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GENERACIÓN DE GRÁNULOS Y AGLOMERADOS MICROALGA - BACTERIA PARA EL TRATAMIENTO DE AGUAS RESIDUALES MUNICIPALES Y PRODUCCIÓN DE METANO.

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Resumen

En el proceso de investigación, se evaluó la influencia del tiempo de retención hidráulica (TRH) y la intensidad de irradiación solar sobre la operación de un reactor microalgal tipo HRAP (por sus siglas en inglés, High Rate Algae Pond) utilizado para el tratamiento de agua residual municipal. En particular se evaluó la remoción de materia orgánica y nutrientes, así como la sedimentabilidad de la biomasa a través de la formación de agregados microalga-bacteria.

Para el estudio se emplearon reactores piloto de 50L inoculados con lodos activados y un consorcio de microalgas nativas, cuya especie dominante fue *Scenedesmus* sp. Se analizaron tres TRH (10, 6 y 2 días) en el reactor HRAP operado en condiciones de laboratorio bajo fotoperiodos de luz - oscuridad de 12:12 y 280 W/m^2 (equivalente a 3300 Wh m^{-2} d^{-1} de energía lumínica suministrada al sistema), complementariamente, las pruebas experimentales en condiciones al aire libre, se enfocaron en el efecto de la irradiación solar sobre la formación de agregados microalga-bacteria; tres condiciones de irradiación solar (6213 ± 1186, 2741 ± 667 y 3799 ± 373 Wh m^{-2} d^{-1}) fueron evaluadas, manteniendo constante el TRH en 10 días. Los resultados demostraron que bajo un TRH mayor o igual a 6 días se presenta una alta remoción de DQO (≥ 90 %), $N\text{-}NH_4^+$ (≥ 95 %) y $P\text{-}PO_4^{3-}$ (≥ 30 %) y una alta actividad nitrificante. Además, se observó la formación de agregados microalga-bacteria con velocidades de sedimentación (Vs) superiores a 1.4 m h^{-1}, 300 veces mayores a los presentados en sistemas microalgales (4x 10^{-3} m h^{-1}).

Sin embargo, al operar el HRAP a TRH de 2 días el sistema colapsó, disminuyendo las remociones de DQO, $N\text{-}NH_4^+$, $P\text{-}PO_4^{3-}$ por debajo del 15%, observándose la disgregación de los agregados. Se caracterizó la comunidad microbiana presente en los agregados formados mediante técnicas de pirosecuenciación.

Los resultados evidenciaron una relación mutualista entre las comunidades de bacterias nitrificantes, bacterias promotoras de crecimiento de plantas (*Porphyrobacer colymbi*, *Hydrogenophaga* sp., *Rhodobacter* sp.) y microalgas (principalmente *Chaetophora elegans*), mostrando que los bajos niveles de irradiación solar (menores a 3800 Wh m^{-2} d^{-1})

promovieron la formación de estructuras granulares de microalga-bacteria dominada por el género *Scenedesmus* sp. Los gránulos formados bajo estas condiciones presentaron una velocidad de sedimentación de 18 m h^{-1}, cercanas a las observadas en gránulos aerobios. Además, los análisis realizados, arrojaron porcentajes de remoción de DQO, nitrógeno y fósforo de 89 ± 3 %, 60 ± 5 %, 28 ± 7 %, respectivamente. Por el contrario, el nivel de irradiancia de 6213 ± 1186 Wh m^{-2} d^{-1} evidenció bajos porcentajes de remoción de DQO y nitrógeno de 50 ± 8 % y 36 ± 12 %, mientras que el porcentaje de remoción de fósforo alcanzó valores de 92 ± 1 %.

Durante este nivel de irradiancia (6213 ± 1186 Wh m^{-2} d^{-1}), el sistema fue dominado por *Scenedesmus* sp., caracterizado por un crecimiento de pequeños cenobios de 4 y 6 células, sin presentar formaciones de estructuras granulares o flocs. Este tipo de crecimiento mostró la velocidad de sedimentación más baja de $4x10^{-3}$ m h^{-1}, similar a las observadas típicamente en cultivos puros del género *Scenedesmus* sp.

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INTRODUCCIÓN GENERAL

Relevancia de la formación de agregados microalgales en el tratamiento de aguas residuales

1.1 Introducción

Los sistemas microalga-bacteria han sido empleados para el tratamiento de agua residual, debido a la alta capacidad fotosintética de las microalgas para la producción de O_2 y consumo del CO_2 generado por procesos de mineralización de la materia orgánica de las bacterias heterótrofas (Muñoz and Guieysse, 2006).

Durante los últimos 60 años la interacción microalga-bacteria para el tratamiento de agua residuales ha establecido nuevos retos tecnológicos basados en alta eficiencia del tratamiento de aguas residuales y la obtención de biomasa con alto valor agregado. La primera aproximación tecnológica de esta interacción microalga-bacteria fue llevada a cabo mediante el empleo de las lagunas facultativas desarrolladas por Caldwell (1946), cuyo diseño era óptimo para la remoción de materia orgánica y nutrientes, pero no para el crecimiento microalgal. Posteriormente, el interés por el uso del contenido proteico de las microalgas contribuyó al diseño de reactores de laguna de alta tasa, conocidos en inglés como High Rate Algae Pond (HRAP) (Oswald y Golueke, 1960, Oswald et al., 1957), donde el objetivo se enfocaba en maximizar la concentración de la biomasa para incrementar la eficiencia de tratamiento de agua residual.

Actualmente, el HRAP es considerado un sistema de tratamiento económico comparado con los sistemas de lodos activados, debido a los bajos costos de construcción (70% menos que los lodos activados) (DOE 2016) y bajos requerimientos energéticos (Shilton et al., 2008; Woertz et al., 2009). Estas características complementadas con la fácil operación de los HRAP**,** hacen a estos sistemas ideales para ser implementados tanto en zonas rurales, periferia de las zonas urbanas**,** como en comunidades ubicadas en zonas remotas donde las restricciones de espacio no son un factor limitante (Acién et al., 2016; Young et al., 2017). El comportamiento de este tipo de reactores ha sido evaluado para el tratamiento de diferentes tipos de aguas residuales como se observa en la Tabla 1.1.Características de los diferentes tipos de agua residual empleados durante el tratamiento biológico microalga-bacteria en reactores , donde se analiza su desempeño basados en la eficiencia de remoción de DQO, nitrógeno y fósforo.

Tabla 1.1.Características de los diferentes tipos de agua residual empleados durante el tratamiento biológico microalga-bacteria en reactores HRAP.

Tipo de agua residual	**Microalgas dominantes**	**Condiciones de operación**	**Características del agua residual (mg L^{-1})**	**REMOCIÓN**			**Referencia**
				% DQO	**% NT**	**% PT**	
Municipal, efluente secundario (St.Andrews, Escocia)	*Phaedactylum tricornutum*; *Oscillatoria* sp	Condiciones ambientales Irradiancia: NR Temp: 4 – 23 ºC pH: 9.5 – 9.8 TRH: 2.5 días Área superficial:0.6 m^2	NT*: 7 mg L^{-1} PT*: 3 mg L^{-} DQO: NR[1]	-	100	100	Craggs (1995)
Urbana, Efluente primario (Barcelona, España)	*Dictysphaeriumpulchellum, Chlorella sp, Micractiniumpusillum, Scenedesmusarmatus*	Condiciones ambientales Irradiancia: 1.700 – 7.300 Wh m^{-2} d^{-1} Temp: 11 – 25 ºC pH: 8.6 – 9.2 TRH: 4-10 días Área superficial:1.5 m^2	NT: 58.2 mg L^{-1} PT: NR DQO: NR	-	57-73	-	Garcia et al., (2000)
Efluente anaerobio, Residuo porcícola, (Veracruz, México)	*Spirulina (Arthrospira)*	Condiciones ambientales Irradiancia: 1700 -2300 µmol m^{-2} d^{-1} Temp: 20-34 ºC pH: 9.5 TRH: 6-7 días, (secuencial por lote) Área superficial: 6 m^2	NT: 28 -30 mg L^{-} [1] PT: 3 – 12 mg L^{-1} DQO: 54 – 83 mg L^{-1}	-	84-96	85-72	Olguín (2003)
Urbana (Barcelona, España)	NR	Condiciones ambientales Irradiancia: 1000 W m^{-2} * Temp: 13-20 ºC pH: 7.8 - 10 TRH:3-10 días	NT: 51 mg L^{-1} PT: 9 mg L^{-1} DQO: 260 mg L^{-1}	38	57-73	32-43	Garcia (2006)

		Área superficial: 1.5 m^2					
Porcicola (Valladolid, España)	*Chlamydomonas spp, Oocytis sp, Chlorella sp, Nitzschia sp*	Condiciones ambientales Sin burbujeo de CO_2 Irradiancia: 4448 – 7062 Wh m^{-2} d^{-1} Temp: 7-21 ºC pH: 8 – 8.8 TRH:10 días Área superficial: 1.5 m^2	NT: 45- 203 mg L^{-1} PT: NR DQO: 526-4346 mg L^{-1}	52-60	25-50	< 10	de Godos et al., (2009)
Porcicola (Valladolid, España)	*Scenedesmus* sp, *Chlamydomonas* sp,	Condiciones ambientales Burbujeo de CO_2 (2-5 %) Irradiancia: 5864 – 6774 Wh m^{-2} d^{-1} Temp: 18-27 ºC pH: 7 – 9.8 TRH:10 días Área superficial: 1.5 m^2.	NT*: 18 mg L^{-1} PT: NR DQO: 342 mg L^{-1}	41-54	96- 99*	NR	de Godos et al., (2011)
Doméstica (Hamilton, New Zeland)	NR	Condiciones ambientales Burbujeo de CO_2 (2 L min^{-1}) Irradiancia: 2100 – 6200 Wh m^{-2} d^{-1} Temp: 15-27 ºC pH: 7 - 11 TRH:4-8 días Área superficial: 31.8 m^2.	NT*: 56 mg L^{-1} PT*: 7 mg L^{-1} DBO_5: 50 mg L^{-1}	84-87	91-97*	70-73	Park et al., (2011a)
Lixiviados (Selangor, Malasia)	*Scenedesmus quadricauda, Chlorella vulgaris*	Condiciones ambientales Sin burbujeo de CO_2 Irradiancia: NR Temp: 21-28 ºC pH: 6 - 9 TRH:25-100 días Área superficial: 0.27 m^2.	NT*: 151 mg L^{-1} PT*: 8.18 mg L^{-1} DQO:4293 mg L^{-1}	70-91	92-99*	75-86	Mustafa et al., (2012)

