) was added as a quencher for ClO·, ·OH and Cl· (Wu et al., 2017). The electrochemical removal efficiency of NH_4^+-N slightly decreased from 99.5% to 90% (Appendix A Fig. S11), indicating little contribution of ClO·, ·OH and Cl· to the removal of NH_4^+-N.

Beside activity, stability is also another important requirement for the catalysts. In the cycling experiments of NH_4^+-N removal, the activity of Fe-SAs/N-C was stable and there was

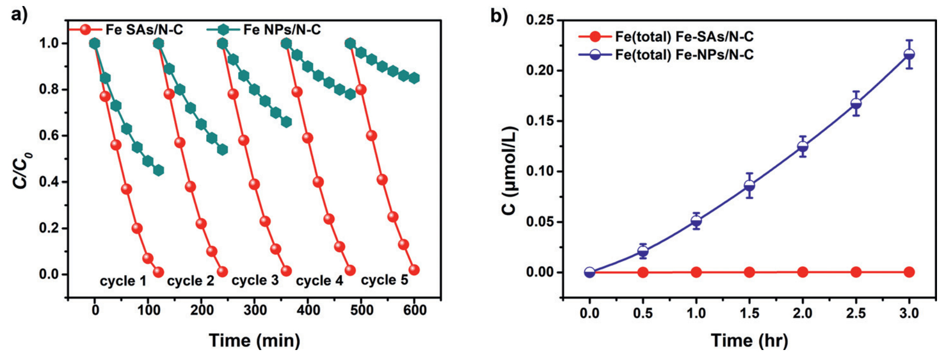

Fig. 4 – (a) Stability of the Fe-SAs/N-C and Fe-NPs/N-C electrode in NH_4^+-N removal in the optimized electrochemical system; (b) The concentrations of total iron during NH_4^+-N removal in Fe-SAs/N-C or Fe-NPs/N-C electrochemical system (pH = 7.0, 0.75 mA/cm^2).

negligible change in the removal efficiency of NH_4^+-N after five cycles. By contrast, the activity of Fe-NPs/N-C decayed evidently (Fig. 4a). Considering that the potential corrosion of anode catalysts might lead to its inactivation, the concentration of Fe ions in the solution was measured to check the iron leaching from Fe-SAs/N-C and Fe-NPs/N-C. There was almost no Fe dissolution from the Fe-SAs/N-C, while the Fe dissolution from the Fe-NPs/N-C increased with the electrolysis time (Fig. 4b).

To understand the specific reaction process on Fe-SAs/N-C, electrochemical impedance and voltammetry were used to compare the electrochemical behaviors of the catalysts. There is no obvious difference in the electrochemical impedance curves of Fe-SAs/N-C and Fe-NPs/N-C (Appendix A Fig. S12), suggesting that the charge-transfer resistance on Fe-SAs/N-C is close to that on Fe-NPs/N-C. Therefore, the charge-transfer resistance is not the main reason for the activity difference. Fig. 5a shows the linear sweep voltammetry (LSV) curves for Fe-SAs/N-C and Fe-NPs/N-C in different solutions. The anodic current of Fe-SAs/N-C in NaCl solution is significantly higher than that in $NaClO_4$ solution, indicating that Fe-SAs/N-C is capable of catalyzing electrochemical oxidation of Cl^- ions. In addition, the chlorine evolution current of Fe-SAs/N-C increases obviously at 1.4 V in NaCl solution. This potential is more positive than that of Fe-SAs/N-C (1.7 V), demonstrating that that the CER is much easier to take place on Fe-SAs/N-C than that on Fe-NPs/N-C.

Furthermore, DFT calculations were undertaken to shed light on the pathway of electrochemical process. The computational details are presented in the supporting information. As we know, the anodic CER process is accompanied by the oxygen evolution reaction (OER). On the potential scale of reversible hydrogen electrode (RHE), the standard reversible electrode potentials of CER ($E°_{CER}$) (Lim et al., 2020; Trasatti, 2000) and OER (E^0_{OER}) (Vos et al., 2018) are 1.36 V (vs RHE) and 1.23 V (vs RHE), respectively. Although OER is thermodynamically more likely to occur than CER, the two-electron process of CER ($2Cl^- \rightarrow Cl_2 + 2e^-$) is kinetically superior to the four-electron process of OER ($2H_2O \rightarrow O_2 + 4H^+ + 4e^-$) (Exner, 2019, 2020a). According to the previous reports, the reaction paths of chlorine (Exner, 2020b; Exner et al., 2016; Sohrabnejad-Eskan et al., 2017) and oxygen evolution (Mavros et al., 2014; Rossmeisl et al., 2005) are proposed as follows.

CER: Reaction pathway 1

1) $^* + Cl^- \rightarrow Cl^* + e^-$
2) $Cl^* + Cl^- \rightarrow Cl_2 + {}^* + e^-$

Reaction pathway 2 (Volmer-Heyrovsky pathway)

1) $O^* + Cl^- \rightarrow ClO^* + e^-$
2) $ClO^* + Cl^- \rightarrow Cl_2 + O^* + e^-$

OER: Reaction pathway

1) $^* + H_2O \rightarrow OH^* + H^+ + e^-$
2) $OH^* \rightarrow O^* + H^+ + e^-$
3) $O^* + H_2O \rightarrow OOH^* + H^+ + e^-$
4) $OOH^* \rightarrow O_2 + {}^* + H^+ + e^-$

According to above paths, CER on Fe-SAs/N-C and Fe-NPs/N-C requires to form Cl^* or ClO^* adsorbents. To check which path is the main reaction path, the free-energy paths of CER and the detail reaction energetics at U = 1.36 V ($E°_{CER}$) were calculated. The free energies in Fig. 5b reveals that the energy barrier needed to be crossed to generate Cl_2 through ClO^* reaction is significantly lower than that required by Cl^* reaction. The above calculation results confirm that the Volmer-Heyrovsky pathway was responsible for CER reaction on Fe-SAs/N-C and Fe-NPs/N-C surfaces. Additionally, the energy barrier on Fe-SAs/N-C (0.32 eV) surface is lower than that of Fe-NPs/N-C (0.54 eV), indicating that Fe-SAs/N-C has better CER activity. This may account for the excellent activity of Fe-SAs/N-C in removing NH_4^+-N.

In addition to CER, the competitive reaction of OER was also analyzed. We calculated the adsorption free energies (ΔG's) of possible adsorbates (i.e., Cl^*, ClO^*, O^*, OH^* and OOH^*) (Exner et al., 2015) to obtain the most stable adsorption structure under applied electrode potential (U = 0 V) and pH = 0 (Appendix A Fig. S13). Then, the adsorption free energy for plausible adsorbates at different voltages was studied by theoretical calculation (Appendix A Fig. S14). The reactants were

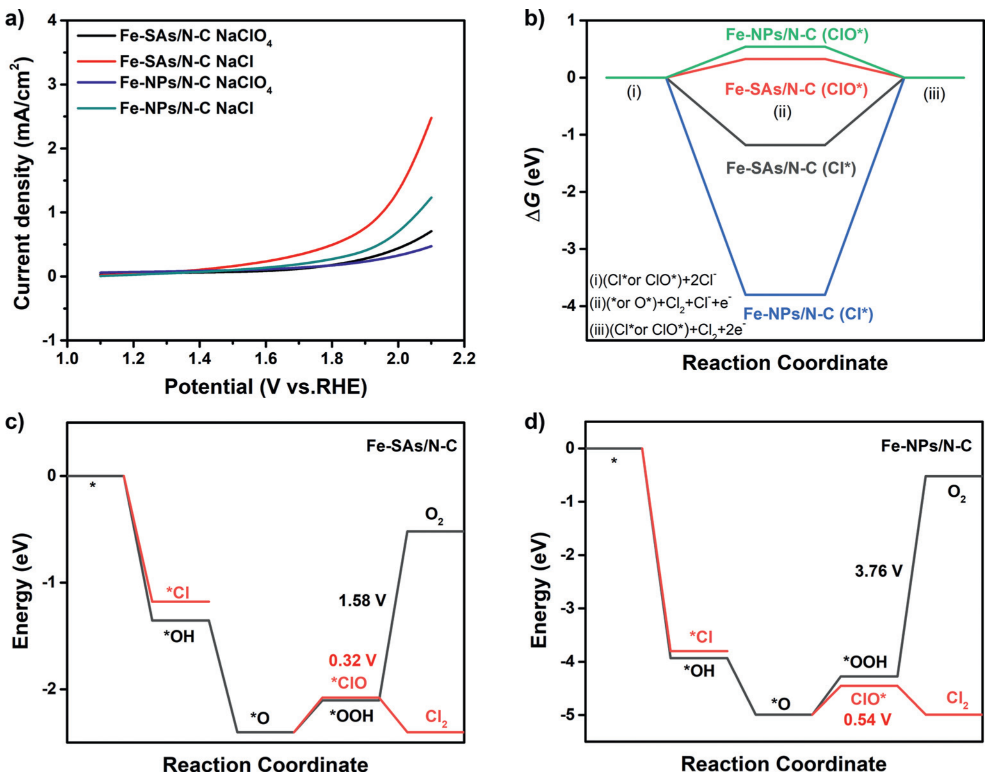

Fig. 5 – (a) LSV curves for Fe-SAs/N-C and Fe-NPs/N-C in the NaCl or $NaClO_4$ electrolyte at a scan rate of 20 mV /sec; (b) Free-energy paths of CER on Fe-SAs/N-C and Fe-NPs/N-C; The Gibbs energy diagram for the CER (red) and for the OER (black) over the Fe-SAs/N-C (c) and Fe-NPs/N-C (d) surface at U = 1.36 V.

adsorbed on the surface of Fe-SAs/N-C and Fe-NPs/N-C, limiting the formation of subsequent products. Subsequently, the free energy diagrams of CER and OER at U = 1.36 V were calculated to compare CER selectivity with the OER on Fe-SAs/N-C and Fe-NPs/N-C. Fig. 5c exhibits that the conversion of *O to ClO* species on Fe-SAs/N-C catalyst is endothermic with the highest reaction energy (ΔG = 0.32 eV). On the basis of the free energetics theory, the conversion of *O to ClO* species should be the rate-determining step (RDS). The RDS of OER is the conversion of *OOH to O_2, and requires 1.58 eV of energy. The RDS energy expenditure of CER (0.32 eV) is much lower than that of OER (1.58 eV), indicating that the CER is more likely to occur on Fe-SAs/N-C. The calculation performed on Fe-NPS/N-C also illustrates that CER is also easier than that of OER (Fig. 5d). But the RDS of CER on Fe-NPs/N-C requires higher energy (ΔG = 0.54 eV) than that on Fe-SAs/N-C (0.32 eV). Therefore, CER is much easier to take place on Fe-SAs/N-C than that on Fe-NPs/N-C.

In previous discussion, acidic condition was found to favor NH_4^+-N removal. In order to further analyze the reasons, the extended surface Pourbaix diagram of the Fe-SAs/N-C surface is obtained from *ab initio* constrained thermodynamics methods and above calculation results (Exner, 2020c; Sumaria et al., 2018), which exhibits the stability window of the electrode as a function of pH and the applied electrode potential. Fig. 6a shows that oxygen (O*) bonded to Fe atoms is very stable over a wide range of potentials and pH. Under condition of U > 1.68 V and pH < 6.8, the terminal adsorption of chlorine on-top of O (ClO*) atoms is more favorable in energy, and CER will take place preferentially. Under condition of U > 1.4 V and pH > 6.8, another surface species (OOH*) is preferentially formed from the perspective of energy, meaning that OER is more likely to occur than CER. The calculated results are in good agreement with the pH experimental results. Compared to Fe-SAs/N-C, ClO* adsorption on Fe-NPs/N-C requires higher energy (U > 1.89 V), indicating that CER is more difficult to occur on Fe-NPs/N-C than that on Fe-SAs/N-C (Fig. 6b). This also explains why the activity of Fe-NPs/N-C is lower than that of Fe-SAs/N-C.

3. Conclusions

In conclusion, a single-atom iron catalyst (Fe-SAs/N-C) was prepared and attempted firstly in electrochemical removal of NH_4^+-N from waste water containing chloride ions, especially that from aquaculture water. With the Fe-SAs/N-C as anode catalyst, 99.3% of NH_4^+-N could be removed in 2 h at a low current density to 0.75 mA/cm^2. Little amount of NH_4^+-N was converted to NO_3^--N (< 0.5 mg/L), and no NO_2^--N was detected in the treated water. The residual NH_4^+-N (< 0.2 mg/L) meets the discharge standard of aquaculture wastewater. The Fe-SAs/N-C catalyst maintained good stability during continuous electrolysis and multiple cycles of reaction without significant iron leaching. Further control experiments and DFT calculations confirmed the crucial role of single-atom iron in produc-

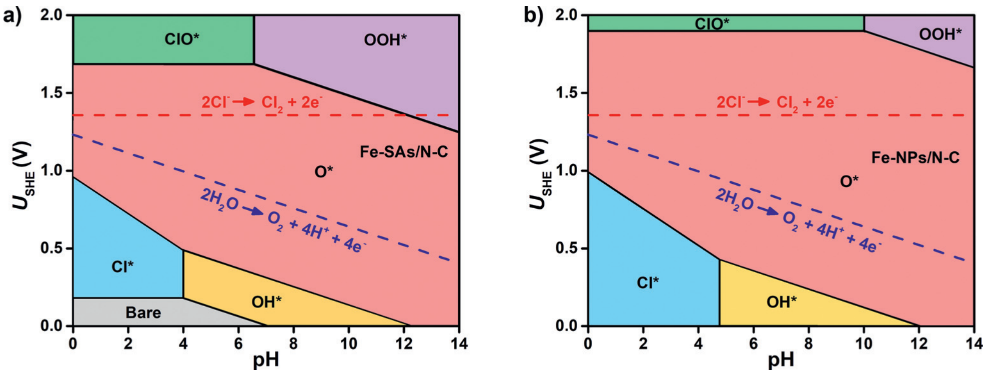

Fig. 6 – Pourbaix diagram of theoretical standard hydrogen electrode potential (U_{SHE}) vs. pH for Fe-SAs/N-C (a) and Fe-NPs/N-C (b) sites in equilibrium with H^+, Cl^- and H_2O at T = 298 K. Red dashed line and blue dashed line represent the equilibrium potential of CER in the SHE scale (U_{eq} = 1.36 V) and OER (U_{eq} = 1.23 V –0.059 pH), respectively. Black solid lines represent the phase boundary where two adsorbate species exist in equilibrium.

ing chloride derived species for efficient NH_4^+-N removal. This work may provide a promising electrochemical method with active, inexpensive and stable electrode to treat aquaculture wastewater.

Acknowledgments

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Appendix A Supplementary data

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jes.2022.03.004.

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ARTICLES FOR FACULTY MEMBERS

AQUACULTURE WASTEWATER

Title/Author	Nutrient consumption of green microalgae, Chlorella sp. during the bioremediation of shrimp aquaculture wastewater / Nasir, N. M., Jusoh, A., Harun, R., Ibrahim, N. N. L. N., Rasit, N., Ghani, W. A. W. A. K., & Kurniawan, S. B.
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Nutrient consumption of green microalgae, *Chlorella* sp. during the bioremediation of shrimp aquaculture wastewater

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ABSTRACT

Aquaculture products are among the biggest contributor to food supplies to meet the global food demands of the growing population over these past few years. For aquaculture to continue developing, an effective wastewater treatment is required to lessen the environmental effects. This study examined the potential of *Chlorella* sp. to reduce nutrients in shrimp aquaculture wastewater and correlate with the growth kinetics of the algae during the bioremediation process. Six different *Chlorella* sp. inoculation dosages ranging from 0 to 60 % (v/v) were used in this study. Marine water wastewater (MW) and Freshwater wastewater (FW) where the two types of shrimp wastewater were employed. Results indicated that the 30 % (v/v) and 40 % (v/v) were the optimum dosage for MW and FW. During the treatment, microalgae cell density increased more than tenfold compared to the initial value. Moreover, batch culture resulted in the specific growth rate concentration of 0.18 k day^{-1} and 0.15 k day^{-1}, respectively. Those dosage also resulting the highest removal efficiencies with removal of ammonia, nitrite and orthophosphate of 96.77 %, 82.07 %, 75.96 % and 90.10 %, 87.09 %, 95.60 %, respectively. The application of FTIR spectroscopy was employed in this study to analyze the functional group in the microalgae biomass. The results of the scanning electron microscopy (SEM) and Energy Dispersive Spectroscopy Analysis (EDS) also included to further illustrate how microalgae biomass was affected by the treatment in this study. Therefore, the research from this study could be used in design novel microalgae treatments that offer a thorough and environmentally beneficial method of treating shrimp aquaculture wastewater.

1. Introduction

The rapid growth of the human population has led to the fast expansion of aquaculture industries to support the global demand. Aquaculture effluent discharge has increased dramatically over the world. Approximately 82 m^3/kg production/year estimation of wastewater generated from aquaculture industries [1]. Wastewater from the aquaculture industry has a large amount of chemical, microbial pollutants, suspended solids and nitrogenous compounds [2]. With concern to the pollution generated by aquaculture, the pollutants discharged from aquaculture industries could destroy the receiving aquatic environment such as eutrophication and deterioration towards the natural ecosystem [3]. Many technologies have been created and applied to minimize the water pollution and one of those technologies that are being developed bioremediation.

Bioremediation uses naturally existing microorganisms and alternative aspects of the natural environment to treat discharged water of its nutrients. It has been demonstrated that bioremediation is more affordable than other technologies for the cleanup of hazardous waste [4]. Algae are used in phytoremediation, a sort of bioremediation, to enhance the water quality. Bioremediation has utilized plant-based remediation such as macro and microalgae. It has been found that microalgae effectively use the nitrogen and phosphorus in wastewater for cell development. Microalgae can take up these chemicals and transform them into biomass that can be used.

Microalgae biomass has become a very promising feedstock in recent

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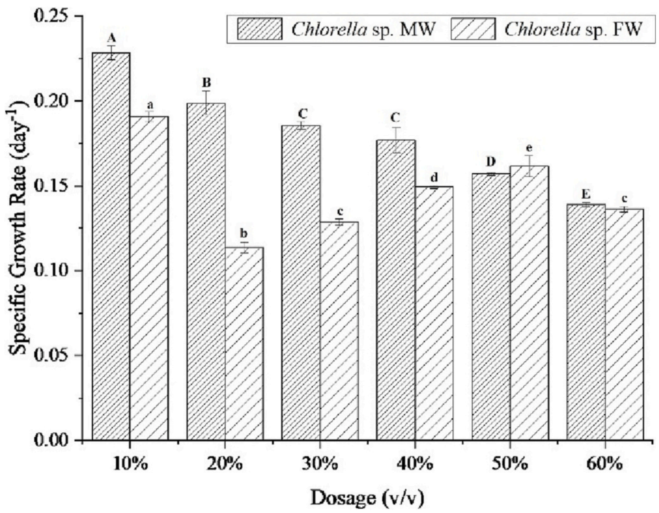

Fig. 1. Specific growth rate of *Chlorella* sp. at exponential phase during bioremediation.
Different capital letters (A-B-C-D-E) indicate significant difference of SGR among used dosage for MW while different lowercase (a-b-c-d-e) indicate significant difference of SGR among used dosage for FW.

years for sustainable biofuels such as biodiesel, bioethanol and biogas [5,6]. The increased cost required for microalgae cultivation is one of the difficulties. This is due to the continued usage of expensive chemicals like Conway or Walne fertilizers to replenish nutrients in growth media [6,7]. The production of microalgae biomass and its nutritional will be significantly influenced by the total nutrient composition and suitable nutrient concentration. Therefore, to meet the suit nutritional needs of microalgae during culture, it is necessary to replace the culture media with macronutrients and micronutrients. One viable substitute for culture media is wastewater.

Microalgae have been recognized as promising agents for improving wastewater quality while collecting nutrients from wastewater at low cost and in an environmentally friendly manner [8–10]. Additionally, heavy metal compounds and pesticides produced by industrial and agricultural wastewaters can be removed using microalgae [11]. Utilizing nutrient-rich of aquaculture wastewater as a growth medium for the development of microalgae could reduce the reliance on chemical pesticides. However, there is currently little research on the simultaneous production of microalgae and bioremediation of aquaculture wastewater. It is also yet to be determined how nutrient uptake and microalgal growth differ between fresh and marine aquaculture wastewater.

This study aims to determine the biomass yield and nutrient uptake by the *Chlorella* sp. microalgae species in aquaculture effluent during bioremediation. The ratio of microalgae and wastewater also considered as an important factor affecting the algae growth and the bioremediation performance. In addition, FTIR spectroscopy was used to examine the functional groups in the biomass of the microalgae. The organic chemical groups -OH, -COOH, NH_2, and C=O were detected in the microalgae biomass by FTIR analysis. SEM was used to characterize the shape of the microalgae cell and EDS was used to examine the chemical characterization of the nitrogen and phosphorus content in the microalgae biomass. The results of this research could enhance microalgae capacity to remove nutrients from different aquaculture effluent. Technologies based on microalgae offer a promising alternative for treating aquaculture wastewater. The success or failure of aquaculture output depends on how well water quality is maintained.

2. Materials and methods

2.1. Wastewater collection

Aquaculture wastewater was collected from the hatchery pond of shrimp, *Penaeus vannamei* for marinewater bioremediation (MW) and *Macrobrachium rosenbergii* for freshwater bioremediation (FW) at Universiti Malaysia Terengganu (UMT), Malaysia. Filtered sterile wastewater was prepared by autoclaved for 20 min at 120 °C. This method was used to ensure unnecessary species were killed. As a result, it remove other microorganisms from samples while preventing changes of the nutrient content in wastewater such as undergo the nitrification process before it was employed in the bioremediation process.

2.2. Microalgae cultivation

Pure cultivation of green microalgae genus *Chlorella* was obtained from Live feed Laboratory, Institute of Tropical Aquaculture Hatchery UMT. It was grown in Guillard's F/2 media for marine species, *Chlorella* sp. UMT LF2 and Bold's Basal Medium (BBM) for freshwater species, *Chlorella* sp. UMT LF1 under sterile conditions. Microalgae were cultures with an initial concentration of 1.0×10^5 cell·mL^{-1} algal cells. Cell density was calculated every two days using an improved Neubauer Haemocytometer. The specific growth rates (μ) of microalgae were determined during the exponential growth phase by the Eq. (1):

$$\mu = \left[\frac{ln N_2 - ln N_1}{t_2 - t_1}\right] \quad (1)$$

where μ is the specific growth rate, and N_1 and N_2 are the biomass at time 1 (t_1) and time 2 (t_2), respectively [12].

2.3. Bioremediation process

Green microalgae genus *Chlorella* was used for the bioremediation of shrimp aquaculture wastewater due to its simple cell cycle, high growth rate and having photosynthetic and metabolic pathways similar to higher plants [13]. *Chlorella* sp. were cultured until early exponential growth phase, Day 4 to Day 6 of the cultivation period. The batch bioremediation process was conducted using six different inoculation dosages: 0, 10, 20, 30, 40, 50, 60 % (v/v) with a total experiment volume of 1.5 L. The growth performance and nutrient analysis of microalgae (removal efficiency) were monitored every 2 days. The removal efficiency (%) of nutrients were determined by the Eq. (2):

$$R\ (\%) = \frac{C_o - C_t}{C_t} \text{ x } 100 \quad (2)$$

where R (%) is the removal efficiency of nutrients, C_0 (mg L^{-1}) is the initial concentration of the nutrient, and C_t (mg L^{-1}) is the final concentration of the nutrient at time t.

Aquaculture wastewater can be a major source of food requirements for microalgae cultivation and contributes to the reduction of nutrient [14]. Therefore, the uptake of nutrients, ammonia, nitrite and orthophosphate in batch bioremediation under sterile condition was studied. The initial NH_3^+, NO_2^- and PO_4^- concentrations for all seven different treatment were approximately 2.80 ± 0.05 mg L^{-1}, 1.5 ± 0.05 mg L^{-1} and 4.1 ± 0.05 mg L^{-1}, respectively for MW and 3.6 ± 0.05 mg L^{-1}, 1.75 ± 0.05 mg L^{-1} and 4.1 ± 0.05 mg L^{-1} respectively for FW before inoculated with *Chlorella* sp.

2.4. Water quality monitoring

The water quality analyses were carried out with the collection of 50 mL of the water sample from each treatment and control at 2-day interval until 14 days treatment period. The water samples were clarified by centrifugation (Hettich Zentrifugen Universal 1200, Germany) at

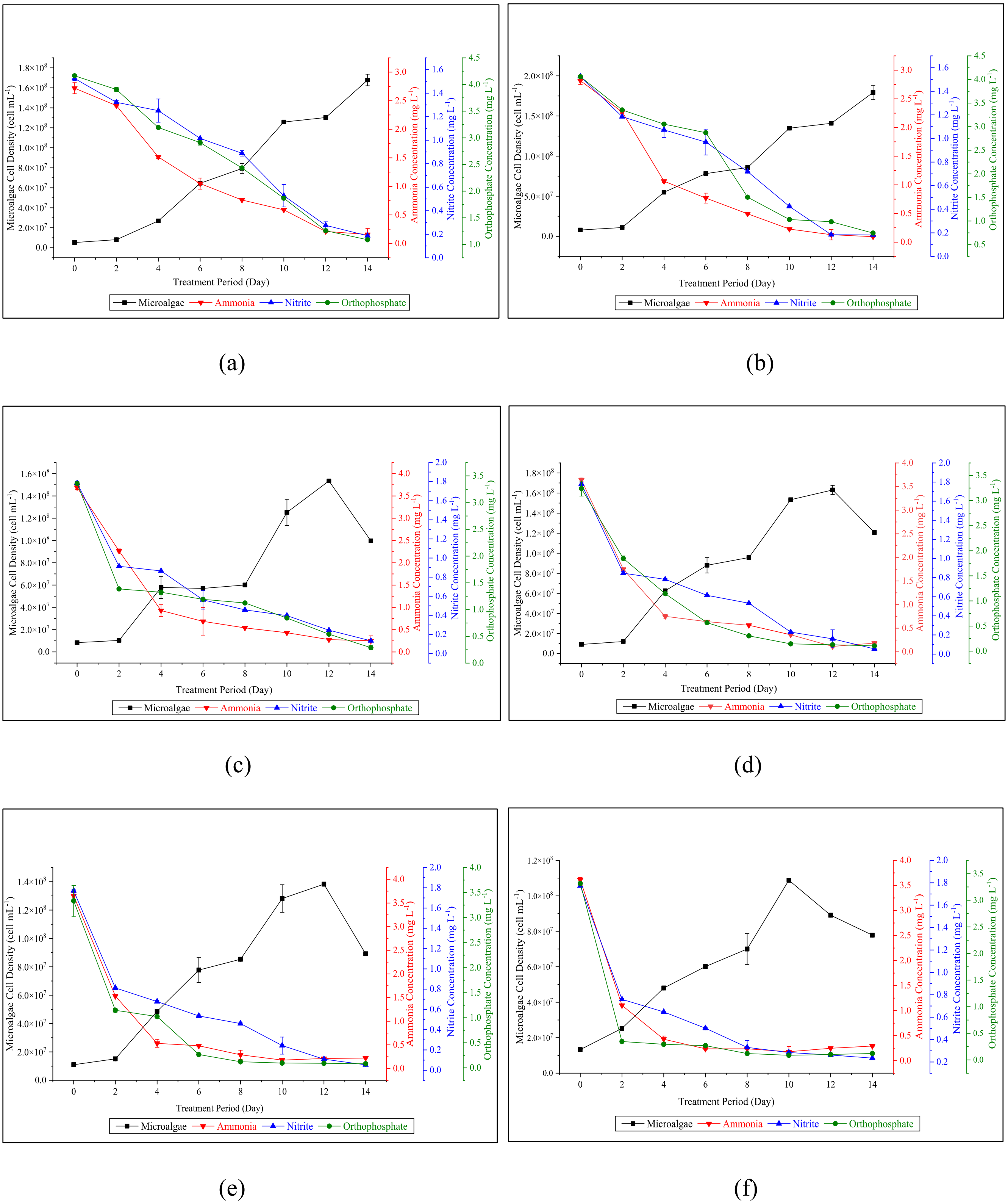

Fig. 2a. Bioremediation performance at (a) 10, (b) 20, (c) 30, (d) 40, (e) 50, and (f) 60 % (v/v) microalgae *Chlorella* sp. inoculation dosages throughout 14-days treatment period for Marine water Treatment (MW).

9000 rpm for 5 min to separate microalgae biomass for producing clear water to perform water quality analysis. The Ammonia (NH_3^+), Nitrite (NO_2^-) and Orthophosphate (PO_4^{3-}) determination were carried out using standard methods, Phenate Method (4500-NH_3.F), Colorimetric Method (4500-NO_2^-.B) and Ascorbic Acid Method (4500-P.E) adopted from APHA (2012). The Dual-Beam UV–Vis Spectrophotometer (Shimadzu UV-1800, Japan) was used to analyze nutrients concentration.

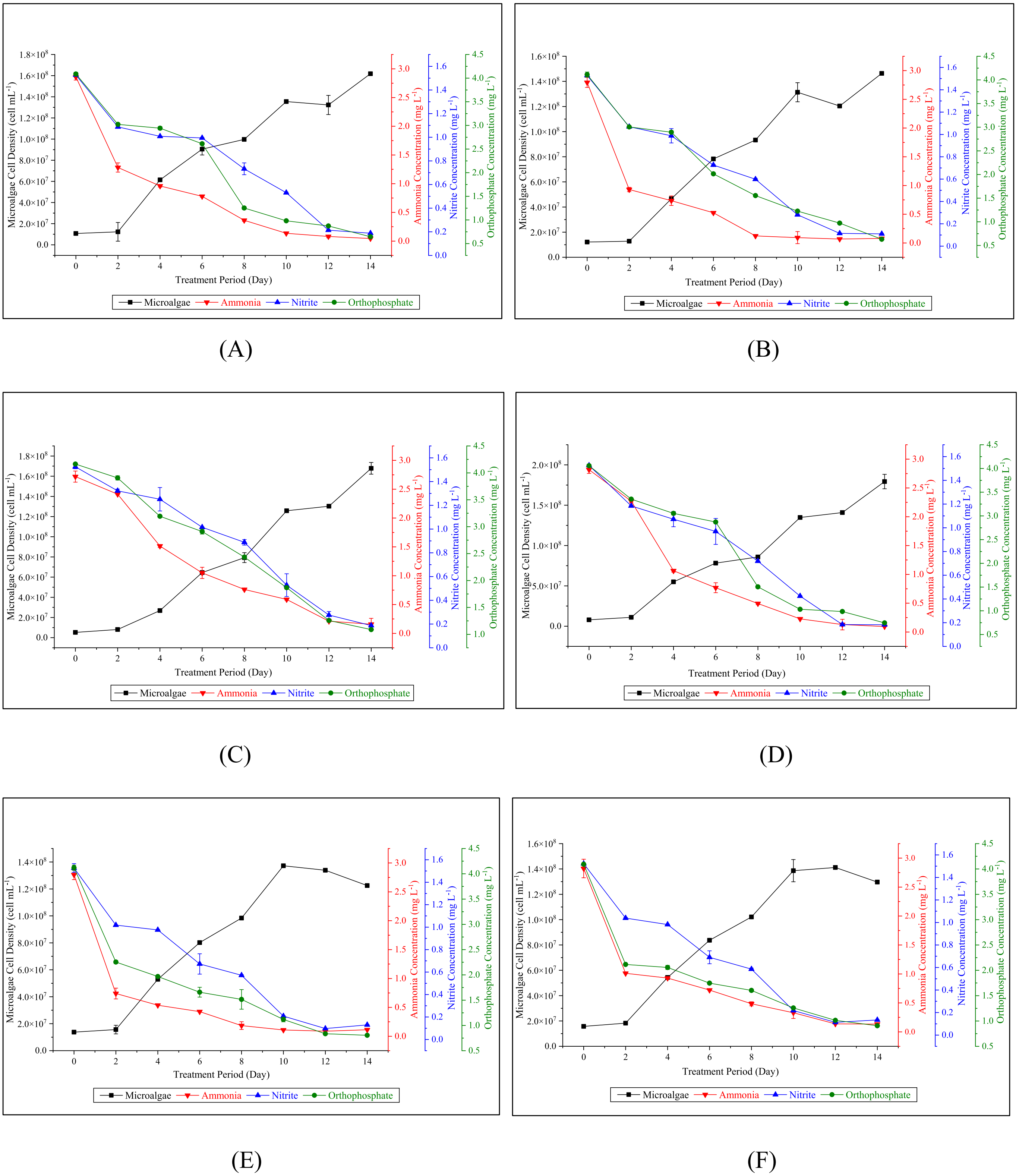

Fig. 2b. Bioremediation performance at (A) 10, (B) 20, (C) 30, (D) 40, (E) 50, and (F) 60 % (v/v) microalgae *Chlorella* sp. inoculation dosages throughout 14-days treatment period for Freshwater Treatment (FW).

2.5. Fourier Transform Infrared (FTIR) analysis

Microalgae cultures were cultured and harvested during late log phase for FTIR analysis. 100 mL of grown microalgae culture was centrifuged at 10,000 rpm for 10 min and the pellet was dried. Both the microalgae cultures, *Chlorella* sp. in media and treatment were processed. Microalgae biomass from marine cultures were rinsed with 0.5 M ammonium formate prior to centrifugation to remove salt from the biomass. The wet microalgae pellets were dried in freeze-dryer using Freezon 4.5 L −50 °C Benchtop Freeze Dryer (USA) for 24 h to form the

Table 1
The removal efficiency (%) of ammonia, nitrite, and orthophosphate for MW and FW at Day 10.

Dosage, % (v/v) / Nutrients	Ammonia (%)		Nitrite (%)		Orthophosphate (%)	
	MW	FW	MW	FW	MW	FW
0	0.77[a]	−3.05[a]	−0.11[a]	−0.17[a]	−0.01[a]	1.05[a]
10	78.36[b]	86.14[b]	65.41[b]	76.89[b]	55.19[b]	56.19[b]
20	91.94[c,d]	88.95[c]	65.23[b]	78.44[c]	74.59[c]	73.49[c]
30	96.77[c]	88.34[d]	82.07[b]	77.76[b]	75.96[c]	74.93[c]
40	95.65[c]	91.93[e]	81.63[c]	87.09[d]	70.19[d]	95.60[d]
50	96.14[c]	93.13[e]	86.42[c]	86.30[e]	72.89[e]	97.14[d]
60	88.32[d]	90.01[d]	85.72[c]	83.96[e]	69.31[d]	97.39[d]

Superscripts letter (a, b, c, d, e) refer to means for group in homogenous subset.

Table 2
The removal rate (k day^{-1}) of ammonia, nitrite, and orthophosphate for MW and FW at Day 10.

Dosage, % (v/v) / Nutrients	Ammonia (%)		Nitrite (%)		Orthophosphate (%)	
	MW	FW	MW	FW	MW	FW
0	0.0008	0.0013	0.0004	0.0013	0.0004	0.002
10	0.21	0.197	0.15	0.182	0.1	0.084
20	0.258	0.237	0.162	0.206	0.131	0.119
30	0.296	0.19	0.149	0.162	0.138	0.139
40	0.273	0.22	0.2	0.218	0.129	0.256
50	0.231	0.199	0.204	0.225	0.108	0.278
60	0.208	0.162	0.195	0.133	0.094	0.201

dried algae powders. The samples were analyzed using FTIR Spectrometer Thermofisher Scientific Nicolet™ iS™ 10 (USA). For this study, a view from the microscope was chosen from the transmission region between 4000 and 400 cm^{-1} wave number range, 4 cm^{-1} resolution and aperture of 20 × 20 μm square aperture, placed over a clear field (background) and 32 scans were taken as spectra.

2.6. Scanning Electron Microscopy (SEM) and Energy Dispersive Spectroscopy (EDS) analysis

The surface morphology of the microalgae was obtained using scanning electron microscopy analysis scan-brand Floor Top Scanning Electron Microscope (SEM) TESCAN/VEGA, CZECH REPUBLIC. The SEM was equipped with EDX BRUKER (Silicon Drift Energy Dispersive Spectrometer model Quantax Compact with XFlash 600Mini). Before the experiment, microalgae biomass was processed using the mentioned technique in FTIR analysis. To perform the analysis SEM, part of the microalgae biomass was bonded to stub with a tape of black carbon and coated with fine thin layer gold, Au to protect the sample and increase the conductivity.

2.7. Data analysis

All experiment data were analyzed in triplicate and graphical analyses were plotted using Origin 2022 software (Origin Lab Corp., USA) for the determination of interactions between factors. Statistical analyses were performed through IBM SPSS ver. 23.0. Normality and homogeneity of variances of the data were satisfied via Shapiro-Wilk test and Levene's test, respectively. Specific growth rate (SGR) of *Chlorella* sp. and removal efficiency of nutrient (%) in different concentrations (10 %, 20 %, 30 %, 40 %, 50 %, and 60 % (v/v)) inoculation in aquaculture wastewater was analyzed by One-Way Analysis of Variance (ANOVA), followed by Tukey HSD test. Results were considered as statistically significant at $p < 0.05$ in this experiment [15].

3. Results and discussion

3.1. Growth performance of microalgae

The performance of microalgae growth primarily governed by nutrients and yields of algae also can be boosted when the nutrients such as nitrogen and phosphorus are readily available in the growth medium [5]. Besides, the growth patterns of *Chlorella* sp. have depicted similar growth pattern at different dosages concentration. The growth kinetics of *Chlorella* sp. throughout bioremediation process suited with microbial growth kinetics by the growth phases of lag, exponential, stationary and declining phases [16]. Fig. 1 illustrated the growth performance of microalgae, *Chlorella* sp. by determining the specific growth rate (SGR) throughout the aquaculture wastewater bioremediation within 14 days treatment period.

The specific growth rate (SGR) of *Chlorella* sp. in this study was determined at exponential phase (Day 10). Fig. 2a and 2b shows that the specific growth rate for *Chlorella* sp. in both treatment, MW and FW. All the different treatment for MW consistently yielded the highest SGR than FW. For MW, the highest SGR (0.228 day^{-1}) was found at 10 % inoculation and lowest SGR (0.139 day^{-1}) was found at 60 % inoculation of microalgae. While for FW, the highest SGR (0.191 day^{-1}) was found at 10 % inoculation and lowest SGR (0.114 day^{-1}) was found at 20 % inoculation of microalgae. SGR for 60 % (v/v) was decreased due to the high competition between microalgae cell for limited available nutrient thus inhibiting effective absorption of nutrient into *Chlorella* sp. biomass [17].