Urbana, efluente de tratamiento secundario (Arcos de la frontera, España)	*Scenedesmus*sp	Condiciones ambientales Sin burbujeo de CO_2 Irradiancia: 100 - 250 W m^{-2}* Temp: 10-25 ºC pH: 9 TRH:10 días Área superficial: 1.92 m^2.	NT: 25 mg L^{-1} PT: 2 mg L^{-1} DQO:79 mg L^{-1}	-14 a 1	55-72	51 - 63	Arbib et al., (2013)
Efluente de acuicultura (Segovia, España)	NR	Condiciones ambientales Sin burbujeo de CO_2 Irradiancia: 5774 Wh m^{-2} d^{-1} Temp: 14-22 ºC pH: 8.6 TRH:5-20 días Área superficial: 1.33 m^2	NT: 31 mg L^{-1} PT: 19mg L^{-1} DQO: 678 mg L^{-1}	64-77	78-85	64-94	Posada et al., (2014)
Efluente acuicultura y agua residual doméstica (50%/50%) (Valladolid, España)	NR	Condiciones ambientales Sin burbujeo de CO_2 Irradiancia: 4677 Wh m^{-2} d^{-1} Temp: 21 ºC pH: 8.7 TRH:7 días Área superficial: 1.33 m^2	NT: 62 mg L^{-1} PT: 15 mg L^{-1} DQO: 545 mg L^{-1}	70 ± 17	88 ± 5	79 ± 15	Posada et al., (2014)
Municipal (Daejeon, República de Korea)	*Stigeoclonium* sp	Condiciones ambientales Sin burbujeo de CO_2 Irradiancia: 130 W m^{-2}* Temp: 22-26 ºC pH: 8.7 TRH:2-8 días Área superficial: 1.33 m^2	NT: 44 mg L^{-1} PT: 5 mg L^{-1} DQO: 109 mg L^{-1}	63-85	90-95	76-95	Kim et al., (2014)
Doméstica (Hamilton, Nueva Zelanda)	NR	Condiciones ambientales burbujeo de CO_2 Irradiancia: NR	NT:19-39 PT: 3-7 DQO: NR	NR	70-80	58-79	Sutherland (2014)

		Temp: 26 ºC pH: 8 TRH:4 días Área superficial: 31.8 m^2					
Efluente de digestión anaerobia de la industria alimenticia (Wavelgem, Bélgica)	*Klebsormidium* y/o *Ulothrix* sp.	Condiciones ambientales modo de operación: SBR burbujeo de CO_2 (5-8 L min^{-1}) Irradiancia: NR Temp: 12-21 ºC pH: 8 TRH:2.5 días Área superficial: 34 m^2	NT:116 ± 26 PT: 15 ± 3 DQO: 635 ± 86	67 ± 8	53 ± 17	31 ± 14	Van den Hende et al., (2016)
Efluente de lodos activados de la industria alimenticia (Wavelgem, Bélgica)	*Klebsormidium* y/o *Ulothrix*sp, *Desmodesmus*sp.	Condiciones ambientales burbujeo de CO_2 (5-8 L min^{-1}) Irradiancia: NR Temp: 12-21 ºC pH: 8 TRH:2.5 días Área superficial: 34 m^2	NT:8 ± 3 PT: 3 ± 1 DQO: 57 ± 36	33 ± 60	37 ± 35	20 ± 18	Van den Hende et al., (2016)
Efluente de digestión anaerobia		Condiciones ambientales burbujeo de CO_2 (5-L min^{-1}) Irradiancia: 5200 Wh m^{-2} d^{-1} Temp: NR pH: 8 TRH:3 días Área superficial: 32 m^2	NT:55 ± 7 PT: 8 ± 1 DQO: 178 ± 11	NR	52 ± 1	57 ± 3	de Godos et al., (2016)
Municipal	*Chaetophora elegans, Navicula sp, Nitzschia sp, Microcystis sp*	Condiciones Laboratorio Sin burbujeo de CO_2 Irradiancia: 3500 Wh m^{-2} d^{-1}	NT:71 ± 2	90-98	40-60	30-50	Arcila y Buitrón, (2016) Capítulo 3

		Temp: 12-21 ºC pH: 8-9 TRH:6-10 días Área superficial: 0.25 m^2	PT*: 15 ± 1 DQO: 593 ± 58				
Municipal	*Scenedesmus sp*	Condiciones ambientales Sin burbujeo de CO_2 Irradiancia: 3800 -6400 Wh $m^{-2}d^{-1}$ Temp: 12-21 ºC pH: 8-10.5 TRH:10 días Área superficial: 0.25 m^2	NT:110 ± 16 PT*: 15 ± 1 DQO: 591 ± 92	50-89	36-60	25-92	Arcila y Buitrón, (2017), Capítulo 4

* máxima irradiancia, calculado basados en el factor de corrección de 1 W/m2 ≈ 4.6 µmole.m2/s.

En cuanto al parámetro DQO, los tipos de aguas residuales domésticas (Park y Craggs, 2011a; Sutherland et al., 2014), municipales (Kim et al., 2014a; Arcila et al., 2016, Arcila et al., 2017), de lixiviados (Mustafa et al., 2012) y aguas provenientes de acuicultura (Posada et al., 2014) muestran remociones superiores al 70%, mientras que los tipos de aguas residuales de origen porcicola (de Godos et al., 2009, 2010) o provenientes de un proceso de tratamiento biológicos secundario como lodos activados (Arbib et al., 2013; Van den Hende et al., 2016) evidencian bajas remociones que no alcanza a superar el 60%. Este comportamiento está relacionado con las características de baja biodegradabilidad que presentan estos tipos de aguas residuales que limitan la eficiencia de remoción de DQO. En el caso de Arbib et al., (2013) los valores negativos de remoción de DQO, están relacionado con la exudación de sustancias poliméricas debido a las condiciones de estrés por los bajos niveles de nutrientes presentes en el sistema.

Uno de los factores relevantes por el cual se emplean los sistemas microalga-bacteria para el tratamiento de aguas residuales, está relacionado con la alta capacidad de remoción de nitrógeno y fósforo que tienen las microalgas (Muñoz and Guieysse, 2006; Olguin, 2012). En el caso del nitrógeno, la eficiencia de remoción de nitrógeno en el sistema de tratamiento depende de los mecanismos de asimilación, nitrificación y volatilización. Estudios contemporáneos a los realizados por Oswald (1957) mostraron que condiciones de pH alto (mayores a 8.5) favorece el mecanismo de volatilización de amonio, siendo el mecanismo dominante de remoción de nitrógeno en el agua residual (alcanzando valores hasta del 45 %) (Craggs et al., 1996; Cromar et al., 1996, García et al., 2006; Olguín et al.,2003).

Por otra parte, el tratamiento de aguas residuales domésticas (Park et al., 2011a), municipales (Arcila et al., 2016, Arcila et al., 2017), residuos porcícolas (de Godos et al., 2009) y efluente de digestión anaerobia (Van den Hende et al., 2016) han mostrado una alta actividad nitrificante que limita la eficiencia de remoción global de nitrógeno alcanzando valores promedio de alrededor de 50 %. Estos estudios sugieren que la proliferación de bacterias nitrificantes se estimula con condiciones de operación como largos tiempos TRH (mayores a 6 días), condiciones de irradiación solar por debajo 3800 Wh m^{-2} d^{-1} (Capítulo 3 y Capítulo 4) o la adición de CO_2 al medio de cultivo (Park y Craggs et al 2011a, 2011b, Van den Hende et al., 2016).

A diferencia de los múltiples mecanismos de remoción de nitrógeno, la remoción de fósforo está asociado principalmente a mecanismos de asimilación y precipitación, este último mecanismo es favorecido a pH altos (mayores a 9) (Larsdotter et al., 2010). Valores típicos en la eficiencia de remoción de fósforo en reactores HRAP para el tratamiento de aguas residuales son superiores al 50 % (Tabla 1); sin embargo, remociones inferiores a 30% son observadas en aguas residuales de origen porcícola (de Godos et al., 2009, 2011), en efluentes de procesos secundarios (Van den Hende et al., 2016) y municipales (Capitulo 3 y 4), las cuales presenta una relación directa con la alta concentración de nitrato en el sistema (mayores a 30 mg L^{-1})

Los resultados favorables para la remoción de nutrientes de aguas residuales, así como el potencial uso de las microalgas en bio-procesos, han direccionado las investigaciones hacia el tratamiento de aguas residuales y la generación de productos de valor agregado utiliizando biomasa de origen microalgal-bacteriana (Fig. 1.1). Una de las primeras apuestas investigativas se enfocó en la extracción de lípidos de microalgas crecidas en aguas residuales para la producción de biodiesel (Chinnasamy et al., 2010; Chisti, 2007; Mata et al., 2010). Sin embargo, de acuerdo a Chisti, (2013), la comercialización de este tipo de biocombustible a partir de microalgas es limitado principalmente por los altos costos de producción a gran escala, que lleva a balances de energía desfavorables (E_{salida} / $E_{entrada}$< 1).

Debido a esta problemática en cuanto a costos energéticos, la migración hacia la obtención de biocombustibles como el biogás (CH_4, H_2), surgió como una alternativa energética para el uso de la biomasa microalga-bacteria (Carrillo-Reyes y Buitrón, 2016; Ward et al., 2014; Weifu et al., 2013), sin dejar de lado el enfoque del tratamiento del agua. Aparte de los biocombustibles, otros productos relacionados como pigmentos (Gong y Bassi, 2016), fertilizantes (Coppens et al., 2016), alimento para peces (De Schryver et 2008), entre otros, se pueden obtener de la cosecha de biomasa microalga-bacteria dependiendo del tipo de agua residual tratada (Figura 1.1). No obstante, la viabilidad económica de estos bio-productos está restringida a la optimización de procesos relacionados con el cultivo (productividad de la biomasa) y cosecha (concentración de la biomasa). Este último proceso consume el 20-30 % del costo total de la producción de biomasa (Molina Grima et al., 2003), debido a la baja sedimentación que presentan las microalgas, limitando la viabilidad económica tanto de los sistemas microalga-bacteria para el tratamiento de aguas residuales, como los procesos

subsecuentes de generación de productos con alto valor agregado (Fernández, 2012; Olguín, 2012; Park et al., 2011). Diferentes tecnologías de concentración han sido implementadas para la cosecha de esta biomasa, tales como centrifugación, filtración, ultrafiltración, floculación química, flotación mediante aire disuelto (Gong y Bassi, 2016; Gutiérrez et al., 2016); desafortunadamente, ninguno de los métodos ha probado ser económicamente viable para ser empleado en gran escala (Barros et al.,2015).