SGR values at various inoculation concentrations (10 %, 20 %, 30 %, 40 %, 50 %, and 60 % (v/v)) were significantly different for FW with ($F = 175.029$, $p < 0.05$), while similar SGR was discovered between the inoculum concentrations of 30 % (v/v) and 40 % (v/v) via Post hoc Tukey's HSD test and Bonferroni test ($p > 0.05$). While for MW ($F = 131.133$, $p < 0.05$) and the SGR were found similar between inoculum concentration, 20 %, 30 % and 40 %. This implies that the microalgae cell growth rate was significantly affected by the amount of cell density that was inoculated [18].

3.2. Effect of microalgae concentration on nutrient consumption

The findings demostrate that microalgae can assimilate the nitrogen from a variety sources, including ammonium, nitrate, nitrite and urea [19]. Additionally, ammonia is the most energy-efficient nitrogen source since less energy is needed for its uptake. Table 1 tabulate the removal efficiency and nutrient availability for different dosages of microalgae for Day 10. According to the analysis, the ammonia concentration were significantly reduced for all different dosages both MW and FW except 0 % (v/v). However, the bioremediation performance of ammonia concentration in MV more effective than FW since the dosage of 20 % of MW already had produced 90 % removal as opposed to 40 % for FW. Generally, ammonium was the predominat nitrogen component in aquaculture, but this study also found that nitrite was present in significant amounts.

Within the first five days of the treatment period, *Chlorella* sp. bioremediation indicated low removal of nitrite and orthophosphate. However, the concentration of nitrite dramatically decreased throughout the treatment and efficiently removed >80 % when the dosages increased from 30 % (v/v) and 40 % (v/v) for MW and FW, respectively. It was noticed that the removal efficiencies were lower at lower dosages concentrations, below than 20 % (v/v). The concentration of nitrite was maintained in this study because the use of sterile microalgae culture and wastewater, without the effects of a complex microbiome that can convert the N and P concentrations. The process of nitrification was suggesting negligible throughout the treatment process. The nitrification process is the process involved the oxidation of ammonia to nitrite by ammonia-oxidizing bacteria, and nitrite to nitrate by nitrite-oxidizing bacteria.

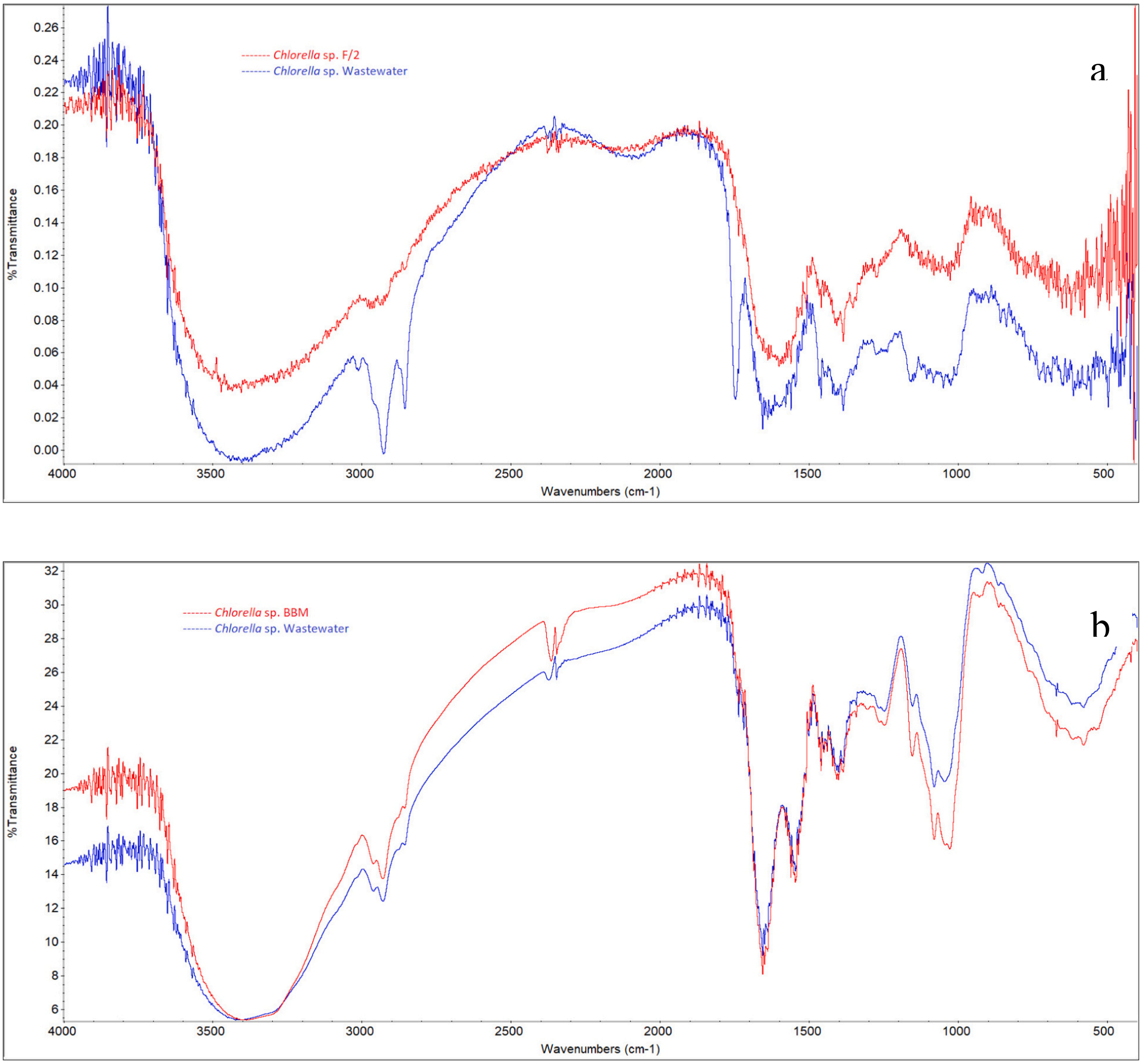

Fig. 3. FTIR Spectral image of *Chlorella* sp. culture in medium (red line) and aquaculture wastewater (blue line). a refer to marine water, Image b refer to fresh water. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3a
Functional group /Assignment of *Chlorella* sp. of Marine Water (MW). References adopted from [28].

Band	Main peak (cm^{-1})	Wave number range (cm^{-1})	Typical band assignment from literature
1.	3854.3	3900–3800	-NH_2 stretching vibration
2.	3401.50	3700–3100	Water v(O—H) stretching Protein v (N—H) stretching (amide A)
3.	2926.58/ 2853.91*	3000–2800	Lipid – carbohydrate Mainly vas(CH_2) and vs(CH_2) stretching
4.	1744.87*	1800–1700	Cellulose–Fatty Acids v(C=O) stretching of esters
5.	1637.09	1700–1600	Protein amide I band Mainly
6.	1458.25	1500–1400	Protein δas(CH_2) and δas(CH_3) bending of methyl, Lipid δas(CH_2) bending of methyl
7.	1155.91	1200–900	Carbohydrate v(C-O-C) of Polysaccharides

(*) Refer to peak present at Bioremediation Process only.

Table 3b
Functional group /Assignment of *Chlorella* sp. of Fresh Water (FW). References adopted from [28].

Band	Main peak (cm^{-1})	Wave number range (cm^{-1})	Typical band assignment from literature
1.	3854.31	3900–3800	-NH_2 stretching vibration
2.	3448.02	3700–3100	Water v(O—H) stretching Protein v (N—H) stretching (amide A)
3.	2927.34	3000–2800	Lipid – carbohydrate Mainly vas(CH_2) and vs(CH_2) stretching
4.	1654.28	1800–1600	Protein amide I band Mainly v(C=O) stretching
5.	1559.70	1600–1500	Protein amide II band mainly δ(N—H) bending and v(C—N) stretching
6.	1458.38	1500–1400	Protein δas(CH_2) and δas(CH3) bending of methyl, Lipid δas(CH2) bending of methyl
7.	1079.73	1200–900	Carbohydrate v(C-O-C) of polysaccharides Nucleic Acid (and other phosphate-containing compounds) vs (>P=O) stretching of phosphodiesters

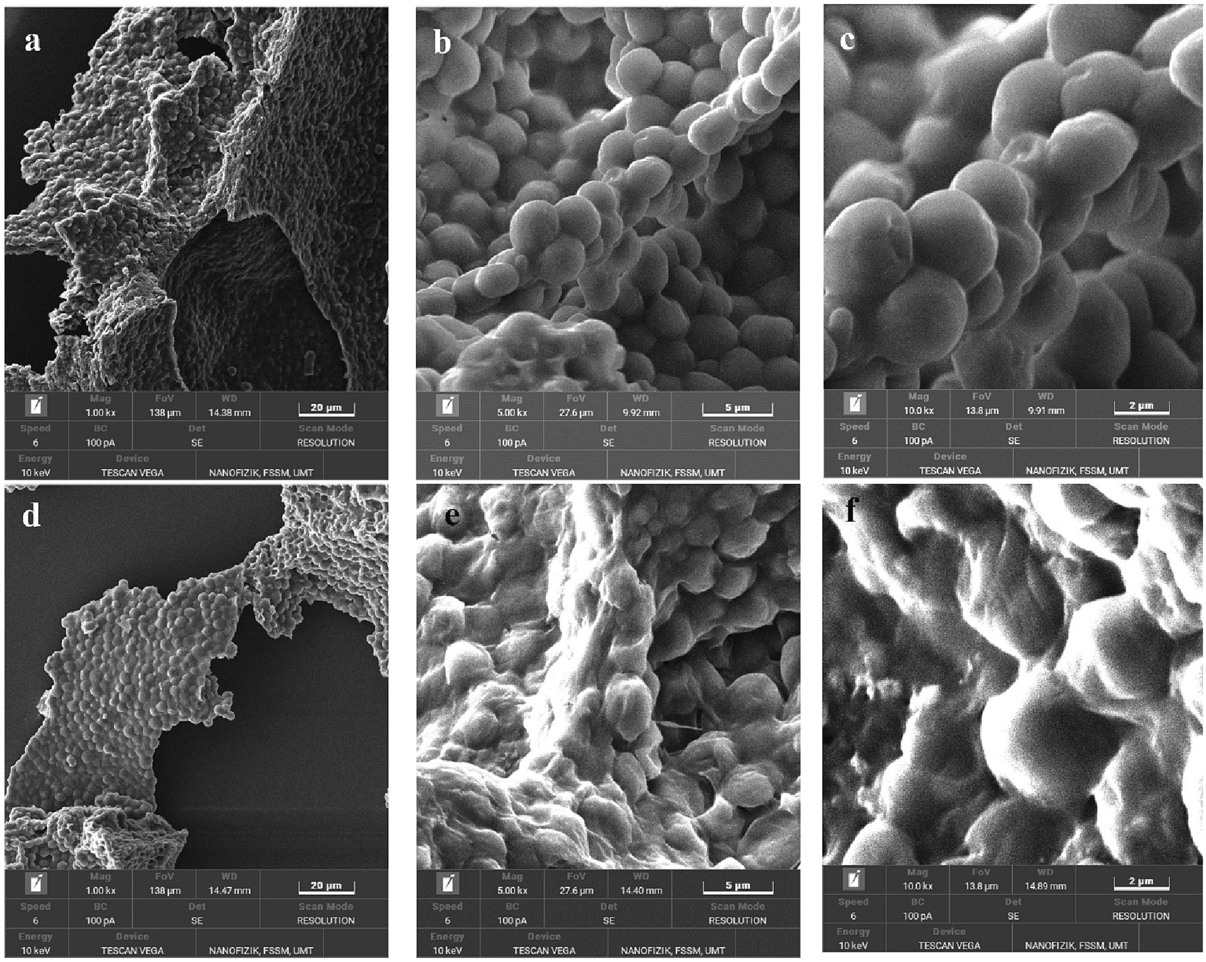

Fig. 4. SEM Image for microalgae magnification x1000, x5000, x10,000. Different letters refer to the genus *Chlorella* culture in different condition at different magnification, (a-b-c) refer to the genus *Chlorella* culture in Guillard's F/2 Media, (d-e-f) refer to the genus *Chlorella* culture in MW, (g-h-i) refer to the genus *Chlorella* culture in BBM, and (j-k-l) refer to the genus *Chlorella* culture in FW.

Phosphorus also crucial component for the growth of microalgae and frequently a major limiting factor for algal growth [20]. Typically, only orthophosphate that is assimilated by phytoplankton and can be utilize for cell development [21]. The absorbed phosphorus is usually retained as polyphosphate granules and will be useful to algae during their growth cycle. A study [22] mentioned that nutrient in the form of orthophosphate was reduced due to absorption by *Chlorella* sp. and stored as polyphosphates within the cells. Additionally, the overall findings show that phosphorus concentration in the form of orthophosphate, PO_4^{3-} for MW was eliminated with a lower removal efficiency <80 %, whereas in FW completely removed from the wastewater. This is postulated due to the green algae species like *Chlorella vulgaris* are capable of absorbing phosphorus only to a limited extent. Similarly, PO_4^{3-} removal in all treatments was higher was compared against the control.

Apart from that, the removal efficiency and removal rate of dosages 0 % (v/v) were the lowest, as could be seen in Fig. 1 and Table 1, and there were no significant differences among the other dosages for all nutrients because it was used as a control treatment and run without a microalgae inoculum.

The removal rate was positively affected by the dosage of microalgae (Table 2). The highest value of removal rate was observed different depending on the nutrients and dosages. The apparent removal rate (k. day^{-1}) at 30 % (v/v) was 0.296 k day^{-1} which is in accordance with the removal efficiency thats suggested as the maximum ammonia removal efficiency for MW. For FW, the removal rate for the 30 % (v/v) is 0.19 k day^{-1} and among the lowest compare to the other dosages. The dosage 20 % (v/v) in FW had achieved the faster rate of ammonia removal at Day 10, 0.237 k day^{-1} which remove about 88.95 % from the water sample.

As the 14 days treatment period, the dosages 30 % (v/v) was selected as the highest performance of nutrient consumption for marine wastewater treatment (MW) based on removal efficiency, with NH_3^+ and NO_2^- were 0.135 mg L^{-1} and 0.274 mg L^{-1} of nutrient availability and the removal efficiency were 96.77 % and 82.07 %, respectively. On the other hand, for the freshwater treatment (FW), the dosage 40 % was choosed as the best dosage, resulting the highest removal efficiency as compared to other dosages which were 90.10 %. 87.09 % and 95.6 % for ammonia, nitrite and orthophosphate, respectively.

For further investigation on the effect of *Chlorella* sp. inoculation concentrations on nutrients removal, the correlation analysis between growth and nutrient were performed individually on Day 10 treatment period for both MW and FW. The findings demonstrated that the positive correlation exists when nutrient concentrations decrease exponentially proportional throughout the treatment as the growth cell density rises and it's complied with the First Order Kinetic Model. This study was confirmed with the assumption make previously that the growth of microalgae was influenced by the reduction of nutrient content in wastewater.

Figs. 2a and 2b showed that the decreasing of nutrients (ammonia,

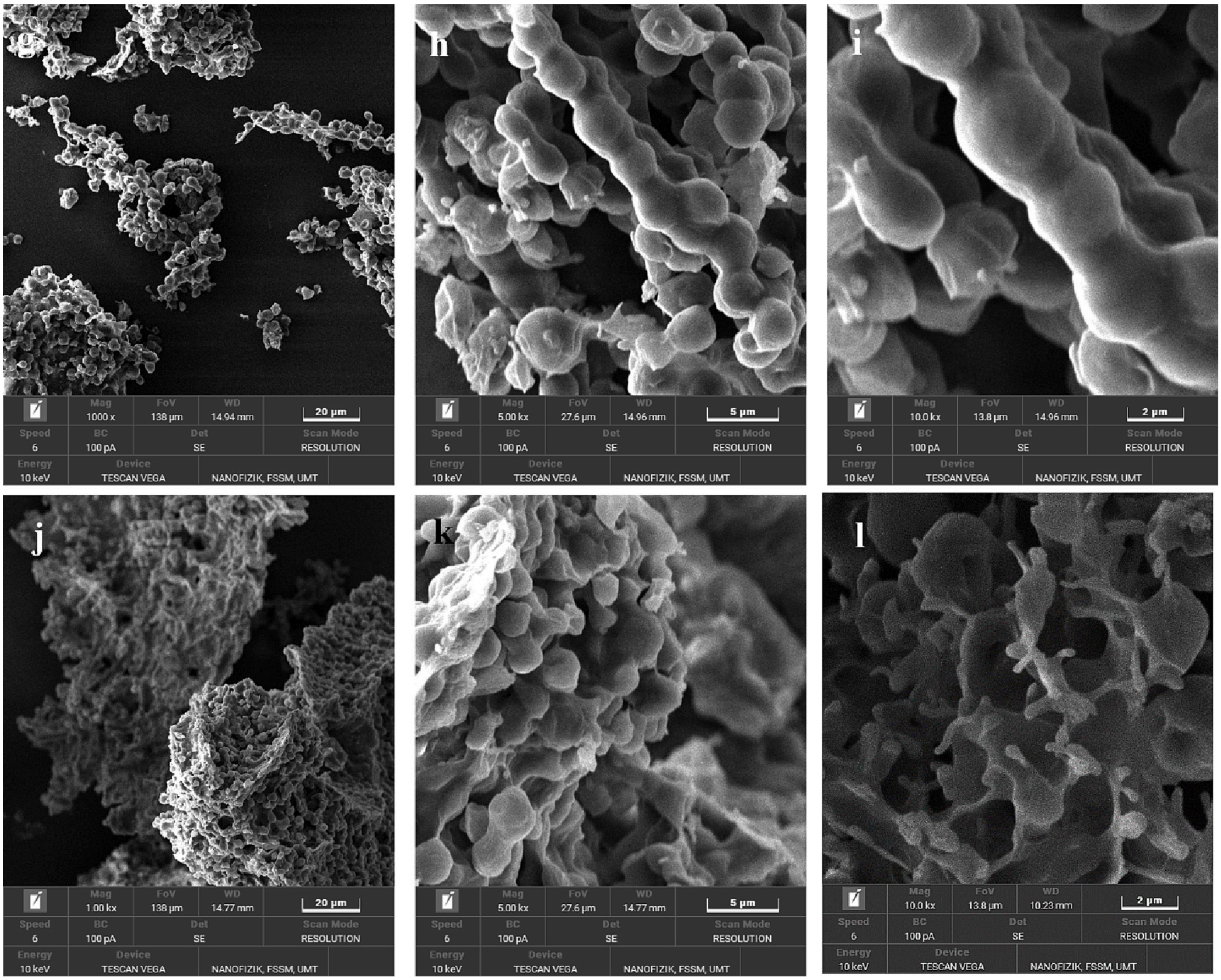

Fig. 4. (*continued*).

Table 4
Elemental identification by EDS.

Biomass sample	Contents of element by weight (%)			
	Chlorella sp. LF-2 Guillard's F/2 Media	*Chlorella* sp. LF-2 MW	*Chlorella* sp. LF-1 BBM	*Chlorella* sp. LF-1 FW
Carbon, C	73.56	58.26	53.27	57.75
Nitrogen, N	4.69	17.39	12.63	6.47
Oxygen, O	9.80	20.16	31.94	18.34
Phosphorus, P	5.87	2.94	0.97	12.18
Sulphur, S	6.08	1.24	1.2	5.17

nitrite, and ortophosphate) was in accordance with the microalgal biomass growth, suggesting the conversion of nutrient into biomass for both marine water (MW) and freshwater (FW) treatment. Similar growth pattern were depicted in the *Chlorella* sp. growth in different treatment with relatively short lag phase in the first two days and followed the exponential phase in the six to eight days. It was observed that the death phase began on Day 12 towards the end of the treatment period except 10 % and 20 % (v/v). As illustrated in Figs. 2a and 2b, the microalgae displayed a brief lag phase of one to two days when the cell concentration were increased about two-folds from the initial biomass density. Therefore, short lag phase revealed that the microalgae had excellent adaption characteristics to the aquaculture wastewater.

In the case of *Chlorella* sp., this species able to utilize both ammonium and nitrite for the syntesize of glutamine and glutamate with the involvement of glutamine synthetase (to gather energy from the breakdown of ammonium) and glutamate synthetase (to produce glutamate using nitrite) [23]. In addition to sufficient nitrogen, P also benefit the lipid content in *Chlorella* sp. followed by the increasing accumulation of poly-P inside cells [24]. The high uptake of ammonia, nitrite, and orthophosphate indicate a good utilization of nutrients by *Chlorella* sp. to support the cell growth [25]. According to the graph, the concentration of ammonia started to increase for all treatments between Days 12 and 14, especially for the 50 % and 60 % (v/v) treatments. Given that microalgae begin to enter the death phase on this day, it might be related to the microalgae's growth phase. As reported from my previous study, bioremediation using *Claries gariepinus* wastewater, this phonemonen happened due to release of absorbed nutrient from microalgae biomass as it experienced early death phase. During this growth phase, the *Chlorella* sp. biomass started to autolyze and degrade.

3.3. Characterization of microalgae morphology

3.3.1. FTIR analysis

FTIR spectroscopy played a crucial role for the characterization of the biochemical composition of phytoplankton [26]. In general, all chemical bonds have a number of bending and stretching vibrations with varying energies, which produce the various absorption bands. In addition, the composition and molecular functional groups can be determined by analysing the position, width, and intensity of infrared light absorption. The results of FTIR transmittance of microalgae biomass from wave number range of 4000–400 cm^{-1} indicates the presence of organic component groups of amine, alcohol, aromatic, alkyne, alkene, acid, ether and alkyl halide groups as well as organic contents such as carbohydrates, proteins, and lipids in *Chlorella* sp. The spectral absorption bands were identified in accordance with information that has been published.

Fig.3 shows the results of FTIR transmittance of four distinct microalgae biomass. Examinations of the infrared spectra of all biomass revealed the presence of the seven uniques bands at 3900–3800 cm^{-1}, 3700–3100 cm^{-1}, 3000–2800 cm^{-1}, 1800–1700 cm^{-1}, 1700–1600 cm^{-1} (MW only), 1600–1500 cm^{-1} (MW only), and 1200–900 cm^{-1}. This indicates that there are variances in the composition of the microlgae biomass despite the fact that they have comprable organic groups. The FT-IR spectrum of *Chlorella* sp. used in this study is similar reported by Ferreira et al. [27].

The typical band assignment from literature is summarized in Tables 3a and 3b. The band contributions were postulated from residual water (band 2), lipids (bands 3 and 6), cellulose (band 4), proteins (bands 5 and 6, 4, 5 and 6), and carbohydrate (band 7). The peaks located at 2853.91 and 1744.87 correspond to the lipid – carbohydrate and cellulose–fatty acids only obtained in bioremediation process of MW, suggesting the presence of additional constituents in nutrients from actual wastewater.

3.3.2. Scanning Electron Microscopy (SEM) and Energy Dispersive Spectroscopy Analysis (EDS)

Advanced microscopy, such as scanning electron microscopy (SEM), is necessary to characterize microalgae [29]. After 14 days of treatment with aquaculture wastewater, the *Chlorella* sp. biomass cells were examined visually using light microscopy, SEM, as well as energy dispersive spectroscopy (EDS) to determine their elemental composition (EDS). In this investigation, SEM microscopy was employed to assess the surface features and morphological changes in the cell wall composition and shape of microalgae biomass after the bioremediation process.

Fig. 4 (a-b-c), scanning electron microscopy (SEM) visualization for all microalgae biomass revealed that cells were attached to each other. According to the findings, the sphericity and surface smoothness of microalgae particles were consistently observed throughout culture in widely used media, Guillard's F/2 Media. In contrasts, the irregular nonporous morphology with cavities on the surface of cells were discovered when subjected to bioremediation process. The *Chlorella* sp. LF1 might be associated with the component presenting in the aquaculture wastewater and created the cell-wall bound substance. This hypothesis was supported by the development of a new peak, which is demonstrable by previously findings, in FTIR analysis.

This result is further confirmed by [30] that the the surface of *Chlorella* sp. had irregular nonporous morphology with cavities on the surface after the treatment. By studying the structure of the particle, the results could serve as a foundation for understanding that the bioremediation using microalgae have affect's the cells of microalgae. The SEM analysis also revealed significant changes in the morphology of the investigated microalgae.

Characterization the chemical composition on cell surface microalgae was analysis using the combination of SEM accomplished with X-ray (EDX) (Table 4). EDS analysis is important to study since its enable to provide valuable information regarding the composition adsorbent surface for a sample. It should be highlighted that SEM provides only a qualitative evaluation of the surface structure and not able to specify the internal structure of cell [31]. When SEM is combined with EDX technique, it can provide valuable input in determining the distribution of various elements on the microalgae biomass. Tables 3a and 3b represented the result of elemental analysis of microalgae biomass. The data in terms of atomic percentages demonstrated the presence of C, N, O, P and S, which are the main components of cellular macromolecules [32].

After the bioremediation process, the percentages of N and O on the surface of the microalgae biomass showed a higher accumulation in MW, whereas C, P, and S were the lowest when compared to microalgae cultivated in Guillard's F/2 Media. In contrast to the microalgae biomass in FW, N and O were less abundant than C, P, and *S. Maximum* absorption peaks in the spectral region of lipids and carbohydrates were also produced by the greater oxygen accumulation in MW [33].

4. Conclusions

As conclusion, the results suggest that different wastewater types require different inoculation dosages for optimal bioremediation efficiency. For MW, the highest bioremediation efficiency was achieved at 30 % inoculation dosage and for FW, the highest bioremediation efficiency was achieved at 40 % inoculation dosage. This study also demonstrated that the varying concentrations of microalgae have significant impact on the growth performance of microalgae. Additionally, microalgae acknowledged able to transform nutrients; nitrogen and phosphorus from wastewater into biomass and bioproducts to boost the sustainability of wastewater treatment. Overall, the successful application of bioremediation approach was accomplished using microalgae-based by nutrient consumption from aquaculture wastewater and is relevant for future application in the aquaculture industry.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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CRediT authorship contribution statement

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Nurfarahana Mohd Nasir: performed the experiment and manuscript writing, Ahmad Jusoh: design, supervise the research project and comments on the critical part of manuscript, Nik Nor Liyana Nik Ibrahim: formatting, conduct on final revision of manuscript comments on the critical manuscript writing, Razif Harun: formatting, conduct on final revision of manuscript comments on the critical manuscript writing, Nazaitulshila Rasit: formatting, conduct on final revision of manuscript comments on the critical manuscript writing, Wan Azlina Wan Abdul Karim Ghani: supervise of the research project and comments on the critical manuscript writing. Setyo Budi Kurniawan: reviewed drafts of the paper.

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ARTICLES FOR FACULTY MEMBERS

AQUACULTURE WASTEWATER

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Review

Wastewater based microalgal biorefinery for bioenergy production: Progress and challenges

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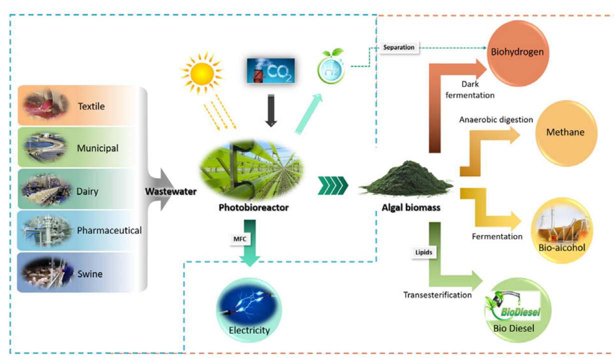
HIGHLIGHTS

- Wastewater treatment and management is required to protect environment.
- Microalgae based wastewater treatment helps in resource recovery from wastewater.
- Need to adopt hybrid technology for microalgae cultivation to improve productivity.
- Recovered biomass can be used to produce bioenergy and commercial products.

GRAPHICAL ABSTRACT



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ABSTRACT

Treatment of industrial and domestic wastewater is very important to protect downstream users from health risks and meet the freshwater demand of the ever-increasing world population. Different types of wastewater (textile, dairy, pharmaceutical, swine, municipal, etc.) vary in composition and require different treatment strategies. Wastewater management and treatment is an expensive process; hence, it is important to integrate relevant technology into this process to make it more feasible and cost-effective. Wastewater treatment using microalgae-based technology could be a global solution for resource recovery from wastewater and to provide affordable feedstock for bioenergy (biodiesel, biohydrogen, bio-alcohol, methane, and bioelectricity) production. Various microalgal cultivation systems (open or closed photobioreactors), turf scrubber, and hybrid systems have been developed. Although many algal biomass harvesting methods (physical, chemical, biological, and electromagnetic) have been reported, it is still an expensive process. In this review article, resource recovery

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Treatment
Feedstock
Resource recovery
Bioenergy

from wastewater using algal cultivation, biomass harvesting, and various technologies applied in converting algal biomass into bioenergy, along with the various challenges that are encountered are discussed in brief.

Contents

1. Introduction

The escalation in industrialization and overexploitation of natural resources in tandem with the increasing global population has adversely affected the environment (Bhatia et al., 2018; Manaf et al., 2019). Different types of wastewater from textile, municipal, dairy, pharmaceutical, swine, and aquaculture, are being produced constantly by respective industries (Kadir et al., 2018; Lellis et al., 2019; Zhang et al., 2017). Wastewater is rich in nutrients; it promotes eutrophication in the ecosystem and poses a threat to the flora and fauna. According to a report, eutrophication causes an annual loss of almost 2 billion US dollars and continues to affect real estate and fishing activities (Aigars and Tālis, 2017). Wastewater contains various unwanted chemical constituents and pathogens which have short and long term effect on the environment and human health. Exposer to untreated wastewater may cause gastroenteritis, skin infection, hepatitis A, and leptospirosis like disease (Tiwari, 2008). Other non-infectious biological substances like endotoxins released by the death of microbes may cause irritation and allergy on contact with skin or induce high fever, intravascular coagulation, multi-organ failure, even death on entrance into host's blood (Fung et al., 2017). The use of untreated wastewater in irrigation also associated with many problems such as excessive vegetative growth, uneven fruit maturity, and reduced quality and yield (Libutti et al., 2018). Traditionally, the main purpose of wastewater treatment is to protect downstream users from health risks. Generally, wastewater is treated using various physical (screening, grit chamber, floatation, etc.) and chemicals methods (neutralization, flocculation, oxidation, disinfection, etc.) (Awaleh and Soubaneh, 2014). Various chemical methods used in the treatment of wastewater are not only expensive but often result in sludge production and increased risk of secondary water pollution (Guimarães et al., 2016). There are other challenges associated with wastewater treatment such as high energy consumption (2–4% of national electric power consumed), shortage of efficient and productive workers, wastewater treatment plants also have environment impact, introduction of new and efficient technology require more money and make the old infrastructure absolute (Sparn and Hunsberger, 2015). In recent decades, an extra desired goal, i.e., resource recovery from wastewater along with wastewater treatment technology, is gaining attention. Pittmann et al. studied polyhydroxyalkanoates (PHAs) production from different wastewater treatment plants and proposed a theory that about 120% of the worldwide biopolymer production (produced in 2016) could be achieved from European wastewater treatment plants (Pittmann and Steinmetz, 2017). Wastewater also have been explored to produce other products like single cell protein and carotenoids etc. (Saejung and Salasook, 2020).

The energy crisis is another issue due to the extensive use of fossil-derived fuels such as petrol, natural gases, and coal, which have been estimated to be exhausted within the next 50 years (Kumar et al., 2020). There is an imperative need to search for new alternative renewable resources of energy to meet future energy demand (Bhatia et al., 2017b; Mehariya et al., 2018). Various technologies have been developed to harness renewable sources of energy i.e. solar, wind, hydraulic, and geothermal energy (Qazi et al., 2019). All these technologies are well developed and available at a commercial scale to fulfill increasing energy demand up to a certain extent. Utilization of wastewater as a resource to produce energy can further help to reduce the burden on other technologies. Microalgae are natural sunlight-driven cell factories that have the ability to assimilate CO_2 and accumulate lipids using solar energy (Lee et al., 2020). Many studies have reported the feasibility of applying microalgal technology in wastewater treatment, which could potentially reduce wastewater-nutrient concentration up to 95% (Ren et al., 2019). Along with nutrient removal capacity from wastewater, microalgae have capability to accumulate lipids, produce biohydrogen and bioelectricity (Bhatia et al., 2017a; Kadir et al., 2018). Utilization of microalgae in wastewater treatment is advantageous as it will not only helps in waste management but can also produce bioenergy simultaneously. Algal biomass is rich in lipids and can be used as an alternative oil source for biodiesel production through transesterification reactions using different acids, alkali, and enzymatic catalysts (Banerjee et al., 2019; Bhatia et al., 2018). Biohydrogen can be produced by using algal biomass using modified strategies such as initial cultivation of algae in wastewater under aerobic conditions and subsequently transferring them into an anaerobic reactor (Vargas et al., 2018). Fresh algal biomass or residual biomass after lipid extraction can be directly used for bioenergy production using dark fermentation (biohydrogen production), fermentation (bio alcohols), and anaerobic digestion (methane) (Sanchez Rizza et al., 2019; Solé-Bundó et al., 2020; Wirth et al., 2018). Use of microalgae has also been reported in the production of bioelectricity using a technology called photosynthetic microbial fuel cell (PhotoMFC). Various reactor designs, such as single chamber, dual chamber, and MFC with water desalination chamber, have been reported for bioelectricity production (Ling et al., 2019; Logroño et al., 2017).

Various traditional (open and closed system) and advanced (turf scrubber and hybrid) microalgal cultivation systems have been developed (Adey et al., 2011; Jankowska et al., 2017). Wastewater-based algal biomass production is affected by various factors such as photobioreactor size, design, operating temperature, mixing, light intensity, organic load and nutrient composition of wastewater, and CO_2 supply (Barros et al., 2015; Kadir et al., 2018). Thus, choosing a suitable reactor design that uses microalgae species based on wastewater composition may help overcome some challenges. After successful cultivation next step is the separation of algal biomass from wastewater. There are many physical, chemical, biological, and electro-magnetic methods of microalgae harvesting have been reported. However, there are certain obstacles that hamper the adoption of this technology. Harvesting algal biomass is a cost-effective process as algae carry a negative charge on the surface that prevents them from forming large particles (Singh and Patidar, 2018). It is estimated that algal biomass harvesting constitutes around 30% of the total production costs (Aigars and Tālis, 2017). A variety of bioproducts such as nutraceuticals, pharmaceuticals, and pigments can also be generated from algal biomass (Galasso et al., 2019). Therefore, the integration of wastewater treatment with algae and the production of bioenergy with other valuable byproducts could help reduce costs in wastewater management (Molino et al., 2020).

Many review articles have been published and provide valuable insights in this field (Acién Fernández et al., 2018; Solon et al., 2019). Despite these valuable contributions, there is still no article that provides a holistic overview of wastewater-based algal biorefinery for bioenergy production. In this study various types of wastewater, advancements in microalgae cultivation and harvesting technologies from wastewater, as well as utilization of algal biomass in bioenergy production (biodiesel, biohydrogen, bioalcohol, methane, and bioelectricity etc.) are discussed. Lastly, the bottlenecks in this field are deliberated for the purpose of improving this technology to be more efficient.

2. Source of wastewater

Daily activity and the production of various resources required for survival and sustainability generate various useless byproducts that finally end up as waste. Depending on the human lifestyle and types of industry present, different types of waste are being produced. The type of sewer system and technology used also significantly affect the ultimate composition of wastewater. In some developed countries, a separate sewer system is often used for different wastes, while in most countries wastewater is discharged into a combined sewer system. Wastewater can be categorized based on their origins such as the textile industry, household or municipal, swine farming, and pharmaceutical. Source of various wastewater and their compositions are discussed below and in Table. 1.

2.1. Textile industry wastewater

Textile industry is a fast growing and one of the largest industries in the world, generating around 1 trillion dollars of annual sales and contributing up to 7% of global export (Lellis et al., 2019). The vast array of synthetic dyes used for coloring fabrics generates huge quantities of colored wastewater. Textiles industries tend to utilize more water compared to other industries resulting in highly polluted discharged wastewater. Water utilization and types of wastewater produced also depend on the steps involved in fabric processing. Textile industry wastewater is mainly composed of dyes, alkalis, acids, surfactants, soap, and metal. It is estimated that around 200 L water kg^{-1} of fabric is consumed by an average sized textile mill every day (Holkar et al., 2016). Fabrics undergo different processing steps, such as desizing, bleaching, mercerization, dyeing, printing, and finishing. Sizing process requires dilute mineral-acid treatment to remove starch residues, and the resulted effluents have high biological oxygen demand (BOD) values ranging from 300 to 450 ppm. To remove the natural color of fibers, bleaching is required and various chemicals like hypochlorite, H_2O_2, and peracetic acids are commonly used. After bleaching, mercerization using NaOH (18–24%) imparts a shine to the fabric. To impart colors to fabrics, various dyes that possess chromophore groups such as azo (-N=N-), nitro (-N=O), carbonyl (-C=O), quinoid, and several auxochromes (sulfonate, hydroxyl, amine and carboxyl) are used, which are responsible for a wide range of dye colors (Benkhaya et al., 2020). Unutilized dye is responsible for pollution and an undesirable color of the textile wastewater. Finishing is the last step used to improve softening, waterproofing, and antibacterial properties of fabric fibers, which further adds pollutants (solvents, resins, and waxes) into wastewater. Textile wastewater has toxic effects on aquatic life as it hinders light penetration, interferes with photosynthetic function of plants, and decreases the activity of microbes. Toxic dyes also act as carcinogenic and mutagenic agents, may enter food chain causing biomagnification, and severely affect organisms at higher trophic levels (Lellis et al., 2019). Therefore, it is absolutely important to treat textile wastewater using various physical, chemical, and biological treatments to protect the environment and ensure sustainability (Piergrossi et al., 2018). Although physical and chemical methods are successfully used, they are neither economical nor ecofriendly and often pose drawbacks including sludge disposal. Biological methods are more advantageous in the treatment of wastewater for nutrient recovery and other resource generation such as biofuel and biochemicals (Kehrein et al., 2020).