Adicionalmente, se evita el uso de procesos químicos de coagulación/floculación debido a la contaminación de la biomasa (Lee et al., 2009; Muñoz and Guieysse, 2006; Xiao and Zheng, 2016). Actualmente, se ha reportado la formación de aglomerados de microalgas y bacterias denominados MABAs (Microalga-Bacteria Agglomerates), cuyo crecimiento se ha estudiado principalmente en reactores por lotes (Tiron et al., 2015; Tricolici et al., 2014) y reactores discontinuos secuenciales (Gutzeit et al., 2005; Valigore et al., 2012; Van Den Hende et al., 2011a)

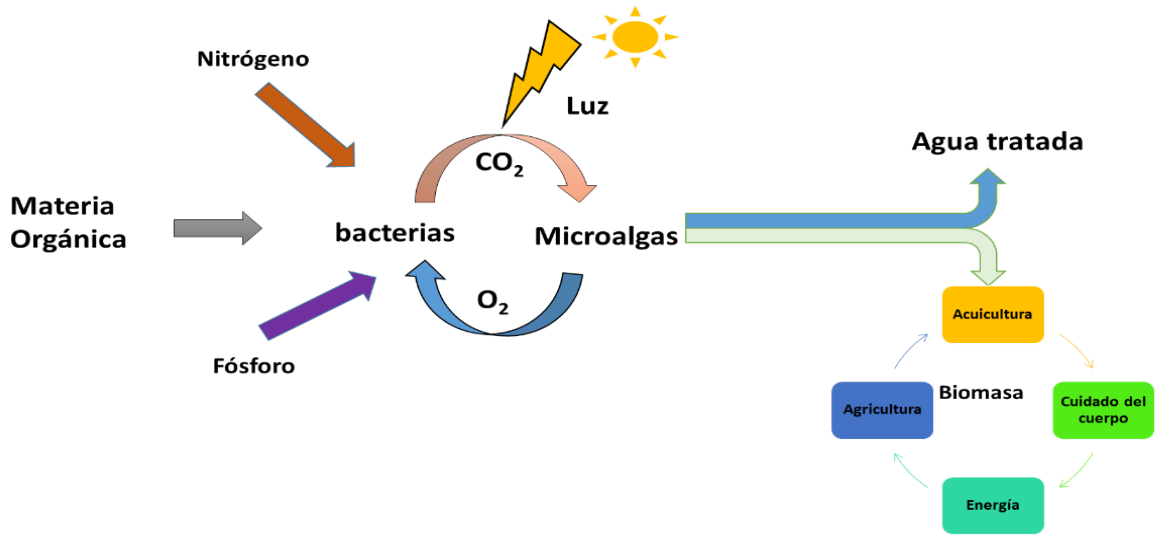

Figura 1.1.Esquema de las variables involucradas en la interacción microalga-bacteria para el tratamiento de agua residual y el potencial biotecnológico de la biomasa recuperada.

En el caso de la operación de reactores en continuo, hasta el momento, los únicos reportes sobre la obtención de estructuras granulares a partir de agua residual han sido desarrollados en nuestro laboratorio producto de esta investigación (Capítulos 2 y 3). Estos sistemas MABAs se caracterizan por tener alta remoción de materia orgánica y nutrientes, así como

excelentes propiedades de sedimentación, permitiendo disminuir los costos de cosecha de la biomasa microalga-bacteria mediante el uso de una sencilla etapa de sedimentación por gravedad. No obstante, la resiliencia de estas estructuras MABAs, depende de diferentes factores como los descritos en la Figura 1.2, que se analizarán a continuación.

Figura 1.2. Descripción esquemática de los principales factores que afectan la formación de las estructuras MABAs

1.2 Factores que influyen en la formación de aglomerados de microalga-bacteria

1.2.1 Factores operativos

Tal como en los procesos biológicos a partir de bacterias, el desacoplamiento de los tiempos de retención hidráulica (TRH), del tiempo de retención de sólidos (TRS) se identifica como un factor clave para la generación de agregados de microalga-bacteria con alta eficiencia de remoción (Medina and Neis, 2007; Valigore et al., 2012; Van Den Hende et al., 2011a). Estrategias de corto tiempo de sedimentación y de reacción en reactores discontinuos secuenciales (SBR), han sido probadas como un proceso selectivo para la formación de agregados de microalga-bacteria. Gutzeit et al. (2005); observó la formación de agregados a partir de un sistema inoculado con *Chlorella vulgaris* y lodos activados de una planta de tratamiento de aguas residuales, alcanzando concentraciones de SST hasta de 18 mg L^{-1} con un TRH de 3 días y un TRS de 25 días, siendo valores muy bajos comparado con los requerimientos de la norma mexicana (NOM 001) respecto a vertimientos en aguas y bienes

de uso nacional (60 -200). Similares remociones de sólidos (16 mg L^{-1}) se obtuvieron por Su et al., (2011), donde la presencia de microalgas filamentosas (Cianobacteria) promovieron la formación de agregados con excelentes propiedades de sedimentación, alcanzando altas eficiencias de remoción de demanda química de oxígeno (DQO), nitrógeno total kjeldahl (NTK) y fosfatos de 98.2 ± 1.3%, 88.3 ± 1.6% and 64.8 ± 1.0%, respectivamente.

La evolución de estos sistemas ha llegado a la formación de flóculos de microalga y bacteria, denominados MAb-flocs (Microalgae-Bacteria flocs), los cuales han sido evaluados en diferentes tipos de aguas residuales industriales (Van Den Hende et al., 2014a, 2016). No obstante, el escalamiento de esta tecnología (MAb-floc) de 4 L a reactores tipo raceway de 12 m^3 operados de manera secuencial, mostraron disminución en la eficiencia de remoción de materia orgánica y nutrientes de 1-3 veces, así como en la productividad de la biomasa con una disminución de 10-13 veces (Van Den Hende et al., 2014b)

Se ha detectado que el régimen hidrodinámico, asociado a las condiciones de mezclado como el esfuerzo cortante (Scarsella et al., 2012), la potencia volumétrica (P/V) (De Schryver et al., 2008) y gradiente de velocidad (Biggs and Lant, 2000), son parámetros relevantes en la formación de agregados de microalga-bacteria. Por ejemplo, Molina-Grima et al., (2010) demostraron que condiciones de energía entre 100 y 300 W m^{-3} causan daños a las paredes celulares debido al esfuerzo cortante. Otro factor asociado al régimen hidrodinámico que afecta la formación de los agregados se relaciona con el régimen de flujo tipo pistón que presentan los reactores tipo HRAP. Estas condiciones de flujo generan líneas de flujo unidireccional, que disminuyen la eficiencia de mezcla, así como la probabilidad de interacción entre microorganismos (Hadiyanto et al., 2013). Debido a esta limitante, el diseño de geometrías nuevas y la utilización de propelas en vez de agitadores tipo Paddlewheel, evidenciaron un mejora respecto a las pérdidas de carga en el sistema (de 43 a 11%), sin embargo su productividad fue 15% menor a la obtenida en los sistemas tradicionales (Chiaramonti et al., 2013).

1.2.2 Factores ambientales

Parámetros como la intensidad lumínica y la temperatura son considerados claves para el desempeño de los reactores HRAP en cuanto a productividad, remoción de materia orgánica y nutrientes (Béchet et al., 2013; Park et al., 2011). Respecto a la formación de aglomerados,

la producción de sustancias expoliméricas (EPS, cuyas siglas en ingles corresponden a Extracellular Polymeric Substances) encargadas de los procesos de agregación y producción de flóculos, son drásticamente afectadas por la intensidad lumínica que incide en el sistema (Ramanan et al., 2015; Weber et al., 2007; Xiao y Zheng, 2016; Zhou et al., 2015). Especies de microalgas como *Microsystis aeruginosa*, *Arthrospira platensis*, and *Nostoc* sp., muestran alta producción de EPS en bajas condiciones de luz (100-180 μmol m^{-2} s^{-1}) (Ge et al., 2014; Trabelsi et al., 2009; Zhen and Fanxiang, 2013). Por otra parte, Tricolici et al., (2014), observaron la formación de agregados de microalga-bacteria bajo condiciones de intensidad lumínica de 820 μmol m^{-2} s^{-1}. Estas estructuras tienen una velocidad de sedimentación de 20% más alta que la producción de EPS a 360 μmol m^{-2} s^{-1}.

En cuanto al desempeño de los sistemas microalga-bacteria en reactores tipo HRAP operados en condiciones al aire libre, las especies que proliferan en estos sistemas también están asociados a condiciones estacionales, siendo las microalgas con difícil sedimentación como *Scenedesmus* sp., *Microcystis* sp., *Chlorella* sp., las especies dominantes a lo largo de un año de experimentación (Assemany et al., 2015; Cho et al., 2015). Sin embargo, las pruebas experimentales realizadas en nuestro trabajo de investigación (**Capítulo 4**) demostraron que una disminución en la irradiación solar, provoca el aumento de proteínas en las EPS asociadas con la formación de estructuras granulares, dominada por la especie *Scenedesmus* sp.

1.2.3 Característica del medio de cultivo

Van den Hende et al., (2011) y Zhou et al., (2015) muestran que factores como la concentración de cationes, el tipo de fuente de carbón y la relación de nutrientes y carbono (N/P, C/N), son relevantes en el desempeño y formación de agregados de microalga y bacterias. En el caso de los cationes (Na^{+}, Ca^{+2}, Al^{+3}, Mg^{+2}, etc.) su presencia contribuye a la formación de EPS. La adición de cationes, Ca^{+2}, Mg^{+2} (10 mM de cada uno), promueve la adhesión de microorganismos como *Chlorella*, *Phaeodactylum tricornutum* y *Bacillus* sp. El Ca^{+2} contribuye a la expresión de proteínas que aumentan la unión de *Chlorella* y *Phaeodactylum tricornutum*, mientras Mg^{+2} regula la formación de polisacáridos en la síntesis de los EPS para la formación de biopelículas en los géneros *Chlorella* (He et al., 2016). No obstante, los altos valores reportados para la producción de agregados de

microalga-bacteria, parecen ser una condición limitante en la producción de estos, debido la alta variabilidad de la dureza en aguas residuales.

Por otro lado, la relación carbono inorgánico a carbono orgánico (CI/CO) es un factor que afecta tanto la remoción de nutrientes, como las propiedades de sedimentación de los agregados (Van Den Hende et al., 2011b). Se ha demostrado que bajo altas relaciones de carbono CI/CO (cercanas a 84), se observan tanto bajas remociones de nutrientes (nitrógeno y fósforo) como de sedimentabilidad; por el contario, relaciones de CI/CO por debajo de 1, muestran un aumento en la sedimentación y la eficiencia en la remoción de nutrientes. Asimismo, se ha observado la formación de agregados microalga-bacteria (500 y 650 μm) bajo condiciones de limitación por nutrientes (C/N/P, 14/1.4/1 y 44/1.4/1)**,** con una remoción de carbono como DQO y nitrógeno mayor a 90% (Zhou et al., 2015).

1.2.4 Factores microbiológicos

La caracterización de las comunidades de microalgas y bacterias, a través de técnicas como DGGE y 454 pirosecuenciación, ha permitido identificar una serie de microalgas resistentes a varios tipos de aguas residuales como son los géneros de *Chlorella* sp., *Scenedesmus* sp., *Nitzschia* sp., así como el dominio de *Proteobacterias* en este tipo de sistemas (de Godos et al., 2009; Ferrero et al., 2012; Ramanan et al., 2016; Su et al., 2012). Se ha detectado que la coexistencia de bacterias en cultivos microalgales están relacionados principalmente a interacciones de mutualismo y parasitismo (Ramanan et al., 2016; Fuentes et al., 2016).

Respecto a la interacción mutualista, dos grupos de bacterias han sido consideradas relevante**s** en el crecimiento y formación de agregados microalgales. El primer grupo está relacionado con bacterias promotoras de crecimiento de plantas (PGPB, plant growth promoting bacteria), las cuales suplen a las microalga de vitaminas como $B_{12,}$ nutrientes, fitohormonas de crecimiento, así como les confieren condiciones de resistencia a patógenos (Ramanan et al., 2015). La mayoría de bacterias pertenecientes a este grupo están asociadas a las α-*Proteobacteria*, incluyendo *Sphingomonas* y *Rhizobacter* (Cho et al., 2015; Kim et al., 2014; Ramanan et al., 2016). En agregados, este grupo de microorganismos mediante interacciones de mutualismo y comensalismo son capaces de aumentar el crecimiento de las microalgas, las cuales brindan a las bacterias un microambiente capaz de protegerlas de parasitismo y estrés abióticos (Grover et al., 2011; Souza et al., 2015).