2.2. Municipal wastewater

Municipal wastewater is mainly composed of discharge from household and domestic activities. It is mostly disposed into drainage systems, lakes, and rivers without any prior treatment and poses obstacles in improving the living standards of the community. Characteristics of wastewater are generally determined based on the

Table 1

Composition of various wastewater and effluent release standard. Data collected from [a](Yaseen and Scholz, 2019), [b](Singh et al., 2017), [c](Mehrotra et al., 2016), [d](Rana et al., 2017), [e](Maggi et al., 2013), [f](https://www.jetro.go.jp/ext_images/thailand/pdf/MOIEffluentStandards2560.pdf). [a]

Parameters	Textile[a]	Municipal[b]	Dairy[c]	Pharmaceutical[d]	Swine[e]	Effluent standard[f]
Temp (°C)	35–45	23.5	18–55	–	–	40
pH	6–10	8.11	4.4–9.5	7.2–8.5	7.7	5.5–9.0
Color (Pt-Co)	50–2500	–	–	–	–	300
COD (mg L^{-1})	150–12,000	0.32	80–95,000	2000–3500	1322	120
BOD (mg L^{-1})	80–6000	98.49	40–48,000	480–1000	945	20
EC (μS^{-1})	–	–	–	–	5.43	–
TS (mg L^{-1})	–	–	135–8500	–	–	–
TSS (mg L^{-1})	15–8000	–	244,500	48–145	–	50
TDS (mg L^{-1})	2900–3100	–	–	–	–	3000–5000
Chlorine (mg L^{-1})	1000–6000	–	–	–	–	–
Chlorides (mg L^{-1})	–	–	48–1930	–	–	–
Free Chlorine (mg L^{-1})	<10	–	–	–	–	<1
TKN (mg L^{-1})	70–80		1–180	80–164	887	100
TN (mg L^{-1})	10–30	–	–	–	–	–
NO_3-N (mg L^{-1})	<5	–	–	–	2.18	–
Free ammonia (mg L^{-1})	<10	–	–	74–116	446	–
Phosphate (mg L^{-1})	<10	–	10–160	18–47	108	–
Sulfates (mg L^{-1})	600–1000	–	–	–	–	–
Sulfides (mg L^{-1})	–	–	–	–	–	1
Oil and grease (mg L^{-1})	10–30	6.22	35–500	–	–	<5
Zinc (mg L^{-1})	<10	0.91	–	–	0.20	<5
Nickel (mg L^{-1})	<10	–	–	–	–	<1
Manganese (mg L^{-1})	<10	–	–	–	–	5
Iron (mg L^{-1})	<10	–	–	–	–	–
Copper (mg L^{-1})	<10	0.15	–	–	0.25	<2
Boron (mg L^{-1})	<10	–	–	–	–	–
Arsenic (mg L^{-1})	<10	–	–	–	–	–
Silica (mg L^{-1})	<15	–	–	–	–	–
Mercury (mg L^{-1})	<10	–	–	–	–	<0.005
Fluorine (mg L^{-1})	<10	–	–	–	–	–
Chromium (mg L^{-1})	–	0.03	–	–	–	<0.25
Potassium (mg L^{-1})	–	–	10–160	–	462	–
Sodium (mg L^{-1})	7000	–	60–810	–	26	–
Calcium (mg L^{-1})	–	–	55–115	–	38.60	–
Lead (mg L^{-1})	–	0.22	–	–	–	<0.2

[a] COD (chemical oxygen demand), BOD (biochemical oxygen demand), EC (electrical conductivity), TS (total solids), TSS (total suspended solids), TDS (total dissolved solids), TKN (total kjeldahl nitrogen), TN (total nitrogen), NO_3–N nitrate–nitrogen.

various physicochemical factors such as pH, chemical oxygen demand (COD), biological oxygen demand, total dissolved solids (TDS), total suspended solid (TSS), total nitrogen, phosphate, potassium, metals, and total microbial load. Wastewater from kitchen sinks, laundromats, showers, or any household activity excluding waste from toilets, is called grey wastewater. Grey wastewater containing toilet waste is called black wastewater (Ibrahim et al., 2020). Management of wastewater is a major challenge in metropolitan cities since disposal of untreated wastewater could cause water and terrestrial pollution and may result in problems in human health, cause epidemics, and eutrophication of water bodies. Therefore, treatment of wastewater is important to ensure it is of low risk prior to rendering it reusable for various purposes. Onay isolated and cultured *Nannochloropsis gaditana* from various municipal wastewater sources, and the recovered hydrolyzed algal biomass was subjected to fermentation using *S. cerevisiae* to produce ethanol 94.4 mgg^{-1} of biomass (Onay, 2018).

2.3. Dairy wastewater

Dairy wastewater is increasing exponentially as the demand for milk and milk products is increasing globally. The global milk production was estimated to be around 818 million tons according to the International Dairy Federation's World Dairy Report 2016 (Yonar et al., 2018). The dairy industry generates milk, yogurt, cheese, cream, butter, ice cream, and other milk products (Davarnejad et al., 2017). Wastewater discharged from diary-processing factories contains high concentration of nutrients, fats, chlorides, sulfates, lactose, COD, BOD, TSS, organic, and inorganic components. Composition of wastewater fluctuates depending on the type of milk products. Nitrogen exists mostly in the form of amino acids in milk protein, while phosphate is present as inorganic phosphates and diphosphates. A study on the effluents from milk-processing facilities indicates a total nitrogen content of 4.2–6% in butter-processing plants, 3.7% of BOD in cheese-making facilities, and a phosphate concentration in the range of 0.6–7% (Kolev Slavov, 2017). These nutrient concentration levels can support microbial growth and enhance the oxidation of dairy wastewater. Cheese effluents have a C/N/P ratio of 200:3.5:1, and the lack of nitrogen component poses a huge problem for aerobic digestion; hence, anaerobic digestion is considered an alternative method (Henze and Harremoës, 1983). In addition, dairy wastewater has low alkalinity and requires more chemicals for pH maintenance during fermentation. Hence, direct discharge of dairy effluents into water bodies without treatment is not recommended as it may cause problems such as rapid depletion of dissolved oxygen and release of toxic volatile compounds, resulting in the destruction of aquatic life and damage to the environment. Dairy wastewater is therefore, generally treated using biological methods like activated sludge process, trickling filters, upflow anaerobic sludge blanket reactors, and anaerobic filters.

2.4. Pharmaceutical wastewater

The global pharmaceutical industry has been growing rapidly since the last few years and contributing to high economic growth, while simultaneously causing severe environmental pollution. Pharmaceutical technology typically uses biological or chemical processes. Biopharmaceutical industry involves various processes, such as microbial fermentation, extraction of organic materials, and addition of antibiotics, vitamins, and amino acids. The discharged effluents contain high levels of antibiotics and microbes, and low C/N ratio (Guo et al., 2017).

Chemical-based pharmaceutical production uses various chemical reactions that produces complex wastewater with high salt content with a low nutrient value that are not easily biodegradable. Wastewater produced by different pharmaceutical industries are not identical and each type of wastewater composition is influenced by the processes used. It is mainly composed of antibiotics, steroids, reproductive hormones, analgesics, beta-lactamides, antidepressant, detergents, as well as unspent solvent and heavy metals (lead, nickel, mercury, chromium, etc.) (Rana et al., 2017). Pharmaceutical wastewater has adverse effects on human health and environment owing to their toxicity, genotoxicity, and mutagenic nature of pollutants. Evidently, pharmaceutical companies are addressing these problems using various strategies including primary (physical, chemical, and physicochemical), secondary (biological), and tertiary (oxidation) methods to treat wastewater.

2.5. Swine and aquaculture wastewater

Global population is increasing exponentially and estimated to be around 9.9 billion by 2050 (Nagarajan et al., 2019). To meet the increasing demand for food and protein resources, there has been a sharp increase in swine farms globally. According to a report, there were around 769.05 million hogs and a global production of 118.8 million metric ton pork was recorded in 2018 (Nagarajan et al., 2019). Operation of swine farming requires veterinary antibiotics and steroidal hormones to decrease mortality and increase production. According to another report, a leading pork producer farm generated around 1300 ton of wastewater per year (Zhang et al., 2017). Wastewater from swine farms contains high levels of ammonia from the urine of hogs; the manure further contributes to a higher COD/BOD ratio that varies depending on the age of hogs, feed composition, farm operation method, as well as temperature and humidity. Cheng et al. reported a typical concentration of various components in swine wastewater as 2–30 gL^{-1} BOD, 0.2–2.0 gL^{-1} total nitrogen, 0.11–1.65 gL^{-1} NH_4-N, and 0.1–0.62 gL^{-1} total phosphorus (Cheng et al., 2020). Discharging swine wastewater into water bodies will lead to eutrophication and algal bloom besides increasing antibiotic load, and further cause the accumulation of pathogenic and antibiotic resistant microbes (*Listeria monocytogenes*, *Salmonella*, *Yersinia*, *Giardia* etc.). Therefore, treatment of swine wastewater is essential before discharging it into the environment (Guan and Holley, 2003).

3. Microalgae cultivation methods

Microalgae cultivation is advantageous compared to other crops as they do not require fertile land, fresh water, herbicides, and pesticides. Microalgae can be easily cultured using various sources of wastewater. The main issues are low-density growth and harvesting of algal biomass. Different technologies have been developed to cultivate microalgae in wastewater, but it is still difficult to achieve high productivity. An ideal culture system should be inexpensive to construct, have adequate light source, be easy to handle, enable effective transfer through liquid-gas barrier, and show a low risk of contamination (Tan et al., 2020). In general, microalgae systems are broadly classified as open and closed systems (Fig. 1). Selection of cultivation system is based on wastewater characteristics, microalgae types, and external parameters.

3.1. Open cultivation system

Open cultivation is the oldest and an inexpensive approach undertaken in an open space for the large-scale cultivation of microalgae (Fig. 1). At an industrial scale, open cultivation is the preferred method due to easy construction, lower energy consumption, and simple operation (Jankowska et al., 2017). Open cultivation includes natural ponds and lakes, as well as special artificially designed water bodies (circular and raceway ponds). Circular pond is the first artificial pond designed in which a rotating agitator is used to ensure adequate mixing and prevent algal biomass sedimentation. The main limitation of this system is size, since larger-sized ponds have stronger water resistance causing mechanical strain on the agitator. A raceway cultivation system is equipped with a paddle wheel to prevent sedimentation and provide recirculation of algal culture for proper CO_2 and nutrient supplies to enhance biomass production. Sapphire Energy's Columbus Algal Biomass (CAB) Farm uses an open raceway algal cultivation system since March 2012 and has been able to produce 520 metric ton of algal biomass to date without any interruption and system crash (White and Ryan, 2015). Dahmani et al. studied *Chlorella pyrenoidosa* cultivation in an open raceway pond using domestic wastewater and were able to achieve an average biomass production of 1.71 gL^{-1} with COD (78%), TN (95%), and TP (81%) reductions (Dahmani et al., 2016). However, the main drawback of the open cultivation system is contamination due to bacteria, protozoa, microorganisms that are introduced by birds, and adverse weather conditions. External contamination and extreme weather conditions could reduce the growth of microalgae and render the pond unfavorable for microalgae. Narala et al. reported that microbial contamination with other microalgae are very common in open pond cultivation systems (Narala et al., 2016). Specifically, the presence of rotifers in the cultures also cause damage to culture of various microalgae *Chlorella*, *Nannochloropsis*, *Tetraselmis*, and *Scenedesmus*. In open pond systems, controlling growth parameters (evaporation, culture temperature) could be challenging. Various advantages and disadvantages of open system are discussed in Fig. 1. Nevertheless, many microalgal species that grow in an open system might achieve low production cost by using large open outdoor facilities to cultivate microalgae for resource recovery from wastewater.

3.2. Close cultivation system

Closed cultivation systems (photobioreactors (PBRs)), are more efficient in controlling and monitoring physiological conditions (Fig. 1). PBRs can be constructed, modified, and designed according to the need of the algal strain of interest. Closed PBRs can be installed in a smaller space, while the optimum conditions for the growth of microalgae could be efficiently managed. In PBRs, irradiation intensity, CO_2 or aeration flow rates, temperature, pH, and mixing can be controlled according to the optimum conditions for microalgal growth. Additionally, in the closed PBRs, external carbon source or organic carbon could be provided for better algal growth, and microalgae can be cultivated under desired conditions (phototrophic, heterotrophic, and mixotrophic) using organic and inorganic carbon sources. Furthermore, closed PBRs have a lower risk for contamination and metabolic pathways can be fine-tuned using different stress conditions. Nevertheless, in closed PBRs there are some difficulties related to biofouling and cleaning and involve very high capital costs in design and operation. However, closed PBRs can be categorized into different types on the bases of their geometry, shape, and materials used for construction such as bubble column, flat-plate, soft-frame, tubular, and hybrid PBRs. Among these, horizontal tubular PBRs are most widely used in microalgal cultivation owing to their higher biomass productivities, whereas airlift and bubble column PBRs are generally used in larger scale industries (Yin et al., 2020). These types of PBRs could be used to enhance microalgae biomass by maintaining optimum conditions. However, for fabrication of different type of closed PBRs, transparent materials such as glass, acrylic, or plastic that allow maximum penetration of sunlight, are preferred. Closed PBRs are the most effective cultivation systems for algal biomass productivity due to the controlled growth conditions and parameters (pH, temperature, CO_2, and O_2). However, the key challenge of PBRs for microalgal biomass production is the high expense in construction and maintenance. Although high costs are impractical for the production of biodiesels, PBRs could be useful in the production of high value-added compounds

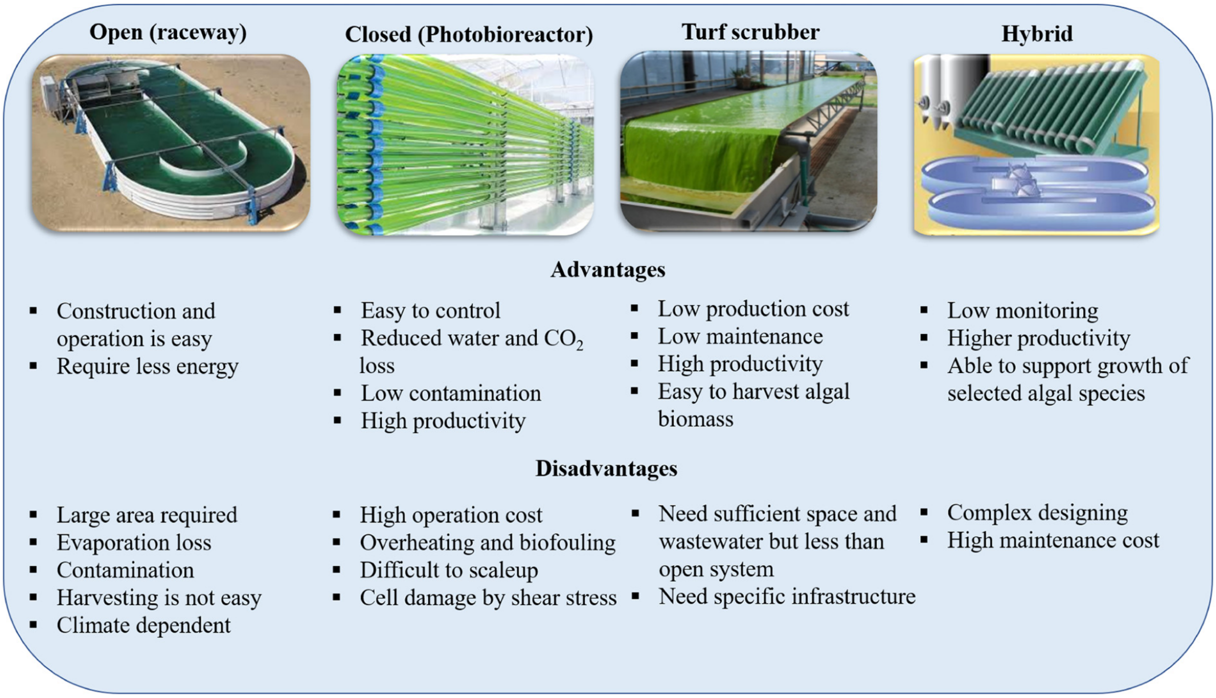

Fig. 1. Different microalgal cultivation systems and their advantages and disadvantages.

of higher commercial value. Nugroho and Zhu, reported that operation costs can be lowered by using efficient and low-cost materials such as wastewater as a feedstock and energy-saving pumps for resource recovery (Nugroho and Zhu, 2019). The second key issue associated with PBRs is that the penetration of light is gradually limited on the surface of PBRs where the algal biofilms form. However, drawbacks due to operational issues can be resolved using better design of bioengineered PBRs of high efficiency and low maintenance cost. Wu et al. designed a novel algal biofilm photobioreactor for *Chlorella vulgaris* growth using hog manure wastewater and reported a high algal growth of 7.37 gm^{-2} which can be easily harvested using a scraping method (Wu et al., 2019). Anaerobically digested wastewater (ADW) generally contains suspended solids and other contaminants which limit light penetration and cause algal death. To solve this issue Chen et al. introduced a hollow fiber membrane (HFM) in the photobioreactor in which nutrients permeate from ADW from an inner chamber in the algal culture medium to an outer chamber through an HFM and restrict other pollutants and eliminate the inhibition resulting from suspended particles (Chen et al., 2018).

3.3. Algal turf scrubber (ATS)

Algal turf scrubber (ATS) consists of an attached algal community (turf) growing on a shallow basin through (raceway) which water is constantly pumped in (Fig. 1). Water is pumped from a water body on the raceway for growing algae to remove nutrients and produce oxygen, and finally, water is released back to the water body (Adey et al., 2011). In the ATS system, algae treats wastewater by up taking organic and inorganic compounds and releases oxygen through photosynthesis. Nutrients removed from the wastewater remain inside the algal biomass and can be easily harvested. ATS cultivation system offers rapid algal growth and higher nutrient-removal efficiency and produces O_2 at a high rate. It can be employed for resource recovery from varieties of wastewater from farm, tertiary sewage, aquaculture, and streams, and can be carried out in a continuous mode without the need to remove the biomass during production (Chia et al., 2018; Hoffman et al., 2017; Martini et al., 2019). Siville and Boeing, studied the potential use of ATSs for resource recovery from wastewater by growing valuable filamentous macroalgal biomass and found that the ATS cultivation system can play an important role in the remediation of high nitrate wastewaters and increase biomass growth with more frequent harvesting (Siville and Boeing, 2020).