En cuanto al segundo grupo de microorganismos, estos se relacionan con bacterias promotoras de los procesos de floculación como B*acteroidetes*, *Flavobacterium* sp, *Sphingobacterium* sp. and *Terrimonas* sp., los cuales han sido reportados en agregados microalgales conformados por especies pertenecientes a los filos de Clorofitas y cianobacterias crecidas en aguas residuales (Lee et al., 2013; Su et al., 2011).

Los experimentos realizado en nuestra investigación sobre las comunidades presentes en el sistema **(Capítulo 4)**, evidencian el domino de bacterias α-*Proteobacteria* pertenecientes a *Porphyrobacter colymbi*, *Hydrogenophaga* sp. en agregados de microalga-bacteria lo cual es contradictorio a lo reportado hasta el momento. Debido a esto el análisis de las comunidades de bacterias durante el inicio de la formación de aglomerados resulta imperante para el entendimiento de los mecanismos de formación de agregados.

1.3 Hipótesis

- Tiempos de retención hidráulicos altos y bajos niveles de intensidad lumínica estimularán la proliferación de estructuras filamentosas que sirven de soporte en la formación de agregados de microalga-bacteria en sistemas HRAP para el tratamiento de agua residual municipal.

- Bajos niveles de irradiancia promoverán la secreción de sustancias poliméricas por parte de las microalgas lo cual favorecerá la formación de gránulos de microalga-bacteria

- La eficiencia de remoción de materia orgánica y nutrientes en los agregados microalga-bacteria para el tratamiento de agua residual municipal será afectado por choques de carga orgánica, ocasionados por una disminución en los tiempos de retención hidráulica (TRH).

- Tiempos de retención hidráulicos altos, asociados a bajas concentración de carga orgánica y nutrientes, estimularán las comunidades fotosintéticas eucariotas y limitarán el crecimiento de comunidades bacterianas anaerobias favoreciendo la remoción de materia orgánica, nutrientes, así como la obtención de biomasa con alta sedimentabilidad y potencial bioquímico de metano.

1.4 Objetivo General

Evaluar la formación de agregados de microalga-bacteria bajo diferentes tiempos de retención hidráulica y condiciones de irradiancia solar, así como su desempeño en cuanto a la remoción de materia orgánica, nutrientes y velocidad de sedimentación empleando aguas residuales municipales en reactores tipo HRAP operados en continuo.

1.4.1 Objetivos específicos

- Comparar el efecto de tres diferentes tiempos de retención hidráulica sobre la formación de agregados de microalga-bacteria, así como su eficiencia de remoción de materia orgánica y nutrientes y la sedimentación en un reactor tipo HRAP, empleando agua residual doméstica

- Analizar la influencia de tres niveles de irradiancia solar sobre la formación de aglomerados de microalga-bacteria y su relación con el desempeño de reactores tipo HRAP operados en continuo utilizados para el tratamiento de agua residual municipal

- Identificar la dinámica poblacional microbiana y sus interacciones ecológicas, presentes en los sistemas microalga-bacteria cuando son sometidas a cambios en los tiempos de retención hidráulica de los reactores tipo HRAP, empleando agua residual municipal

2

Microalgal-bacterial aggregates (MABAs): applications and perspectives for wastewater treatment

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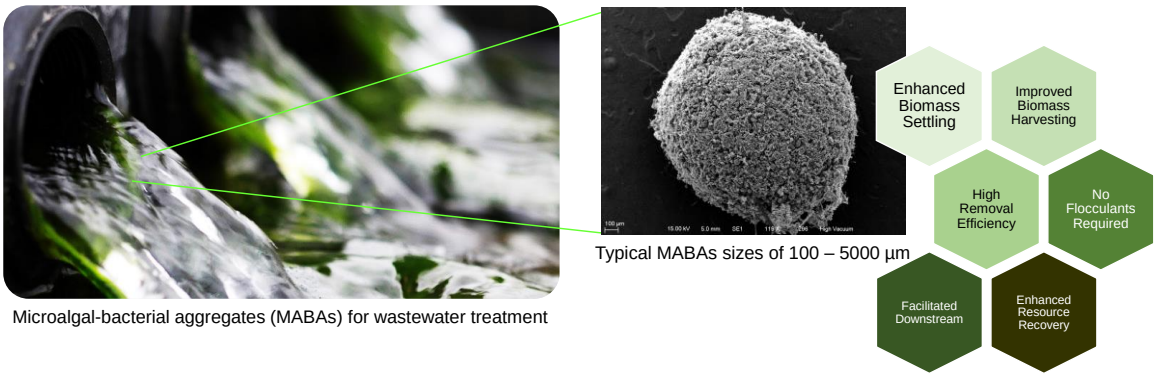
Abstract

Research on wastewater treatment by means of microalgal-bacterial processes has become a hot topic worldwide during the last two decades. Owing to the lower energy demand for oxygenation, the enhanced nutrient removal and the potential for resource recovery, microalgal-based technologies are nowadays considered as a good alternative to conventional activated sludge treatments in many instances. Nevertheless, biomass harvesting still constitutes one of the major challenges of microalgal-bacterial systems for wastewater treatment, which is hindered by the poor settleability of microalgal biomass. In this review, the use of microalgal-bacterial aggregates (MABAs) to overcome harvesting issues and to enhance resource recovery is presented. The fundamentals of MABAs-based technologies, the operational strategies and factors affecting the formation of MABAs, the microbiology and the methanogenic potential of the aggregates are addressed and critically discussed. The most recent findings and the challenges facing this technology towards its consolidation are also presented.



Microalgal-bacterial aggregates (MABAs) for wastewater treatment

Keywords: Biomass harvesting; High rate algal ponds; Microalgal-bacterial aggregates; Resource recovery; Wastewater treatment.

Nomenclature

BMP	Biomethane potential ($mLCH_4\ gVS^{-1}$)
D_{CO2}	Diffusivity coefficient of CO_2 in water ($cm^2\ s^{-1}$)
D_{O2}	Diffusivity coefficient of O_2 in water ($cm^2\ s^{-1}$)
EPS	Extracellular polymeric substances
G	Gravitational acceleration ($m\ s^{-2}$)
HRAP	High rate algal pond
MABA	Microalgal-bacterial aggregate
R	MABAs radius (µm)
SBR	Sequential batch reactor
SVI	MABAs volume index ($mL\ g^{-1}$)
t_D	Characteristic time for mass transfer (s)
V_s	settling velocity ($m\ h^{-1}$)
P/V	Volumetric power input ($W\ m_{reactor}^{-3}$)
Greek letters	
Δ	Thickness of the EPS film between bacterial and microalgal cells (µm)
ρ_p	MABAs density ($g\ mL^{-1}$)
ρ_f	density of the culture broth ($g\ mL^{-1}$)
µ	Dynamic viscosity of the culture broth ($g\ cm^{-1}\ s^{-1}$)

2.1 Microalgal-bacterial systems for wastewater treatment

Wastewater treatment by means of microalgal-bacterial systems is a technology that has been in use for more than 60 years. The pioneering studies of Oswald in California set the fundamentals of wastewater treatment in the so-called “high-rate algal ponds” (HRAPs), which were originally focused on removing organic matter and nutrients (Oswald, 1973, 1962). Later on, numerous studies demonstrated that added-value products and biofuels can be obtained concomitantly with wastewater treatment by microalgal-bacterial processes. Pigments, nutraceuticals, fertilizers, lipids as well as poultry and fish feeds can be obtained from the harvested microalgal-bacterial biomass depending on the type of wastewater treated and the environmental conditions (De Schryver et al., 2008; Gong y Bassi, 2016;

Subashchandrabose et al., 2011). Moreover, gas biofuels such as CH_4 and H_2 can be further obtained from the anaerobic digestion of microalgal-bacterial biomass (Alzate et al., 2014; Cardeña et al., 2017; Carrillo-Reyes y Buitrón, 2016). Conventional aerobic activated sludge processes or anaerobic technologies for wastewater treatment still present important economic or technical limitations related with their high energy requirements and poor nutrient removal performance, respectively (Posadas et al., 2013; Tiron et al., 2015). Microalgal-bacterial processes are nowadays regarded as sustainable platforms for wastewater treatment as they exhibit a lower energy demand for oxygen supply due to the photosynthetic oxygen production of microalgae, while supporting efficient nutrient removal and resource recovery (Posadas et al., 2017; Van Den Hende et al., 2014; Xiao y Zheng, 2016). A general framework of the wastewater treatment by microalgal-bacterial systems is depicted in Fig. 2.1. The fundamentals of the treatment process are the same than those early presented by Oswald (1962). However, recent studies have shown that the resource recovery from the harvested biomass is necessary for making economically feasible the microalgal-based wastewater treatment compared to conventional aerobic/anaerobic processes (Gong y Bassi, 2016; Olguín, 2012; Vulsteke et al., 2017).

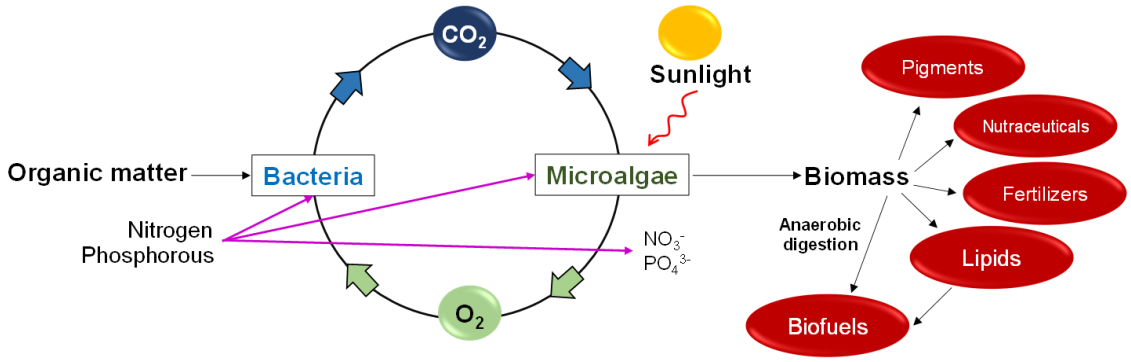


Figura 2.1. Wastewater treatment by means of microalgal-bacterial processes and resource recovery. Oxygen is photosynthetically produced by microalgae in the presence of light and CO_2, which is used by bacteria to oxidize organic matter, producing in return CO_2 for microalgae photosynthesis. The growth of microalgal biomass involves a significant removal of nutrients as they require high amounts of nitrogen and phosphorous for proteins (45–60% of microalgae dry weight), nucleic acids and phospholipids synthesis (Muñoz and Guieysse, 2006).

It must be noted that although a successful removal of organic matter and nutrients has been consistently achieved in HRAPs, the harvesting of the microalgal-bacterial biomass still constitutes one of the key operational limitations of this technology (Gutiérrez et al., 2016; Tiron et al., 2015). The poor settleability of microalgae substantially increases the biomass harvesting cost, this being widely reported as one of the major limitations to the economic viability of wastewater treatment by means of microalgal-bacterial processes (Acién-Fernández et al., 2012; Olguín, 2012; Park et al., 2011). Biomass harvesting accounts to 20-30% of the total cost of biomass production, which is necessary for the subsequent resource recovery (Molina-Grima et al., 2003). When fine chemicals such as pigments and nutraceuticals are recovered, harvesting costs might increase the total downstream processing costs (including harvesting, extraction and purification stages) representing up to 60% of the total biomass production cost (Olguín, 2012; Van Den Hende et al., 2011a). Therefore, many studies have focused on developing biomass harvesting methods including centrifugation, filtration, ultrafiltration, chemical flocculation and chemical flocculation-flotation processes (Gong y Bassi, 2016; Gutiérrez et al., 2016). Unfortunately, none of these harvesting methods have been proven to be economically suitable for large-scale microalgal removal, the resource recovery being also hindered when chemical flocculating/coagulating agents are used due to biomass contamination (Lee et al., 2009; Muñoz and Guieysse, 2006; Uduman et al., 2010; Xiao y Zheng, 2016). Several authors have reported the formation of microalgal-bacterial aggregates (MABAs) during wastewater treatment. In this review, the term MABAs includes the "bio-flocs", "MAB-flocs", "granular activated algae", "bioflocculent algal-bacterial biomass" and "algal-bacterial aggregates" previously reported in the literature and refers to aggregates of bacteria, microalgae and/or cyanobacteria (Arcila y Buitrón, 2016; De Schryver et al., 2008; Gutzeit et al., 2005; Tiron et al., 2015; Van Den Hende et al., 2012, 2011a, 2011b). Likewise, the term "microalgae" will be used to refer both microalgae and/or cyanobacteria to ease reading. MABAs, besides maintaining a high organic matter and nutrient removal performance, they also present excellent settling characteristics, which allows for efficient and inexpensive biomass harvesting by gravity sedimentation (Arcila y Buitrón, 2016; Van Den Hende et al., 2015, 2014, 2011a). The tremendous potential of MABAs for wastewater treatment has been identified as one of the most interesting research fields towards consolidating microalgal-bacterial processes for wastewater treatment (Lee et

al., 2009; Muñoz y Guieysse, 2006; Ramanan et al., 2016; Van Den Hende et al., 2011b). This work constitutes an updated state-of-the-art review on the use of MABAs for wastewater treatment. The fundamentals, the most recent findings and the research niches relevant for consolidating the process design of this technology at full scale are identified and critically discussed.

2.2 MABAs for wastewater treatment

2.2.1 Main characteristics of MABAs

As above mentioned, dispersed microalgal biomass settles slowly due to well identified factors: (a) their small cell size (ranging between 5 and 50 μm), (b) the relatively low cell density achieved in wastewater treatment processes (in the order of 0.4-0.5 kg m^{-3} of dry biomass), and (c) the negative charge of microalgal cell surface that prevents aggregation of microalgal cells in suspension (Molina-Grima et al., 2003; Uduman et al., 2010). In the case of wastewater treatment, bacteria play a key role in the removal of organic matter and it has been observed that they might produce extracellular polymeric substances (EPS) that mediate their aggregation with microalgae and cyanobacteria (De Schryver et al., 2008). The resulting MABAs present sizes ranging from 100 to 5000 μm, depending on the operating conditions (Arcila y Buitrón, 2016; Gutzeit et al., 2005; Lee et al., 2013; Tiron et al., 2015; Van Den Hende et al., 2016). Such increase of up to three magnitude orders in the size of biomass particles improves dramatically the harvesting without requiring the addition of flocculants as in the case of dispersed biomass systems (Gutzeit et al., 2005).

The presence of other organisms such as protozoa and small metazoans in the surroundings of MABAs has been consistently observed during wastewater treatment (De Schryver et al., 2008; Gutiérrez et al., 2016) (Fig. 2.2). De Schryver et al. (2008) proposed that protozoa and metazoa play an important role in the size of MABAs as they are associated with bacterial grazing from the surface of the aggregates. Interestingly, studies performed with axenic microalgal cultures revealed that the aggregates formed only with microalgal EPS settled slowly in the absence of bacteria, which highlighted the key role of the bacterial EPS on the formation of stable and settleable MABAs (Gärdes et al., 2011; Grossart et al., 2006; Lee et al., 2013; Powell y Hill, 2014; Ramanan et al., 2016). The chemical nature of the bacterial

EPS within MABAs is expected to vary with the type of microbial communities, the type of wastewater and the prevailing environmental conditions. However, it was pointed out that the presence of proteins and uronic acids in the EPS is an important factor towards the formation of settleable MABAs by stabilizing the negative charges of the microalgal biomass surface (De Schryver et al., 2008; Grossart et al., 2006; Xiao and Zheng, 2016). The production of EPS in microalgal-bacterial systems also influences the viscosity of the culture broth. The impact of microalgal growth on the viscosity of the culture broth has been comprehensively studied with 110 microalgal strains, apparent viscosities of up to 6.55 cp being recorded in the culture broths maintained for 84 days (Mancuso-Nichols et al., 2009). However, most microalgal cultures exhibited viscosities in the range of 1.50 to 3.18 cp. Likewise, a viscosity value of 1.90 cp was reported for *Chaetoceros muelleri* cultures (Michels et al., 2010). This information is useful for estimating the settling performance of MABAs, which will be further discussed in the section 2.3.

2.2.2 Operational strategies and factors affecting the formation of MABAs

In this section, the specific operational strategies that induce the formation of MABAs and influence their performance are reviewed and discussed. It is important to remark that there is a lack of systematic studies on the experimental conditions that favor the formation of stable and settleable MABAs during the wastewater treatment, which certainly constitutes an important research niche. The following lines lump the information available in the literature on this matter.

Bioreactor operation mode

The operation mode of the treatment system as a sequential batch reactor (SBR) is likely the most recurrent strategy to generate MABAs. Operating the HRAP as an SBR allows for selecting those microalgal and bacterial communities able to aggregate and settle, while the rest of microbial communities in suspension are washed-out (Fig. 2.3). SBR operation at hydraulic retention times (HRTs) of 1.4-8.0 days have been reported (Gutzeit et al., 2005; Medina y Neis, 2007; Valigore et al., 2012; Van Den Hende et al., 2016, 2015, 2014, 2011b).

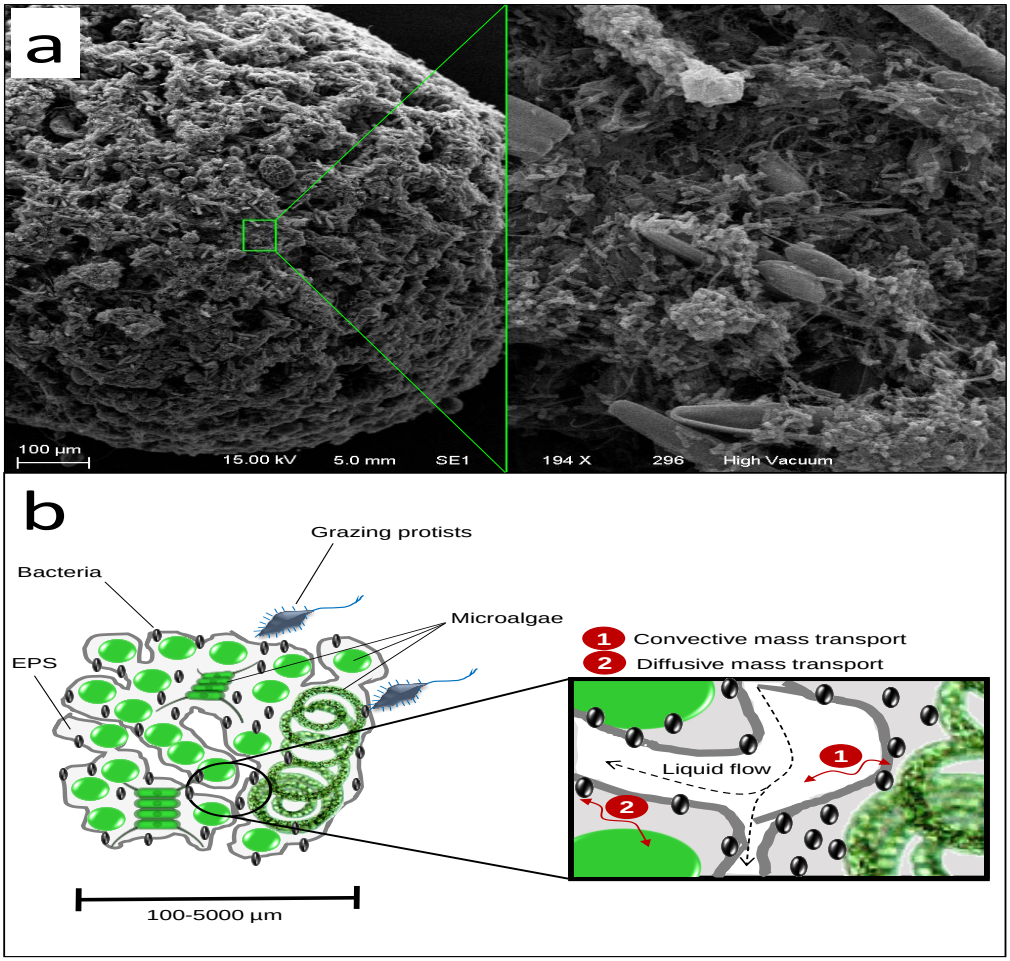


Figura 2.2. MABA depicted in (a) scanning electron image taken in our laboratory where bacterial and microalgal cells are clearly observed in the aggregate (SEM images obtained in a Zeiss EVO-50 microscope equipped with a Leica EM-ACE200 camera), and (b) schematic representation. MABAs are porous and permeable to fluids, which allows for diffusive and convective mass transport of CO_2, O_2, organic matter and nutrients within the aggregates (De Schryver et al., 2008).

When active MABAs already formed are used to start a bioreactor, HRTs as short as 0.67 days could be used (Van Den Hende et al., 2011b). Although SBR operation can be permanently set for the wastewater treatment, this operation mode may also be used only during the startup to generate the MABAs and then be switched to continuous operation (Gutzeit et al., 2005). It must be noted that the SBR operation is not the only way to generate MABAs. In our laboratory MABAs were formed during the treatment of municipal wastewater by starting the HRAP in batch mode for 10 days and then switching to continuous operation. We observed the formation of MABAs within approximately 1 month under this

operational strategy (Arcila and Buitrón, 2016). A high removal performance of organic matter (78-91% COD removal) and nutrients (70-99% nitrogen removal) has been recorded in continuous MABAs-based HRAPs operating at HRTs of 6-10 days (Arcila and Buitrón, 2016; Gutiérrez et al., 2016; Gutzeit et al., 2005).