3.4. Hybrid cultivation system

Hybrid cultivation system refers to a combination of two or more cultivation systems for efficient resource recovery from wastewater (SundarRajan et al., 2019). Hybrid cultivation systems can be small and closed PBRs, which can be functionally integrated with open ponds and ATS (Fig. 1). The main benefits of hybrid systems are maximizing the benefits of each process. The hybrid cultivation system is appropriate for coupling wastewater with microalgal cultivation, with a potential to decrease the production cost for every kilogram of algal biomass (Salama et al., 2017). Narala et al. compared the microalgae cultivation in PBRs, open raceway ponds, and a two-stage hybrid system, and found that hybrid system process have ability to increase algal lipids productivity (Narala et al., 2016). A hybrid anaerobic baffled reactor and photobioreactor (HABR-PBR) was designed by Khalekuzzaman et al. in which HABR was able to remove most organic and solid loads (>90% COD and TSS) and produce feedstock ($N{:}P = 3{:}1$) with higher lipid production (44.1%) using a coculture of *Chlorella vulgaris*, *Chlorella sorokiniana*, and *Scenedesmus simris* 002 (Khalekuzzaman et al., 2019). Nevertheless, cultivation of desired algal strains against invader algae in an open pond is a challenge. To overcome this issue Chang et al. used a photobioreactor PBR-open raceway pond (ORP) hybrid system where the PBR acted as a continuous source of inoculum of desired algae to sustain algal growth in an ORP. The process resulted in 40 to 60% increase in algal biomass and lipid content (Yun et al., 2018). Similarly, Adesanya et al. reported that hybrid system coupled with airlift tubular photobioreactors using raceway ponds in a two-stage process achieves higher biomass and lipid production (Adesanya et al., 2014). Furthermore, they proposed a model for 1 ton of biodiesel production, in which the hybrid cultivation system and hypothetical downstream process would have reduced global warming (42%), and fossil-energy requirements (38%), potential respectively. A process flow diagram of different microalgal cultivation system with main advantages and disadvantages are detailed in Fig. 1 (Yin et al., 2020).

4. Traditional and advanced methods of harvesting microalgae

In order to further enhance the application of microalgae in wastewater treatment and nutrient removal for maximum efficiency, recovery of algal biomass for use as feedstock for bioenergy and biochemical production is an essential step. Several physical, chemical, biological, and electromagnetic methods of algal harvesting have been proposed and used routinely to attain efficient biomass recovery. However, biomass harvesting from wastewater is a major economic hurdle for algal refineries. Therefore, an ideal harvesting process needs to be developed and made applicable for all types of microalgal species, which ensures high biomass concentration as well as energy and cost effectiveness (Barros et al., 2015; Liu et al., 2017). The integration of two or more methods could obtain a higher biomass harvesting rate at lower operation costs. Schlesinger et al. highlighted that the combination of flocculation and sedimentation followed by centrifugation could minimize harvesting costs and improve biomass recovery (Schlesinger et al., 2012). Various biomass harvesting methods are discussed in detail in the following subsections.

4.1. Physical/mechanical harvesting

Physical methods also known as mechanical biomass harvesting methods are most reliable and efficient for algal harvesting. These methods involve techniques such as gravity sedimentation, flotation, centrifugation, and filtration. Gravity sedimentation is an energy-efficient solid-liquid separation method, where concentrated slurry is removed using gravitation force and microalgal biomass density (microalgal settling rates is ~0.1–2.6 cm h^{-1}), while the supernatant forms a clear liquid layer (Barros et al., 2015; Mathimani and Mallick, 2018). Mathimani and Mallick reported that microalgal sedimentation is affected by shape, and microalgae having needle-like or long, cylindrical shapes and motility (like *Euglena*, *Chlorogonium*) are difficult to be removed, while spiral (*Spirulina*) *and* large-sized colonial algae (*Micractinim*, *Scenesdesmus*) *can be easily* removed using gravity sedimentation (Mathimani and Mallick, 2018).

In flotation or inverted sedimentation process, aeration is maintained to form gas bubbles to provide the lifting force needed for particle transport and separation. Flotation is frequently employed for wastewater treatment processes and is more effective compared to sedimentation due to the low density of microalgal biomass. Generally, the following flotation techniques are available: i) dissolved air flotation (DAF–bubble diameter <100 mm); ii) dispersed air flotation (DiAF–bubble diameter 100–1000 mm); iii) electrolytic flotation; and (iv) ozonation-dispersed flotation (ODF) (Barros et al., 2015). Recently, de Souza Leite et al. investigated the potential of pH-modulated dissolved air flotation (DAF) harvesting method at optimized operational parameters (pH, velocity gradient, mixing time, and flotation) to harvest *Chlorella sorokiniana* from wastewater (de Souza Leite et al., 2020). A maximum biomass recovery yield of 96.5–97.9% was obtained under operating conditions such as velocity gradient of 500 s^{-1}, mixing time of 30 s, pH 12, and 20% recirculation rate (de Souza Leite et al., 2020). Zhang and Zhang reviewed the potential of foam flotation for harvesting microalgal biomass and concluded that it is a promising technique for microalgal harvesting owing to its high efficiency and low energy consumption (Zhang and Zhang, 2019). The perspectives of microalgal harvesting using foam flotation are presented in Fig. 2.

Filtration is another method ideal for harvesting biomass from growth media which utilizes the pressure difference between two sides of a membrane to force through the fluid. The membrane-based biomass harvesting can be employed at an industrial scale for the recovery of algal biomass with risk free external contamination. Marino et al. investigated the potential of membrane-based harvesting method for the recovery of *Scenedesmus almeriensis* biomass using a polyvinylidene fluoride (PVDF) membrane (pore size of 3 μm) such that the growth medium could pass through (permeate) and the algal biomass was retained (Marino et al., 2019). Molino et al. carried out biomass harvesting using vacuum membrane filtration using a membrane with pore size of 0.45 μm for lutein extraction from *Scenedesmus almeriensis* biomass (Molino et al., 2020). Recently, magnetically induced membrane vibration (MMV) system has been introduced for efficient harvesting of *Dictyosphaerium* sp. biomass without breaking the cells. The study suggests that an intermittent cycle time of 4 min with 50% vibration rate is optimum for harvesting microalgae (Zhao et al., 2020). Membrane filtration using nanofiber membrane was found to be efficient for harvesting microalgae; however, it is highly limited due to its weak mechanical strength (Mat Nawi et al., 2020).

Centrifugation is the fastest physical harvesting method and easy to handle but consumes high energy and makes it the most expensive method. However, this method is commonly used at an industrial scale for biomass harvesting for the recovery of various high-valued compounds (Tan et al., 2020). Wang et al. compared centrifugation and flocculation harvesting methods and reported that *S. obliquus* biomass harvested using centrifugation showed higher bio-oil yield; however, flocculation is the recommended method due to its relatively lower harvest cost (43.2% lower than centrifugation) (Wang et al., 2019).

4.2. Chemical harvesting method

Chemical harvesting are carried out using various chemicals (flocculant and coagulant [$FeCl_3$, $Al_2(SO_4)_3$, and $Fe_2(SO_4)_3$]) and considered as an economical approach to harvest for microalgae from wastewater (Mohd Udaiyappan et al., 2017). Microalgae have a negative charge on their surface and density almost similar to that of the growth medium, and thus, always remains in a dispersed state (Singh and Patidar, 2018). Caetano et al. investigated the effects of pH variation and/or addition of $CaCl_2$ as flocculants on the harvesting of microalga *Arthrospira maxima* and observed that a pH >10 and $CaCl_2$ 0.2–2.0 gL^{-1} at a 1:30 ratio (*v*/v) of $CaCl_2$/microalgae culture could be applied to harvest biomass efficiently (Caetano et al., 2020). Chemical flocculation can be applied to large volumes of culture without disrupting cellular structure and is considered as a reliable, high-yielding process. Furthermore, these chemicals are easy to use, inexpensive, and do not pose any substantial risk of contamination to the recovered microalgae biomass. Therefore, chemical harvesting methods can contribute to the economical harvesting of microalgae biomass for various applications.

4.3. Biological harvesting method

Biological harvesting refers to auto or bioflocculation, which occurs by secreting biopolymers, especially by extracellular polymeric substances (EPS). The bioflocculants produced by microbes can be utilized for microalgae harvesting from wastewater to produce biofuel, which can be a significant economical approach for algal biorefineries. Ndikubwimana et al. used poly-γ-glutamic acid (γ-PGA) produced by *Bacillus licheniformis* CGMCC 2876 for the harvesting of *Desmodesmus brasiliensis* and reported >98% flocculation efficiency (Ndikubwimana et al., 2016). Yang et al. studied *Scenedesmus acuminatus* EPS for flocculation of same algae and reported that Low-MW (<3 kDa) EPS which is composed of higher contents of glucose and mannose influence the interactions of the algae and the alum coagulant and have potential to reduces the chemical cost from $282 per metric ton to $71 per metric ton harvested dry biomass (Yang et al., 2020). Bioflocculants can reduce the demand of chemical flocculants, but the co-cultivation of microalgae with bacteria, fungi, or flocculating microalgae could result in microbial contamination. This possibility of microbial contamination limits the

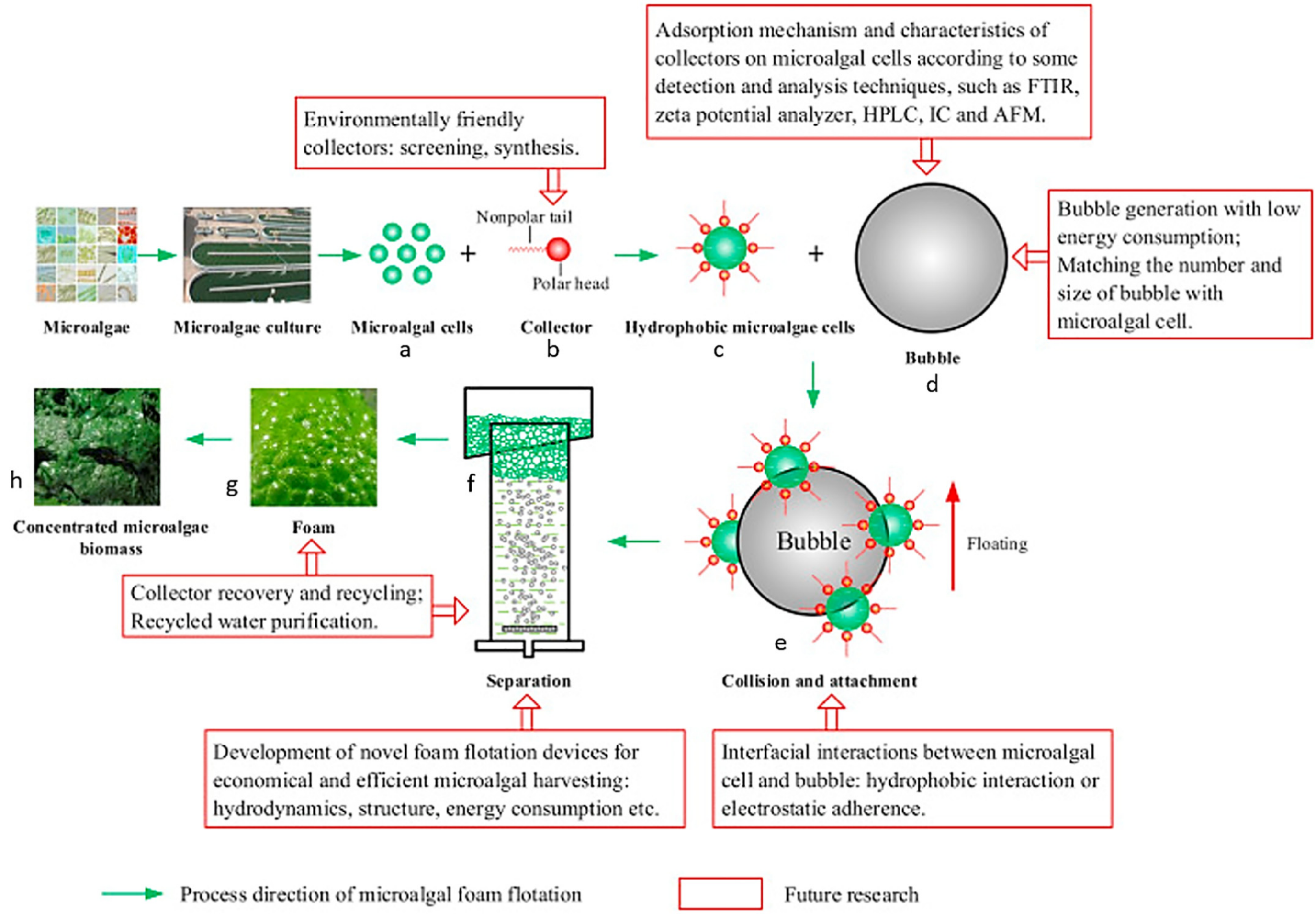

Fig. 2. Illustration of microalgal harvesting process using foam flotation (a) microalgal cells (b) collector (c) hydrophobic microalgae cells (d) bubble (e) interfacial interaction between microalgae and bubble (f) foam development (g) microalgae recovery (h) concentrated microalgal biomass. Reproduced with permission from (Zhang and Zhang, 2019) (Copyright 2020, Elsevier).

application of harvested microalgal biomass in the food or feed sector, but can be a potential source for biofuel production (Vandamme et al., 2013). However, co-cultivation of other microbes can enhance lipid productivity (Salim et al., 2011). Jiang et al. reported that oil-containing microalgae *Chlorella pyrenoidosa* can be harvested using *Citrobacter freundii* (No. W4) and *Mucor circinelloides* as bioflocculants. Several factors such as the initial pH, bacterial, fungal, and glucose dosages could significantly influence the flocculation efficiency and achieve a 97.45% flocculation efficiency at optimum conditions (Jiang et al., 2020). The potential use of activated sludge derived-extracellular polymeric substance (ASD-EPS) derived bio-flocculants for microalgae harvesting was explored by Choi et al. and they report that among the three tested algal strains (*Chlorella vulgaris*, *Chlamydomonas asymmetrica*, and *Scenedesmus* sp.) *Chlorella vulgaris* showed the highest bioflocculation efficiency with high lipid production using ASD-EPS (Choi et al., 2020). Pugazhendhi et al. reviewed the potential of biopolymer-based flocculants for microalgae biomass harvesting and concluded that bio-flocculation is a cost-effective method for biofuel production (Pugazhendhi et al., 2019). Therefore, bioflocculation harvesting methods can be considered for wastewater treatment as well as for microalgae biomass harvesting for biofuel production.

4.4. Electrical and magnetic harvesting method

The electrical harvesting method does not need any flocculants because in this process negatively charged microalgae moves toward the anode, loses charge, and forms aggregates (Mubarak et al., 2019). Kim et al. developed the continuous electrolytic microalgae harvest system for the recovery of *Nannochloris oculata* (KMMCC-16) and showed a better biomass recovery using an aluminium and dimensionally stable anode (Al–DSA) compared to an Al-platinum (Al-Pt) electrode (Kim et al., 2012). Harvesting efficiency could be further improved by optimizing the polarity exchange timing. Furthermore, Shuman et al. developed an electro-coagulation-flocculation (ECF) reactor to reduce treatment time and energy input and were able to recover *Nannochloropsis* sp. in 120 min at a 90% harvesting efficiency (Shuman et al., 2014). Furthermore, Fayad et al. used aluminium and iron electrodes to harvest *Chlorella vulgaris* using electro-coagulation-flocculation (ECF) and reported that ECF have no effect on the amount of microalgal lipid and pigment production (Fayad et al., 2017). Mubarak et al. reviewed and summarized that harvesting efficiency of the microalgal biomass was 95% with a current consumption of 0.3 kW hm^{-3} from algal suspensions containing blue-green algae, green algae, and some diatoms, when the electrolytic flocculation method was used (Mubarak et al., 2019).

The magnetic method of harvesting is performed using functionalized magnetic particles in the presence of an external magnetic field. A cationic polyelectrolyte bridge is needed between the algal cells and magnetic particles, as both are negatively charged (Kim et al., 2012). After the successful coupling of algal cells and magnetic particles, the external magnetic field is applied to separate the complex from aqueous solution (Kim et al., 2012). Wang et al. optimized harvesting factors such as optimal mass ratio (0.14–0.18), stirring speed (85–120 rpm), stirring time (70–95 s), and adsorption time (5.5–7 min) for *Microcystis aeruginosa* 1343 (M1) and 905 (M9) based on polyethylenimine (PEI)-coated iron oxide nanoparticles (IONPs) and reported harvesting efficiencies of 93.3% and 97.5%, respectively (Wang et al., 2020). Chu et al. used nanosized Fe_3O_4 immobilized in polyvinyl alcohol (PVA)/sodium alginate (SA) as a flocculant (Fe_3O_4/PS) to harvest Nannochloropsis oculata and showed a 96% harvesting efficiency with reusability up to five cycles (residual efficiency of 80%) (Chu et al., 2020). It can be concluded that magnetophoretic harvesting using immobilized magnetic iron oxide as a particle-based flocculant is a potentially useful method to reduce challenges related to costs involved in harvesting microalgae. Merits and demerits of different harvesting methods are discussed in Table. 2.

5. Biotechnological methods of resource recovery from wastewater cultured microalgae

Microalgae biomass is an efficient resource for the production of different types of biofuel such as biodiesel, bioalcohol, biohydrogen, methane, and bioelectricity. Microalgal biomass can be converted into fuel using physical, chemical, or biological methods depending on the characteristics of algal biomass and byproducts. Biodiesel production from algal biomass is an attractive option but extraction of oil requires various solvents, which add to costs and are not ecofriendly. Similarly, bioethanol is another biofuel candidate produced from the fermentation of algal biomass, but pretreatment of biomass is a prerequisite step. Microalgal growth is related to environmental factors, and its lipid, carbohydrate, and protein content directly depends on the ratio of various media components (nitrate, phosphate, etc.) (Choudhary et al., 2020). Integration of wastewater treatment using microalgal cultures can be an attractive approach for wastewater treatment and energy production simultaneously. Since microalgae assimilate nutrients from wastewater and recycle CO_2, there is no requirement for fertilizer and herbicides and the produced biomass can be processed into various energy resources. This synergy between wastewater treatment and microalgal culture effectively reduce the processing costs and thus benefits both sectors.