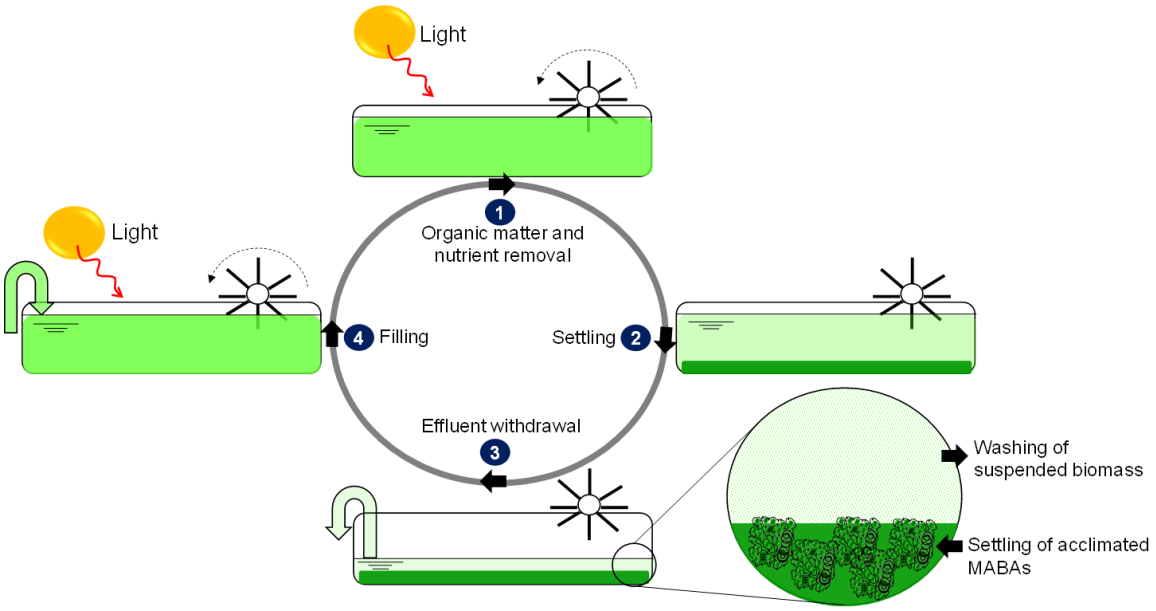


Figura 2.3. SBR operation for MABAs formation. MABAs acclimated to the specific wastewater characteristics are obtained after some operation cycles, depending on the type of wastewater and the operating conditions.

Light intensity

The light intensity has been consistently considered as a key parameter in the evaluation of microalgal biomass productivity (Béchet et al., 2013; Park et al., 2011). This parameter has been reported to impact the production of EPS in microalgae, which are required in the formation of MABAs (Ramanan et al., 2016; Weber et al., 2007; Xiao y Zheng, 2016). Microalgal species such as *Microsystis aeruginosa*, *Arthrospira platensis*, and *Cyanobacteria Nostoc* sp. exhibited a high production of EPS relatively low-intensity light conditions (e.g. 100-180 μmol m^{-2} s^{-1}) (Ge et al., 2014; Trabelsi et al., 2009; Zhen y Fanxiang, 2013). The same behavior was also observed in *Chlorella vulgaris* and *Graesiella* genus, which supported the highest production of EPS at light intensities below 120 μmol m^{-2} s^{-1} (Mezhoud et al., 2014; Shen et al., 2015). Nevertheless, Tricolici et al. (2014) demonstrated

that MABAs can be formed during wastewater treatment even at a light intensity of 820 μmol m^{-2} s^{-1}.

The MABAs obtained by these authors exhibited a settling rate 20% higher that observed in the same system operated at 360 μmol m^{-2} s^{-1}. Other studies also confirmed the formation of MABAs during wastewater treatment at light intensities ranging from 200 to 600 μmol m^{-2} s^{-1} using artificial LED light or solar irradiance (Arcila and Buitrón, 2016; Kim et al., 2014). Moreover, the outdoor experiments carried out in our laboratory using municipal wastewater demonstrated that a decrease in the solar irradiance from 5027 ± 178 to 2257 ± 76 μmol m^{-2} s^{-1} boosted the production of EPS as well as the formation of MABAs with *Scenedesmus* as the dominant genus (>90%) (Arcila et al., 2017). As discussed in section 2.1, bacterial communities can also produce EPS that mediates the formation of stable and readily settleable MABAs. Based on the available literature, MABAs can be formed under a wide range of light intensities (e.g. 200- 2257 μmol m^{-2} s^{-1}) even when high light intensities might hinder the EPS production by microalgae.

Mixing conditions

Mixing is an important parameter for MABAs formation during wastewater treatment. According to Tiron et al. (2015), a relatively high mixing favors the bacterial and microalgal cell aggregation. They formed stable and settleable MABAs at mixing rates of up to 150 rpm in a stirred tank operated as a SBR. Unfortunately, these authors did not provide details of the impeller geometry to estimate the volumetric power input (P/V). Schryver et al. (2008) reported that P/V values in the range of 0.1 to 10 W m_{water}^{-3} are suitable for MABAs formation, which correspond to typical power consumptions in open HRAPs (raceway configuration) that ranges from 2 to 40 W m_{water}^{-3} (Acién-Fernández et al., 2012; Hadiyanto et al., 2013). Excessive stirring may lead to microalgal cells damage. Molina-Grima et al. (2010) observed that P/V values from 100 to 300 W m^{-3} generate a damaging shear stress to microalgal cells.

In this context, there is a need for systematic studies addressing the impact of P/V on the formation and size of MABAs. As further discussed in sections 2.3.1 and 2.3.3; P/V influences the biomass settleability and the CO_2/O_2 mass transfer within the aggregates by

affecting the MABAs diameter. Hence, the effect of P/V on MABAs size and performance constitutes an important research area to be pursued.

Presence of divalent cations

The presence of divalent cations such as Ca^{2+} and Mg^{2+} play an important role in the formation of MABAs by promoting the aggregation of microalgal and bacterial cells (Lee et al., 2013; Powell y Hill, 2014, 2013; Xiao y Zheng, 2016). Ca^{2+} and Mg^{2+} cations mediate multiple cross-linkages among polysaccharide, sugars and protein chains in EPS, which confers a high consistency and stability to the resulting MABAs (Xiao y Zheng, 2016). The mechanisms underlying the formation of microbial aggregates have been under study for many years and it is widely accepted that divalent cations are required for the gelation of EPS, process that confers the structural stability of the cell aggregates (Sutherland, 2001).

Experiments performed by Powell and Hill (2013) showed that approximately 70% of microalgal biomass samples (composed by *Nannochloropsis oceanica*, *Tetraselmis chuii*, *Tetraselmis sucia*, *Phaeodactylum* sp., *Neochloris oleoabundans*, *Nitzschia angularis* and *Cyclotella cryptica*) formed MABAs when Ca^{2+} and Mg^{2+} were present at concentrations of 80 and 190 g m^{-3}, respectively. As a reference, domestic wastewater influent might contain Ca^{2+} and Mg^{+2} at concentrations of approximately 55 and 15 g m^{-3}, respectively (Schönborn et al., 2001). However, the presence of these cations will depend on the local hardness of the drinking water.

The data available in the literature suggests that a limitation of divalent cations for MABAs formation is unlikely to occur in wastewater treatment processes. Attention must also be paid to the presence of chelating agents (e.g. EDTA and EGTA) or surfactants (e.g. SDS). These compounds, commonly present in wastewater, might inhibit the formation of MABAs even in the presence of divalent cations. It was observed that MABAs formation was inhibited by either EDTA or EGTA both at a concentration of 50 mM, while SDS inhibition was recorded at a concentration of 34 mM by Powell and Hill (2014, 2013). These authors recorded inhibition of MABAs formation by SDS, EDTA or EGTA at Ca^{2+} and Mg^{2+} concentrations of up to 10 mM and 4 mM, respectively.

Inorganic/organic carbon concentration

Van Den Hende et al. (2011b) showed that the inorganic/organic carbon concentration ratio also influences the MABAs characteristics and performance. These authors tested C-$KHCO_3$ concentrations of 84, 42 and 0 g m^{-3} combined with C-sucrose concentrations of 0, 42, 84 g m^{-3}, yielding inorganic/organic influent carbon ratios of 84, 1 and 0, respectively. It was observed that the MABAs obtained with a high inorganic/organic carbon ratio settled slowly and exhibited low nitrogen removal efficiencies. On the contrary, MABAs with a good settling performance and high nitrogen removal were obtained at low inorganic/organic carbon ratios. Therefore, the addition of inorganic carbon (e.g. bicarbonate) to wastewater in order to enhance the performance of the HRAP must be carefully done. More research on this subject is still necessary using real wastewater effluents to confirm the role of the inorganic/organic carbon ratio. Information on this matter will be useful for the design, operation and optimization of MABAs-based technologies.

2.2.3 Key advantages of MABAs for wastewater treatment

Among the potential advantages of MABAs for wastewater treatment, three of them are worth highlighting: (i) the efficient biomass harvesting by gravity sedimentation without the addition of flocculants; (ii) the facilitated downstream processing for resource recovery from biomass compared to chemically-enhanced harvesting; (iii) the efficient mass transfer performance within the aggregates. The following lines describe and discuss the above mentioned aspects.

Enhanced harvesting without addition of flocculants

Regarding the biomass harvesting by gravity sedimentation, the settling velocity (V_s) is a key parameter for comparing the performance of different MABAs-based treatments. In addition, V_s is a useful parameter for design and optimization purposes. As a reference, dispersed microalgal cells are characterized by V_s values ranging from 0.001 to 0.026 m h^{-1} (Choi et al., 2006). Although there are few reports on this subject, some authors already determined the V_s values of MABAs. For instance, Gärdes et al. (2011) observed that MABAs mainly constituted by *Thalassiosira weissflogii* and heterotrophic bacteria exhibited an average V_s of 2 m h^{-1}. Likewise, Arcila and Buitrón (2016) reported that MABAs formed during the

treatment of municipal wastewater exhibited V_s values of up to 8.3 m h^{-1}. In this context, Gutiérrez et al. (2016) determined the V_s of microalgal biomass from urban wastewater after the addition of the commercial flocculant "Tanfloc SG". They observed that 70-80% of the total biomass presented V_s values of 6.5 m h^{-1} or higher. Similar V_s values were reported for *Chlorella vulgaris* biomass, settling rates ranging from 1.4 to 21.6 m h^{-1} being recorded when the biomass was flocculated with chitosan, aluminum sulfate, cationic starch or by means of electro-coagulation-flocculation using aluminum anodes (Vandamme et al., 2014). These studies showed that the formation of MABAs enhance the settling performance of the microalgal biomass by several magnitude orders relative to dispersed biomass. Furthermore, MABAs exhibit comparable V_s values than those reported in the literature for microalgal biomass harvested with flocculants, which improves the economics and the sustainability of the treatment process. Several studies on wastewater treatment with MABAs also report either the density (ρ_p) of the aggregates or their settling volume index (SVI), parameter related with the density of the MABAs according to Equation 2.1 (Kirkpatrick-Price, 1991):

$$SVI \cong \frac{100}{\rho_P} \qquad (2.1)$$

Table 2.1. Summarizes the densities of MABAs obtained by several authors. Under optimal operating conditions MABAs exhibit densities of up to 1.77 g mL^{-1}, which is in agreement with the large settling velocities recorded in MABAs-based treatments. Considering the Stoke's law (Eq. 2.2), the size of the MABAs is the parameter that most affect their settling velocity, however, the density differences between the culture broth and the aggregates also impact V_s according to:

$$V_S = \frac{2}{9}\frac{r^2 g(\rho_P - \rho_f)}{\mu} \qquad (2.2)$$

Tabla 2.1. Average density values of settleable MABAs reported in the literature and bioreactor operating