5.1. Biodiesel production

Biodiesels are fatty acid alkyl esters (FAAEs) produced through the transesterification of oils with alcohols in the presence of catalysts. Microalgae have immense potential in wastewater treatment, nutrient recovery, biomass growth, and lipid accumulation (Table. 3). It is estimated that around 760 ton ha^{-1} $year^{-1}$ biodiesel can be produced from microalgae cultured at small scale under controlled conditions, while using oil seeds yielding only 0.4 ton.ha^{-1} $year^{-1}$ for soybean and 0.7 ton.ha^{-1} $year^{-1}$ for canola oil (Mehrabadi et al., 2016). Growth, nutrient recovery, and lipid accumulation capacity vary among microalgal species apart from season, light intensity, and nutrient content of wastewater (Table. 3). Liu et al. studied the cultivation of four different microalgae in the effluent of wastewater treatment plant under continuous supply of CO_2 and reported biomass and lipid production for *Scenedesmus obliquus* (0.76 gL^{-1}, 17%), *Chlorella pyrenoidosa* (0.71 gL^{-1}, 24.4%), *Scenedesmus dimorphu* (0.58 gL^{-1}, 30.8%), and *Chlorella vulgaris* (0.36 gL^{-1}, 29.1%) with little difference in nitrogen removal, while *Chlorella vulgaris* showed a higher removal of P (Liu et al., 2020). Mehrabadi et al. investigated the year-round potential of wastewater treatment plant for biodiesel production and reported variation in biomass (2.0–11.1 g $VSSm^{-2}d^{-1}$) and lipids (0.5–2.6 $gm^{-2}d^{-1}$), respectively (Mehrabadi et al., 2016). Light intensity has a significant effect on algal growth and lipid accumulation. Nzayisenga et al. studied the effect of light intensity on the growth of different algal species and found that fatty acid content is related to protein and not to carbohydrate, and an increase in light intensity leads to more oleic acid (18,1) and lower linolenic acid (18,3) production (Nzayisenga et al., 2020). Fan et al. studied the growth of two microalgae, *Spirulina platensis* and *Scenedesmus obliquus*, in wastewater and found that *S. obliquus* requires high light intensity and daily illumination time for better growth and lipid accumulation (Fan et al., 2020). The composition of nutrients in municipal wastewater depends on the effluent treatment stages, namely, primary and secondary. Primary effluents are rich in organic materials and other nutrients (N and P) but may inhibit microbial growth as there is competition due to heterotrophic bacteria and lower light intensity. A medium with lower N content is suitable for maximum lipid accumulation. Wastewater from food and beverage industries is considered more suitable for microalgal culture as it has low content of heavy metals and other toxic chemicals. Soybean processing wastewater (SPW) is rich in saccharides, lipids, organic acids, proteins, phosphate, calcium, iron, and other nutrients and acts as a suitable medium for microalgal culture without affecting microalgae. Hongyang et al. cultivated *Chlorella pyrenoidosa* in SPW without any additional nutrients and was able to remove COD (77.8%), TN (89.1%), and TP (70.3%) in 120 h with a biomass and lipid productivity of 0.64 gL^{-1} h^{-1} and 0.40 gL^{-1} h^{-1}, respectively (Hongyang et al., 2011). Livestock waste (dairy waste) are rich in nitrogen and phosphorus, and algal cultivation in this medium results in increased carbohydrate content of (24.8–27.7%) biomass, while lipid content decreases to 45–34% (Choudhary et al., 2020). Some pollutants provide color to wastewater and inhibit algal growth by limiting light intensity. Cheng et al. used TiO_2 photocatalysts to pretreat swine wastewater and studied the culture of two algae *Tribonema* sp. and *Synechocystis* sp. and reported a higher nutrient removal efficiency in pretreated compared to nontreated wastewater. They report an increase in lipid accumulation by 40–42.4% and 23.9–26.3% for these species, respectively (Cheng et al., 2020). Microalgae-bacteria symbiotic cultures have the potential to increase biomass production and lipid accumulation. Microalgae provide oxygen to aerobic heterotrophic microbes, and in return, obtain nutrients by decomposing organic matter from wastewater. Excessive nutrients in wastewater causes eutrophication and affects microalgae growth. Zhou et al. studied the coculture of *Chlorella pyrenoidosa* using different ammonia-oxidizing bacteria in municipal wastewater and reported increases in biomass (14.8%) and lipids (13.6%) (Zhou et al., 2020). To remove the excess phosphate from municipal wastewater, Wang et al. cocultured *Chlorella pyrenoidosa* with phosphate-accumulating bacteria (*Klebsiella* sp.) and was able to increase growth and lipid yield by 13.6 and 90.1%, respectively (Wang Q. et al., 2019). Leong et al. studied microalgal-bacterial cocultivation using activated sludge and synthetic wastewater and found that microbes present in the activated sludge help in the removal of nitrogen, which results in increased biomass production (27%) and lipid accumulation (38%) (Leong et al., 2019). Some microalgae have weak adaptability to wastewater, which results in slow growth and low lipid accumulation. Various techniques (selective breeding and mutation breeding etc.) have been used to improve their potentials. Yang et al. used the 3MeVC2^{+} beam to mutate *Nitzschia* sp., and the mutant strain showed a 9.8% increase in accumulating lipids compared to control (Yang et al., 2013). Similarly, Tu et al. used an N^{+} ion beam to improve *Chlorella pyrenoidosa* and the improved strain produced a 35% higher lipid content containing higher levels of polyunsaturated fatty acids (Tu et al., 2016).

5.2. Biohydrogen

Hydrogen is a sustainable and clean source of energy and has the potential to replace fossil- based energy resources owing to its high energy yield of 141.65 $MJkg^{-1}$. Biohydrogen is produced using biological methods and include various microbes such as algae, cyanobacteria, dark fermentative bacteria, and photosynthetic bacteria that utilize various metabolic pathways. Microalgae as photosynthetic microorganisms are suitable candidates for biohydrogen production owing to their capability to fix CO_2, simple nutrients, and sunlight as source of energy from the environment. A detailed mechanism of biohydrogen production from

Table 2
Advantages and disadvantages of various microalgal harvesting methods.

Harvesting method	Advantages	Disadvantages	References
Gravity sedimentation	Easy and inexpensive method. Requires low energy.	Require a long time and large space. Risk of biomass deterioration. The concentration of the algal cake is low.	(Barros et al., 2015; Mubarak et al., 2019)
Flotation	Process requires short operation time. Require a small area and easy to scale up. More effective than sedimentation.	Chemicals required as flocculants. Not applicable for marine microalgae harvesting. Expensive process and requires high particulate size of algal biomass.	(Pragya et al., 2013)
Filtration	Biomass recovery efficiencies is high. Suitable for shear sensitive species. Selective filtration is possible.	Membrane fouling/clogging problem, Operational cost is high. Membrane replacement and requirement of pumping associated with high costs.	(Uduman et al., 2010)
Centrifugation species.	Fast method of separation. Recovery efficiency is high. Suitable for almost all microalgal	High shear forces may cause cell damage. Expensive method and suitable only for the recovery of high-value products. Requires high energy input.	(Mubarak et al., 2019)
Chemical coagulation/flocculation	Fast, easy, and applicable to large volume. Method. No energy requirements.	Chemical based flocculants are expensive and toxic. Recycling of culture medium is limited.	(Chen et al., 2011; Mubarak et al., 2019)
Bioflocculation	Inexpensive and easy method. Culture medium can be recycled and reused. Non-toxic to microalgal biomass.	Changes in cellular composition. Microbial contamination and not suitable for food related applications.	(Mubarak et al., 2019)
Electrical based	Applicable to different types of microalgal species. There is no need of any chemical flocculants.	Poorly disseminated. Process requires high energy and costly equipment.	(Barros et al., 2015; Mubarak et al., 2019)
Magnetic separation	Low running cost, energy saving and simple operation.	Fabrication was complex and expensive, practical limitability.	(Barros et al., 2015; Xu et al., 2011)

microalgae is discussed elsewhere (Khetkorn et al., 2017). Hydrogenases (H_2ase) and nitrogenases (N_2ase) are the two key enzymes involved in biohydrogen production using various microbes. Hydrogen production in microalgae is directly associated with the biophotolysis process that occurs when an algal culture is exposed to light after a dark period of anaerobic adaptation. Wild type algal [FeFe]-hydrogenase is functional only in an anaerobic environment and oxygen produced during the photosynthesis inactivates the binding site of enzymes. The main challenge is to keep cells alive while suppressing photosynthesis. H_2 production occurs by utilizing stored material under anaerobic conditions (Fan et al., 2016). Different approaches have been adapted to overcome challenges such as depletion of nutrients (sulfur, nitrogen, phosphorus, and magnesium etc.), acetate regulation, and coculture using other bacterial strains (Wirth et al., 2018). Bacteria utilize oxygen and help create anaerobic conditions to allow H_2 production in an algal system. Further H_2 production can be improved by using bacteria with low H_2 uptake capacity.

5.2.1. Direct biohydrogen production from algae

Vargas et al. utilized a two-step strategy for biohydrogen production using *Chlamydomonas reinhardtii* (CC425) and *Chlamydomonas moewusii*. In the first step, algae were cultured in aerobic conditions to attain exponential phase growth. In the second stage, the culture was transferred to a closed anaerobic photobioreactor under sulfur-induced limited conditions (Vargas et al., 2018). Olive oil mill wastewater (OMW) is rich in various phenolic compounds and requires aerobic and anaerobic processes for treatment. Papazi et al. used a combinational approach of OMW-microflora and the alga, *Scenedesmus obliquus*, to treat the phenolic compound and produce hydrogen (Papazi et al., 2019). OMW microflora degrade phenolics and create an anoxic environment for increased biohydrogen production when *Scenedesmus obliquus* is used. Various reduced meta-substituted phenolic compounds mimic electron transporters in chloroplasts. Mitochondria can inhibit photosynthesis system II, but they increase the activity of hydrogenase in photosynthesis system I and lead to higher H_2 production. Kose et al. studied the effects of various photobioreactor designs (tubular and panel) on algal biomass growth and H_2 production, and concluded that tubular reactors provide better illumination during aerobic growth, which results in higher biomass production, as panel photobioreactors are better for H_2 removal during the process and leads to increased production (Oncel and Kose, 2014). Hwang et al. investigated acetate- and butyrate-rich wastewater effluents for *Micractinium reisseri* YSW05 and reported a higher H_2 production under continuous light conditions compared to a dark/light cycle (Hwang et al., 2014). Generally, hydrogenase activity and hydrogen production decreases significantly with increasing headspace oxygen, but *M. reisseri* YSW05 hydrogenase was active under different partial pressures of oxygen and could help produce H_2 under different atmospheric conditions.

5.2.2. Anaerobic digestion of algal biomass for biohydrogen

Direct production of H_2 using microalgae is not very efficient, and to overcome this obstacle, researchers have explored the utilization of algal biomass and residual algal biomass as raw materials for microbial fermentation to produce biohydrogen. The yield of biogas depends on the cellular structure of algae. Algae like *Dunaliella salina* lacks cell wall, the cell wall of *Chlamydomonas reinhardtii* is rich in easily biodegradable proteins, which results in a higher yield of biogas (Wirth et al., 2018). Other algae species such as *Chlorella kessleri* and *Scenedesmus obliquus*, which have cell walls rich in hemicellulose, are resistant to hydrolysis (Dębowski et al., 2013). Algal biomass requires various pretreatment steps (physical, chemical, and biological) to disrupt cell wall before subjecting them to microbial fermentation. Ferreira et al. studied the cultivation of *Scenedesmus obliquus* in different wastewater effluents (poultry, swine, cattle, brewery, and dairy) and reported that resulting biomass comprised 31–53% proteins, 12–36% sugars, and 8–23% lipids, regardless of the type of wastewater. Produced biomass was further subjected to dark fermentation using *Enterobacter aerogenes* and resulted in 50–390 mL $H_2 g^{-1}$ of volatile solids. Batista et al. cultured *Chlorella vulgaris*, *Scenedesmus obliquus*, and a natural algal *Consortium C* using urban wastewater. After depletion of nutrients, the microalgal biomass remained in the photobioreactor for two weeks and induced sugar accumulation (22–43%). *Enterobacter aerogenes* was further used to convert the collected algal biomass into H_2 by using dark fermentation and resulted in 56.8 mL H_2 gvs^{-1} (Batista et al., 2015). A mixed microalgal consortium (*Scenedesmus* and *Chlorella* species) was cultured using swine wastewater and biomass, and pretreated using ultrasonication and an enzymatic method. Pretreated biomass was inoculated into a sludge and a hydrogen yield of 116 mL H_2 gTS^{-1} was achieved (Kumar et al.,

Table 3
Nutrient removal, resource recovery and lipid accumulation potential of various microalgae from different wastewater.

Wastewater	Microalgae	Comments	COD (%)	N (%)	P (%)	Biomass	Lipids content (%)	Reference
Dairy effluent	*Chlorella vulgaris*	Biodiesel produced was in good agreement with international standards.	80.62	85.47	65.96	0.175 mg $L^{-1}D^{-1}$	–	(Choi, 2016)
	Scenedesmussp. ASK22	Algae was isolated from domestic and dairy effluent wastewater.	90.5	100	91.24	1.22 g cdw L^{-1}	30.7	(Pandey et al., 2019)
	Ascochloris sp. ADW007	Outdoor cultivation results in higher biomass and lipid productivity.	95.1	79.7	98.1	–	34.98	(Kumar et al., 2019)
Swine wastewater	*Tribonema* sp.	Algae have auto-flocculating ability and wastewater was pretreated to remove color and increase availability of light.	56.6	89.9	72.7	–	42.4	(Cheng, P. et al., 2020)
	Synechocystis sp		68.6	75.8	71.4		26.3	
	Tribonema sp.	A weak electric field was applied to decolorize the effluent.	52.5	100	68–74	2.04 g L^{-1}	55.4	(Huo et al., 2020)
	Tribonema sp.	Titanium dioxide (TiO_2) and pulse intense pulsed light (T-IPL) was used for decolorization.	55.6	89.9	72.7	–	42.4	(Cheng, P. et al., 2020)
	Synechocystis sp.		68.6	75.8	71.4	–	26.3	
Piggery wastewater	*Chlamydomonas mexicana*	Different microalgal species were screened *Ourococcus multisporus*, *Nitzschia* cf. *pusilla*, *Chlamydomonas mexicana*, *Scenedesmus obliquus*, *Chlorella vulgaris*, and *Micractinium reisseri*.	–	62	28	0.56 g dwt L^{-1}	33.0	(Abou-Shanab et al., 2013)
	Desmodesmus sp. PW1	Produce EPS and have self-flocculating activity.	–	90	70	–	29.4	(Chen et al., 2020)
Domestic wastewater	Mixed algal culture with diatoms	Filamentous cyanobacteria dominant in the summer while during the rainy and winter filamentous algae are dominant.	–	2.52 g $m^{-2}d^{-}$	1.25 g $m^{-2}d^{-}$	34.83 g cdw $m^{-2}d^{-1}$	14–22	(Marella et al., 2019)
	Chlorella vulgaris	Wastewater needs pretreatment to avoid decay of algal biomass and contamination.	–	85	35	–	32.7	(Lam et al., 2017)
Poultry wastewater	*Chlorella minutissma*	Integrated process simultaneously led to production of 7.21 mg $L^{-}D^{-}$ lutein.	–	93	90	292 mg $L^{-1}D^{-1}$	–	(Bhowmick et al., 2019)
	Dunaliella sp.	Process used for simultaneous production of β-carotene.	64.1	63.8	87.2	0.678 g L^{-1}	–	(Han et al., 2019)
Municipal wastewater	*Anabaena* sp.	Facultative heterotrophic strains were isolated from surfactant mediated wastewater.	98.6	~100	96.5	215.7 mg L^{-1} D^{-1}	7.24	(Hena et al., 2015)
	Botryococcus braunii	–	98.0	~100	98.1	158.9 mg L^{-1} D^{-1}	41.98	
	Chlamydomonas sp.	–	98.5	~100	98.4	236.8 mg L^{-1} D^{-1}	21.92	
	Chlorella vulgaris	–	98.3	~100	97.4	129 mg L^{-1} D^{-1}	26.41	(Hena et al., 2015)
	Chlorella zofingiensis	Mixing with pig biogas slurry resulted in increase in lipids by 8%.	–	93	90	2.5 g L^{-1}	21.6–25.4	(Zhou et al., 2018)
	Scenedesmus obliquus	Additional basal medium was used to increase lipid content.	–	78.5	95.2	0.529 g L^{-1}	21.9	(Eida et al., 2018)
	Scenedesmus obliquus	Used N^{+} ion implantation for mutagenesis and able to increase lipid content by 24%.	85.43	80.30	95.72		46.92	(Qu et al., 2020)
	Scenedesmus sp.	Anaerobic-digested effluent from cattle manure combined with municipal wastewater.	90	90	79–88	4.65 g L^{-1}	–	(Luo et al., 2019)
	Scenedesmus sp. HXY2	Able to grow under high content of organic carbon and ammonia.	96.0	96.6	94.5	–	15.56	(Ye et al., 2020)
	Chorella minutissima	A stainless steel photo cavity reactor was used to prevent the scattering of light.	89.4	92	90	0.995 g $L^{-1}D^{-1}$	14.0	(Fatima et al., 2019)
	Cenedesmus abundans	–	90.5	96.7	83.6	1.2 g $L^{-1}D^{-1}$	21.3	(Fatima et al., 2019)
Palm mill effluent	*Chlorella sorokiniana* CY-1	A novel photobioreactor with triangular bottom makes microalgae harvesting easy by sedimentation.	93.7	98.6	96	5.74 g L^{-1}	14.43	(Cheah et al., 2020)
	Chlorella vulgaris	Cocultured with *Pseudomonas* sp. to decolorize the effluent.	53.7	55.6	77.3	2.04 g L^{-1}	16.0	(Cheah et al., 2020)
	Chlorella sorokiniana C212	Filter sterilized media results in high biomass productivity	45.08	–	–	1.07 g L^{-1}	–	(Nwuche, 2014)
Textile wastewater	*Chlorella* sp. Wu G23	High content of FAME is produced when algae cultured at higher pH 9–11 under aeration.	75	75	–	58 mg $L^{-1}D^{-1}$	16.6	(Wu et al., 2017)
	Anabaena ambigua	All the studied microalgae have the potential to grow in 100% textile wastewater	50	52.95	63.05	11.61 mg $L^{-1}D^{-1}$	–	(Brar et al., 2019)
	Chlorella pyrenoidosa		85	74.43	28	12.97 mg $L^{-1}D^{-1}$	–	
	Scenedesmus abundans		86.87	68.86	70.79	10.80 mg $L^{-1}D^{-1}$	–	
Pharmaceutical wastewater	*Tetraselmis Indica* BDU 123	Anaerobic treatment of wastewater was performed first with MFC; then aerobic treatment with microalgal was performed.	66.30	67.17	70.03	46.85 mg $L^{-1}D^{-1}$	16.40	(Nayak and Ghosh, 2020)

(continued on next page)

Table 3 (*continued*)

Wastewater	Microalgae	Comments	COD (%)	N (%)	P (%)	Biomass	Lipids content (%)	Reference
Molasses wastewater	*Scenedesmus* sp. Z-4	Biomass is more affected by the change in temperature as compared to lipid accumulation.	87.2	90.5	88.6	–	28.9	(Ma et al., 2017)
	Monoraphidium sp. FXY-10	Melatonin phytohormone was used to increase the lipid accumulation.	92.33	80	86	1.21 g L^{-1}	92.33	(Dong et al., 2019)

2018). There are many examples on the conversion of algal biomass into H_2 using dark fermentation; however, limited literature is available for microalgal grown in wastewater that are used as raw material for dark fermentation to produce H_2.