ρ_s (g mL^{-1})	Process	Operating conditions	SVI (mL $gTSS^{-1}$)	Bioreactor configuration	Reference
1.17	Aggregation experiments of *Thalassiosira weissflogii* and heterotrophic bacteria in filtered seawater	Light intensity: 115 μmol m^{-2} s^{-1} Temperature: 18 ºC Light periods: 12 h Light source: not specified.	-	1.15-L rolling tanks	(Gärdes et al., 2011)
~1.00*	Municipal wastewater treatment	Light intensity: 2,000 μmol m^{-2} s^{-1} Light periods: 10 or 12h Light source: metal halide lamps with quartz Temperature: 20-30ºC	80 – 120	30-L continuous reactor with mechanical mixing (40 rpm).	(Gutzeit et al., 2005)
~1.10*	Synthetic wastewater treatment	Light intensity: 2,000 μmol m^{-2} s^{-1} Light periods: 12h Light source: metal halide lamps with quartz Temperature: 20-30ºC	56 – 156	30-L SBR with mechanical mixing (40 rpm)	(Medina and Neis, 2007)
~1.30*	Synthetic wastewater treatment. The ρ_s value corresponds to settleable MABAs obtained by the authors.	Light intensity: 100 μmol m^{-2} s^{-1} Light periods: 15 h Light source: fluorescent lamps Temperature: 23-29ºC	76-990	1.5-L SBR reactors magnetically stirred (500 rpm)	(Van Den Hende et al., 2011b)

~1.77*	Combined secondary sewage and flue gas treatment	Light intensity: 100 μmol m^{-2} s^{-1} Light periods: 24h Light source: fluorescent lamps Temperature: 20-30ºC	43-158	4-L SBR. Mixing provided by flue gas sparging at a flow rate of 0.6-1.2 L h^{-1} and liquid recirculation at 20 L h^{-1}	(Van Den Hende et al., 2011a)
~1.02*	Treatment of aquaculture, food processing, manure and chemical production wastewaters.	Light intensity: 152-172 μmol m^{-2} s^{-1} Light periods: 12h Light source: halogen lamps Temperature: 20ºC	54- 1274	4-L SBR with mechanical mixing, ranges from 150 – 200 rpm.	(Van Den Hende et al., 2014)

*Obtained from the average SVI value using Equation 2.1.

Where, r, g, ρ_f and μ represent the average MABAs radius, the gravitational acceleration, the density and the dynamic viscosity of the culture broth, respectively. A screening of the expected V_s values calculated from Eq. 2.2 is shown in Figure 2.4 considering the densities and sizes of MABAs reported in the literature. A culture broth viscosity of 2 cp was used for the calculations based on experimental measurements available in the literature (Mancuso-Nichols et al., 2009; Michels et al., 2010). It is observed that the biomass density plays an important role in determining the critical MABAs radius at which the V_s is comparable to the reported values in chemical flocculation (values above 5 m h^{-1} approximately). Thus, for biomass with a similar density than that of the culture broth (e.g. ρ_p =1 g mL^{-1} vs ρ_f =0.998 g mL^{-1}), the critical radius for achieving a good settleability is ~900 μm. On the contrary, MABAs with an average density of 1.8 g mL^{-1} require a critical radius of ~50 μm for achieving a good settling performance. However, it must be stressed that although MABAs with density of up to 1.77 g mL^{-1} have been reported in the literature, the aggregates formed during wastewater treatment without external CO_2 addition present typical densities in the range of 1.02 to 1.30 g mL^{-1}. For such densities, MABAs with average radiuses of 80-300 μm are expected to support V_s > 5 m h^{-1}. In this context, Equation 2.2 has assumptions that must be fulfilled to yield realistic V_s values such as the sphericity of the particles, the lack of interaction among particles and the prevalence of a laminar regime (MABAs Reynolds numbers <0.4). Deviations from these assumptions may lead to over- and under-estimated V_s values. Nevertheless, the MABAs settling velocities showed in Fig. 2.4 are in good agreement with the experimental V_s values so far reported in the literature, being therefore useful to figure out the impact of MABAs size and density on their settleability.

Facilitated resource recovery from biomass

Wastewater treatment with biomass forming aggregates might facilitate in large extent the downstream processing for resource recovery compared with chemically-enhanced harvesting. In this regard, Van Den Hende et al. (2014) pointed out that MABAs can be dewatered in a filter press with large pores (e.g. 200 μm). They observed that this simple and inexpensive process allowed for the recovery of 79-99% of the MABAs generated during the treatment of several industrial wastewaters (e.g. aquaculture, manure treatment, food processing and chemical production) in a SBR. The filter press yielded a biomass cake with

12-21% dry matter. Depending on the type of wastewater treated, the resulting biomass cake could be further valorized as a shrimp and poultry feed, fertilizer, pigment/nutraceuticals extraction or CH_4/H_2 production by anaerobic digestion (Vulsteke et al., 2017). On the contrary, coagulant/flocculant compounds might contaminate the biomass and severely hinder its application as feed, fertilizer and the recovery of fine chemicals such as pigments and nutraceuticals. This is the case of aluminum sulfate, polyaluminum chloride, poly γ-glutamic acid and chitosan, which have medium to high contamination risks (Gutiérrez et al., 2016).

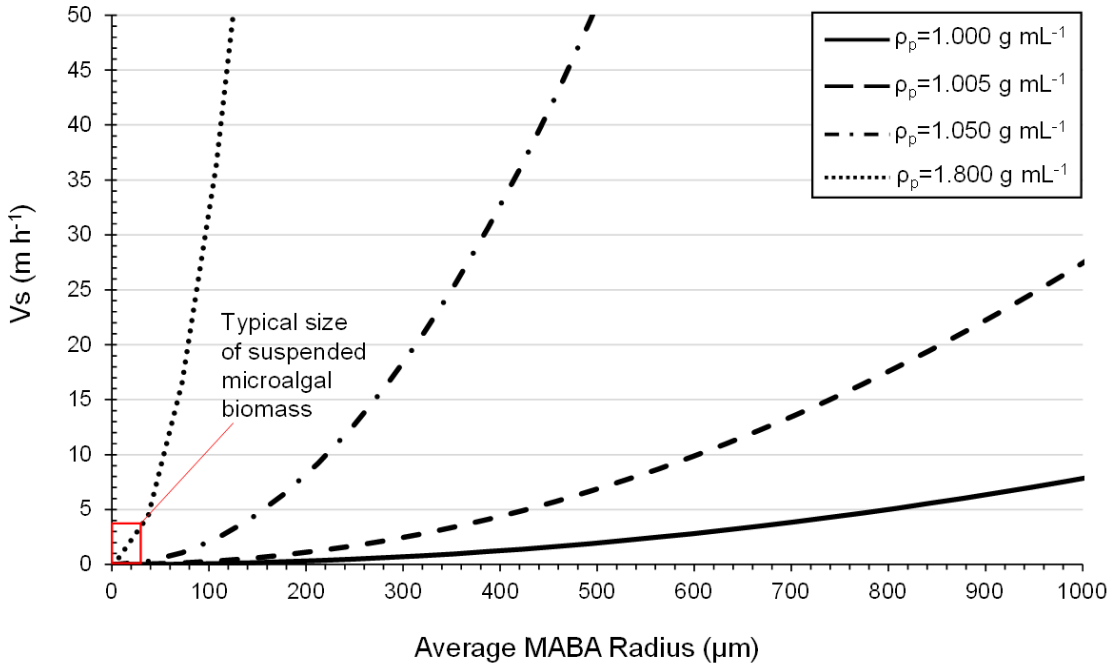


Figura 2.4. MABAs settling velocities calculated from Eq. 2.2 for different aggregate sizes and densities. The following density and viscosity values of the culture broth were used for the calculations: ρ_f = 0.998 g mL^{-1} (water density at 20°C) and µ=2 cp.

Mass transfer performance within MABAs

The dimensionless Henry's law constant of CO_2 is 1.22 at 20°C and 1 atm (Sander, 1999). Considering the CO_2 concentration in the atmosphere of 0.035% (corresponding to 6.4×10^{-4} gCO_2 L_{air}^{-1}), the resulting CO_2 equilibrium concentration in water is around 0.5 mg CO_2 L_{water}^{-1}. Likewise, with a dimensionless Henry's constant of 30 at 20°C (Sander, 1999), the dissolved O_2 concentration in equilibrium with air is approximately 8 mg L^{-1}. Based on the

low dissolved concentrations of CO_2 and O_2, several authors have pointed out that microalgal growth in HRAPs is prone to be limited by mass transfer, mainly when no additional CO_2 supply is provided (Acién-Fernández et al., 2012; Kumar et al., 2010). Nevertheless, the main CO_2 source for microalgal growth in wastewater treatment comes from the oxidation of organic matter. In the case of MABAs, bacterial and microalgal communities grow adhered closely each other. The major component of EPS (the glue for cells adhesion in MABAs) is water, which accounts for approximately 95% of the total mass of EPS (Flemming y Wingender, 2001; Sutherland, 2001). Nuclear magnetic resonance studies demonstrated that the self-diffusion coefficient of water within microbial EPS is only 15% lower than that in free water, non-charged molecules with molecular mass <10,000 Da being able to diffuse practically with no limitation (Flemming and Wingender, 2001). Therefore, the diffusion coefficients for CO_2 and O_2 in EPS are expected to be only slightly lower than those in free water. Equation 2.3 describes the characteristic time for mass transfer (t_D) of a molecule diffusing through an EPS film of a given thickness (δ) (Fig. 2.5):

$$t_D = \frac{\delta^2}{D} \qquad (2.3)$$

Where, D is the diffusion coefficient of the molecule in the EPS. As above mentioned, D for CO_2 and O_2 in the EPS is expected to be similar to the diffusivity value in free water. However, it is important to assess the impact of reduced diffusion on the characteristic time for the transport of these gas substrates within MABAs. Equation 3 was used to estimate the t_D of CO_2 and O_2 for different diffusivity values and EPS film thicknesses between microalgal and bacterial cells (Fig. 2.6). Diffusivities 15, 30, 50 and 70% lower than that in free water were considered as well as the diffusivity coefficients in free water as reference values. It is observed that for EPS film thicknesses below 10 µm, the mass transfer of both CO_2 and O_2 is practically instantaneous as t_D values < 0.5 s were obtained regardless the diffusivity value tested. Based on the experimental observation of MABAs sizes and their spatial configuration (see Fig. 2.2), it is reasonable to consider EPS film thicknesses ranging between 0.1 and 5 µm in MABAs. However, in extreme (and unlikely) cases were microbial and bacterial cells are separated by distances of 50 µm, the t_D for CO_2 mass transfer ranges from ~1.50 to ~4.50

seconds, while the t_D for O_2 mass transfer ranges from ~1.25 to ~3.50 s. Such characteristic times suggest that CO_2 or O_2 mass transport limitations within MABAs are unlikely to occur.