5.3. Methane

Wastewater grown algal biomass is considered a suitable biomass for biogas production. Photosynthetic algae are able to convert freely available nutrients (CO_2, and nutrients in wastewater) in the presence of light into lipids, proteins, carbohydrate, and other compounds. It is considered as a potential candidate for wastewater treatment and nutrient recovery. An integrated process that combines microalgal-based wastewater treatment and methane production may be a suitable approach to reduce production costs. Methane fermentation is a complex process involving various steps such as hydrolysis, acidogenesis, acetogenesis, and methanation carried out by different groups of microbes (Bhatia and Yang, 2017). Methane yield is affected by various factors such as biomass composition, pH, temperature, hydraulics, and solid retention time (explained in detail elsewhere) (Bhatia et al., 2020; Sarker et al., 2019). Biomass of higher C/N ratio results in rapid consumption of nitrogen and leads to lower methane yield, while biomass with lower C/N causes accumulation of ammonia and increases in pH which is toxic to the methanogen (Ajeej et al., 2015). Nitrogen and phosphorus content in wastewater affects the content of carbohydrate, protein, and lipids of microalgae. Parazzoli et al. studied the effect of nutrient starvation on *Scenedesmus* spp. based on the chemical composition of swine wastewater. *Scenedesmus* spp. cultured in swine wastewater produced carbohydrate (27.6%), protein (57.6%), and lipids (3.9%). Harvested biomass was inoculated into an N/P free medium and a shift in algal biomass composition was reported for carbohydrate (54.6%), protein (24.1%), and lipids (16.9%), respectively. Anaerobic digestion of algae cultured in N/P free nutrient medium showed higher methane production, i.e., 103.5 CH_4 (kg biomass)$^{-1}$ as compared to 44 CH_4 (kg biomass)$^{-1}$ (Perazzoli et al., 2016). Passos et al. studied the effect of microwave pretreatment on the solubilization and anaerobic digestion of microalgae cultivated in municipal wastewater and reported an increase in methane production up to 12–78% (Passos et al., 2013). For methane production, a wastewater with higher content of lipids is preferred as compared to carbohydrates. Yu et al. studied the phycoremediation and methane-generating potential of *Diplosphaera* sp. MM1 in winery and dairy wastewaters. *Diplosphaera* sp. MM1 was cultured in a 50% winery wastewater containing limited nitrogen (25 mgL^{-1}) and 30% dairy wastewater containing high content of nitrogen (107 mgL^{-1}). Nitrogen-limited winery wastewater media resulted in higher lipid accumulation (43.07% TS) and lower carbohydrate content (9.35% TS), while nitrogen-rich dairy wastewater exhibited lower lipid (16.98% TS) and higher carbohydrate (29.39% TS) content. Dark fermentation of algal biomass rich in lipid content (winery wastewater) results in higher methane yield (218.51 $NmL^{-1}g^{-1}VS$) compared to algal biomass from dairy wastewater (129.39 $NmL^{-1}g^{-1}VS$) (Liu et al., 2016). Juarez et al. studied various pretreatment methods (acid, basic, bead milling, steam explosion, and ultrasounds) for microalgae biomass grown in pig manure treatment plant waste; maximum methane (234%) production was recorded using alkaline pretreated biomass (Martín Juárez et al., 2018). Different pretreatment methods solubilize different components, and Passos et al. reported that thermal and alkaline pretreatment solubilized glycoprotein and protein, while acidic pretreatment mostly solubilized hemicellulose, and methane yield increased by 82 and 86%, respectively (Passos et al., 2016). High protein content in microalgae also affect anaerobic digestion as it leads to excess ammonia production and results in the accumulation of volatile fatty acids, which further reduce methane production. Mahdy et al. used two biocatalysts (carbohydrases and protease) to solubilize algal biomass and reported that protease addition shows a higher biomass hydrolysis (54%) which leads to higher methane yield (6.3 fold) (Mahdy et al., 2016). The anaerobic digestion of algal biomass remains a challenge due to unbalanced nutrient components and low biodegradability. To improve methane production, co-digestion of waste is considered a promising approach. Siddique et al. studied co-digestion of algal biomass using sewage sludge and catering waste leachate and reported a 39.31% higher methane production (Siddique and Wahid, 2018). Co-digestion improves proliferation of methanogens by inducing a multiphase digestion of different waste. Similarly, Sole-Bundo et al. studied the co-digestion of microalgae using sludge, fat oil, and grease, and reported a 15% increase in methane yields (Solé-Bundó et al., 2020).

5.4. Bioalcohol

Presently, most of the bioalcohol are produced from the first generation feedstocks (food crops). In view the global food security issue, there is an urgent need to search for cheap, alternative feedstock. Microalgae cultivated in wastewater can be a suitable feedstock for microbial fermentation and bioalcohol production owing to their higher photosynthetic activity, favorable chemical composition, and structural properties as compared to other terrestrial feedstocks. Cultivation of microalgae also requires nutrients, and this issue can be solved by using wastewater. Sanchez Rizza et al. used semi-closed loop microalgae cultivation platform for cyanobacteria (assimilate atmospheric N_2) and was able to accumulate 60% *w*/w carbohydrate. Microalgal biomass was harvested, pretreated with dilute H_2SO_4 and fermented using yeast culture to produce ethanol (50 gL^{-1}) (Sanchez Rizza et al., 2019). *Hindakia tetrachotoma* ME03 cultured in various concentrations of wastewater (0–100%), was pretreated using various methods (alkali, acidic, and enzymatic treatment), evaluated for ethanol production using *S. cerevisiae*, and maximum ethanol production (11.2 gL^{-1}) was recorded using enzymatic pretreated biomass (Onay, 2018). Chavan and Mutnuri used microalgal consortia for domestic wastewater treatment and the recovered biomass was used for lipid extraction. The residual biomass was subjected to fermentation to produce ethanol (1.31 gL^{-1}) (Chavan and Mutnuri, 2020). Castro et al. used sulfuric acid pretreated wastewater algae as feedstock for *Clostridium saccharoperbutylacetonicum* N1-4 and was able to produce 5.23 gL^{-1} acetone, butanol, and ethanol containing 3.74 gL^{-1} butanol (Castro et al., 2015).

5.5. Bioelectricity

Microbial fuel cell (MFC) is an attractive technology and can be used for wastewater treatment and bioelectricity production (Fig. 3). Microorganisms in MFC biodegrade organic compounds into CO_2, water, and energy through various metabolic pathways. A general design of MFC composed of two chambers, i.e., anodic, and cathodic chambers.

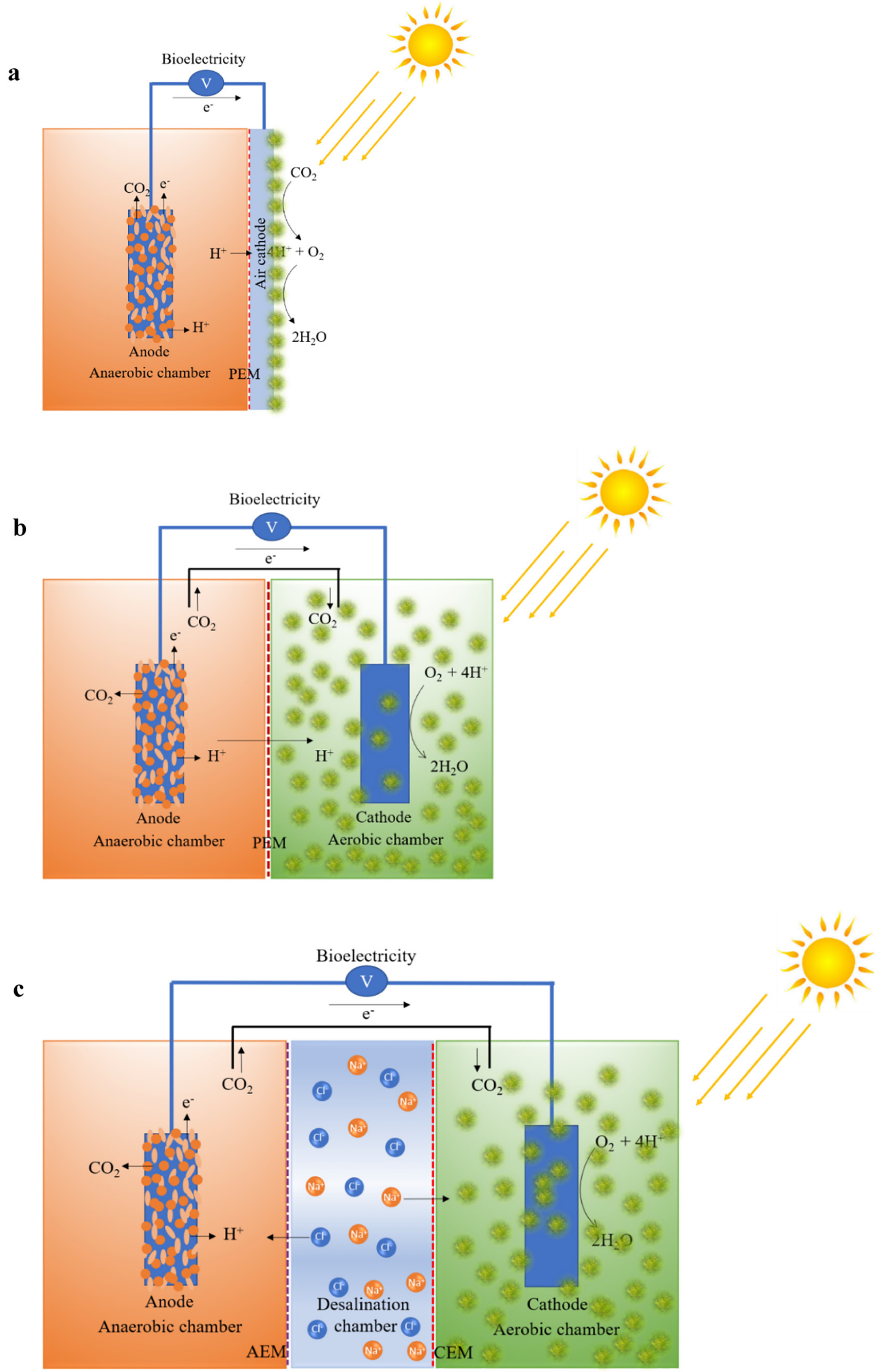

Fig. 3. Microalgal based photosynthetic microbial fuel cell (a) single chamber (b) dual chamber (c) microbial fuel cell with desalination chamber.

Microorganisms help in the oxidation process of substrates in the anodic chamber and produce electrons and protons (Bhatia et al., 2019). Electrons produced in the anodic chamber travel through external circuit and simultaneously, protons pass through the proton exchange membrane (PTE) to the cathodic chamber and react with reducing agent (oxygen) to produce water. Microalgae-based photosynthetic microbial fuel cell (PhotoMFC) is also composed of anode and cathode chambers, and microalgae can be used in either the anodic or cathodic chamber. Subhash et al. studied microalgae mediated bio-electrocatalytic fuel cell for bioelectricity generation under oxygenic photomixotrophic mode using atmospheric CO_2 and wastewater as a carbon source, and microalgae as an anode biocatalyst and reported a good electrogenic activity (3.55 μWm^{-2}) (Venkata Subhash et al., 2013). Behl et al. used *Chlamydomonas* sp. TRC-1 for textile wastewater treatment and the recovered biomass was further evaluated for bioelectricity production, and showed a good power 1.83 m^{-2} production as compared to fresh biomass due to the better electron transfer (Behl et al., 2020). Use of metal-based (platinum) and commercial carbon-based (graphene) electrodes and other electron acceptor (potassium ferricyanide) is an expensive approach. In contrast, use of algae-based biocathode is advantageous in PhotoMFC as in the presence of light and $CO_{2,}$ it produces $O_{2,}$ which together with electron and proton (produced by oxidation in the anodic chamber) produces water to complete the cathodic cycle (Gouveia et al., 2014). Electricity production is higher in a microalga-assisted MFC as compared to air-based cathode, which is associated with increased dissolved oxygen levels. However, microalgae biocathode-based MFC has limitations since its performance is affected by the growth of microalgae. A study reported that voltage output dropped from 260 mV to 70 mV, when microalgae growth entered dead phase and oxygen content decreased (22.5 to 3.7 mgL^{--1}) (Hou et al., 2016). Ling et al. used a partially submerged microalgae biocathode to solve this issue, and piggery wastewater was used as the anolyte and anaerobically digested swine wastewater as the catholyte and was able to achieve 50% higher voltage (297 mV) as compared to conventional MFC (Ling et al., 2019). Mohamed et al. studied *Oscillatoria* sp. and *Scenedesmus* sp. for food processing wastewater treatment and electricity production in MFC, and reported a power density of 32.5 and 28.5 mW m^{-2}, respectively (Naina Mohamed et al., 2019). Logrono et al. developed a single chamber MFC using an air exposed cathodic microalgal biofilm and used it for simultaneous biodegradation of textile dye wastewater and electricity production, showing a 18–43% higher power production as compared to control (Fig. 3a) (Logroño et al., 2017). González del Campo et al. studied a dual chamber photosynthetic MFC where oxygen in the cathode is provided by microalgal culture not through aeration and observed oxygen level was not the same throughout the day as when in dark phase it started to consume oxygen and the same was observed for voltage change (Fig. 3b) (González del Campo et al., 2013). Khazraee Zamapour et al. integrated MFC with saline removal system, two different MFC i.e. one with air cathode and other with biocathode having *Chlorella vulgaris* were compared. MFC with biocathode resulted in maximum power density of 20.25 mWm^{-2} with a salinity removal rate of 0.341 gL^{-1} day^{-1} (Fig. 3c) (Zamanpour et al., 2017). Bioelectricity production potential of different microalgal species is discussed in Table 4.

6. Potential, challenges, and future perspectives

Integration of wastewater treatment with microalgae culture for bioenergy production will help to improve the wastewater treatment cost and energy production simultaneously. Wastewater is rich in organic content and microalgae capture these nutrients, and are able to grow in wastewater without additional requirement of nutrients. Microalgae capture CO_2 and produce oxygen during the pretreatment process which helps in the growth of aerobic microorganisms and reduces energy consumption required for mechanical mixing and results in increased removal of organic load (Khazraee Zamanpour et al., 2017). Sludge management is a challenge as wastewater treatment requires additional amount of various chemicals which lead to sludge formation and discharge into the environment, while the use of microalgae in wastewater do not require any chemicals and the produced sludge is mostly composed of algal biomass. Along with wastewater treatment and resource recovery from waste, microalgae could also help in the

Table 4
Electricity production potential of different microalgae in different type of MFC from various wastewater.

Microalgae	Wastewater	MFC type	Comments	Power	Reference
Blue green algae	Domestic wastewater	Single chamber	Process also helps to remove microcystins.	114 mWm^{-2}	(Yuan et al., 2011)
Mixed algal culture	Landfill leachate	Double chamber	Landfill leachates require appropriate dilution.	50 mWm^{-2}	(Nguyen et al., 2017)
Botryococcus braunii	Sugar industry wastewater	Double chamber	*Saccharomyces cerevisiae* culture supplemented with methylene blue was used as the anolyte.	7.27 μWm^{-2}	(Manchanda et al., 2018)
Chlorella vulgaris	Wastewater activated sludge	Double chamber	Biocathode was used to eliminate the mechanical air supply.	13.5 mWm^{-2}	(González del Campo et al., 2013)
	Wastewater	Double chamber	Anodic off gas was supplied to the cathodic chamber.	5.2 WM^{-3}	(Wang et al., 2010)
	Wastewater	Photosynthetic microbial desalination cell	Process led to simultaneous wastewater treatment and desalination.	660 mWm^{-2}	(Arana and Gude, 2018)
	Dairy wastewater	Photosynthetic microbial desalination cell	Use of high saline solution have positive effect on power generation due to higher conductivity and less resistance.	20.25 mWm^{-2}	(Zamanpour et al., 2017)
	Wastewater and industrial effluent	Double chamber	Able to remove COD by 71.0 and 78.6% in anodic and cathodic chamber respectively.	327.67 mWm^{-2}	(Huarachi-Olivera et al., 2018)
Chlorella and *Phormidium*	Synthetic wastewater	Double chamber	An MFC with covered anodic chamber showed higher voltage, power density, coulombic efficiency and specific power than the one without covered anodic chamber.	1.65 mWm^{-2}	(Juang et al., 2012)
Golenkinia sp.	Food wastewater	Double chamber	TP and TN removal efficiency was 90% with 55.85% lipid production in cathodic chamber.	400 mWm^{-3}	(Hou et al., 2017)
Scenedesmus quadricauda SDEC-8	Domestic wastewater	Double chamber	Process led to 6.26 $mgL^{-}d^{-}$ lipid production with COD (80.2%), TN (96.0) and TP (91.5%) removal.	62.93 mWm^{-2}	(Yang et al., 2018)
Spirulina platensis and *Chlorococcum* sp.,	Tapioca wastewater	Double chamber	Main purpose was to study Tapioca utilization by microalgae for electricity production.	44.33 and 30.2 mWm^{-2}	(da Costa, 2018)
Synechococcus sp.	Kitchen wastewater	Double chamber	Gas produced in the anodic chamber was supplied to cathodic chamber.	41.5 mWm^{-2}	(Naina Mohamed et al., 2020)

assimilation of greenhouse gas (CO_2). Microalgae are able to fix CO_2 at 10–15 times faster than terrestrial plants and approximately 183 tons of CO_2 are required for the production of 100 tons of algal biomass. However, in order to have a more efficient algal technology, there are many challenges to be overcome. Wastewater contamination (bacteria, protozoa, and fungi) affects and inhibits microalgal growth. Pretreatment is a prerequisite step which could be done by using filtration and autoclaving at small scale but not feasible at commercial scale. Different types of wastewater have different compositions which also affect microalgae growth, hence, there is a need to identify and select suitable microalgae species which are more robust, resistant to various environmental factors, able to withstand high nutrient load, and can fulfill the desired outcomes (high nutrient removal capacity, high lipid accumulation etc.). Wastewater C/N ratio affects biomass production, as well as lipid, carbohydrate, and protein contents. Low C/N is more favorable for biomass and carbohydrate accumulation while high C/N ratio is required for lipids accumulation. Internal shading is also an issue, as wastewater are rich in nutrients to support rapid growth of microalgae in the presence of light. Microalgae grow on the surface of water and at high culture density, it inhibits light penetration inside the water, hence, restricting microalgae culture. There is an urgent need to design new bioreactors to improve the efficiency of reactors. Wastewater turbidity and suspended solids also restrict light penetration which further affects microalgae growth and productivity. Algae growth is affected by temperature and light availability; hence, the use of this technology is not beneficial at area of high altitude where most seasons have low temperature and shorter daylight hours. Another challenge is the recovery of algal biomass from wastewater. Although many techniques have been introduced, the harvesting process is still costly. To overcome all these issues, extra steps are required which increase cost, so it is imperative to develop technology that is more efficient and economical.

Despite all these challenges, there are still many benefits offered by the algal based wastewater treatment technology. Algal biomass recovered from wastewater treatment plant or residual algal biomass from commercial production can be transformed into biofertilizer to as supplement nutrients and to improve water holding capacity of soil. Microalgae are rich in nutrient content (protein, lipids, and carbohydrate) and easily digestible and hence, considered a suitable feed for aquaculture. In view of all these advantages of algal biomass integration with wastewater treatment and in combination with other emerging technology, higher revenue and improved overall economics of wastewater treatment process could be achieved.

7. Conclusion

Integration of wastewater treatment with microalgal based resource recovery is gaining attention. Although many new methods of microalgae cultivation and its downstream processing to produce various commercial products have been reported, there are many hurdles to be overcome to make this technology more economical and efficient. There is an urgent need to find microalgal strains that are more robust, able to grow under pressure due to higher nutrient content and containing self-flocculating ability to simplify harvesting process. The use of hybrid technology could be a promising approach to overcome many drawbacks of using the single culture system. In conclusion, the human community is in a dire need that requires more integrated approaches for wastewater treatment, microalgae cultivation, production of bioenergy as well as other valuable compounds, to achieve an overall streamlined process for enhanced industrial feasibility.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CRediT authorship contribution statement

Shashi Kant Bhatia: Conceptualization, Writing - Review & Editing, **Sanjeet Mehriya**: Writing - Review & Editing, Visualization, **Ravi Kant Bhatia**: Writing - Review & Editing, **Manu Kumar**: Writing - Original Draft, **Arivalagan Pugazhendhi**: Writing - Original Draft, **Mukesh Kumar Awasthi**: Writing - Original Draft, **A.E Atabani**: Writing - Review & Editing, **Gopalakrishnan Kumar**: Writing - Original Draft, **Wooseong Kim**: Review & Editing, **Seung-Oh Seo**: Review & Editing, **Yung-Hun Yang**: Conceptualization, Writing - Review & Editing, Supervision.

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