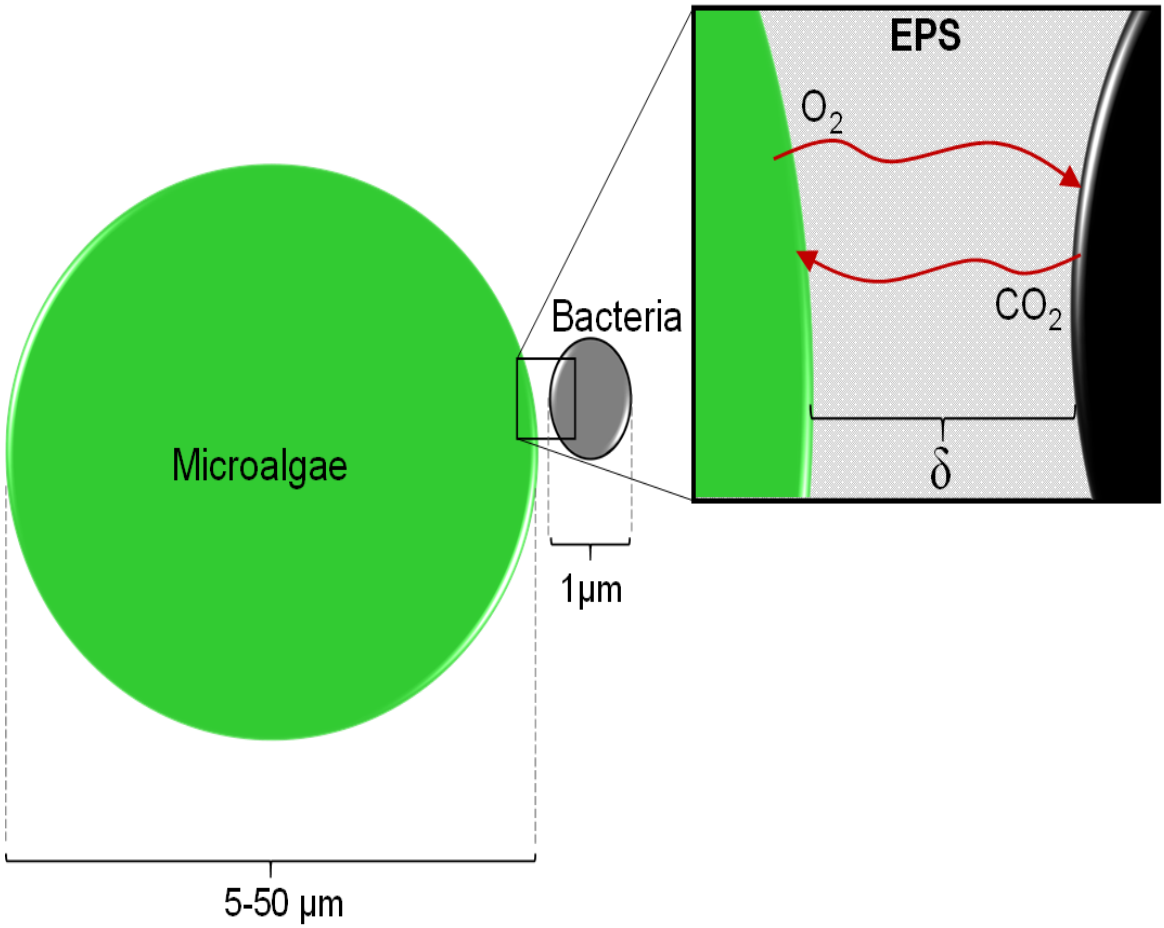


Figura 2.5.-schematic representation of CO_2 and O_2 mass transfer in the EPS film of thickness δ between microalgal and bacterial cells in MABAs

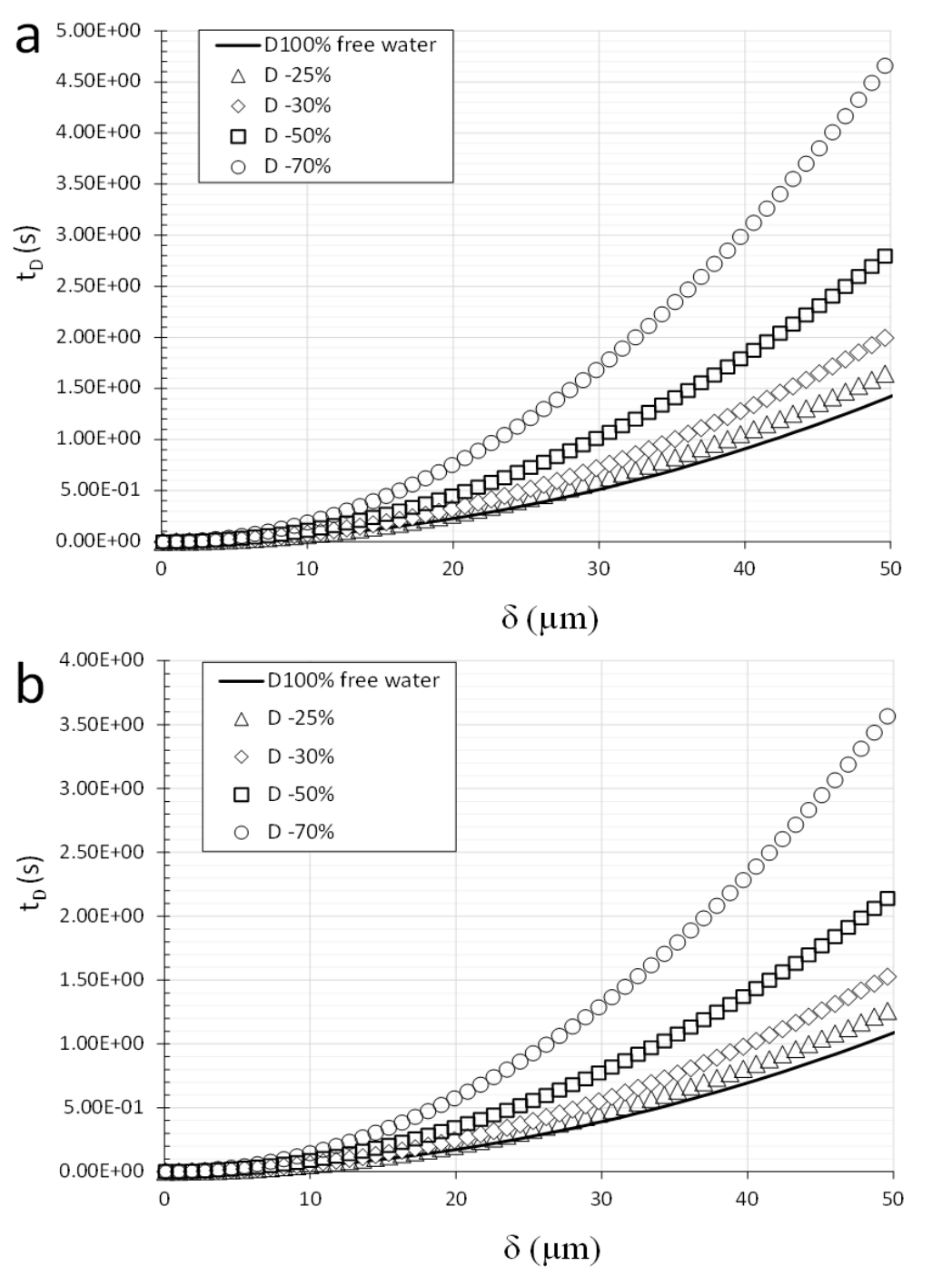


Figura 2.6. Characteristic time of mass transfer within MABAs at varying EPS thicknesses for (a) CO_2 and (b) O_2. Diffusivity values in free water at 20°C were considered: D_{CO2}= 1.76×10^{-5} cm^2 s^{-1} and D_{O2}= 2.30 ×10^{-5} cm^2 s^{-1} (Tamimi et al., 1994; Wise and Houghton, 1966). D-25%, D-30%, D-50% and D-70% refers to diffusivity values 25, 30, 50 and 70% lower than that in free water (D100%).

2.3 Microbial populations in MABAs

To understand the population dynamics triggering the formation of MABAs during wastewater treatment, knowledge on the microbial communities present in MABAs becomes of paramount relevance. In this regard, available studies characterizing the microbial communities in MABAs have been traditionally focused on understanding the algal blooms and how the "marine snow" (MABAs formed in upper waters that fall to the deep ocean) contributes to the formation of sediments and their role in carbon and nitrogen cycles (Schwenk et al., 2014). However, insights on MABAs composition can be obtained from these studies despite they are not related to wastewater treatment. For instance, the characterization of MABAs from the North Sea by means of DGGE 16rDNA analysis showed that bacterial communities are not strictly species-specific for microalgae (Sapp et al., 2007). Thus, the same bacteria can be attached to different microalgal strains and vice versa. These authors identified the phyla of bacteria able to attach the following microalgae: *Guinardia delicatula*, *Pseudonitzschia pungens*, *Thalassiosira rotula*, *Skeletonema costatum*, *Ceratium horridum* and *Akashiwo sanguinea*. They found that bacteria belonging to the phyla *Firmicutes*, *Bacteroidetes* and *Proteobacteria* were predominant, being able to attach to most microalgae tested.

The characterization of MABAs from municipal wastewater treatment by means of DGGE 16rDNA analysis revealed that bacteria belonging to the class *Bacteroidia* and filamentous blue-green algae were predominant, although *Flavobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* were also present (Su et al., 2011). Likewise, MABAs formed with a *Chlorella vulgaris* strain obtained from swine wastewater characterized by means of DGGE 16rRNA analysis showed the predominant presence of *Sphingobacteria*, *Flavobacteria*, *Terrimonas* and *Hyphomonas* (Lee et al., 2013). It is worth noting that most bacterial cells identified in MABAs from wastewater treatment are related to the phyla *Bacteroidetes* and *Proteobacteria*, which were also identified as predominant bacteria in MABAs from seawater. Furthermore, these predominant bacteria were identified from different gene libraries based either on 16S rDNA or 16SrRNA fragments, which confirms the key role of these bacteria on MABAs formation. Finally, it must be remarked that there is a lack of

studies focused on the microbial characterization of MABAs formed during wastewater treatment, which indicates that this is an important niche opportunity for future research.

2.4 Methanogenic potential of MABAs

The anaerobic digestion process has been considered as one of the most favorable technologies for microalgal biomass valorization through CH_4 production (Arcila y Buitrón, 2016; Passos et al., 2015; Wieczorek et al., 2015). Studies on the digestion of MABAs showed that the operational conditions prevailing during the wastewater treatment impact on the resulting biomethane potential (BMP, mL of CH_4 produced per gram of volatile solid consumed). Two specific aspects have been pointed out to impact the BMP of MABAs, leading to significant differences in the CH_4 production (Table 2.2). According to Van den Hende et al. (2015), the seasonal variability affects the composition of the microbial communities within MABAs, resulting in significantly different BMP values during the year. On the other hand, Arcila and Buitrón (2016) observed that the BMP values of MABAs increased with the HRT. They observed an efficient wastewater treatment at HRT of 6 and 10 days and the BMP of the resulting biomass at HRT=10 days was 20% higher than that recorded at HRT=6 days. Overall, the above mentioned studies concluded that the following factors affected negatively the BMP values: (i) the predominance of microalgae with low biodegradability such as *Ulotrhix* sp., *Klebsormidium* sp., *Nitzschia* sp. and *Navicula* sp., and (ii) the low C/N ratio in the biomass due to the reduction of carbohydrates and increase of proteins in MABAs. The most common pretreatment methods applied to the harvested microalgal biomass in order to increase the CH_4 yield include: ultrasound treatment, french pressing, microwave treatment, thermal hydrolysis and enzymatic treatments (Alzate et al., 2012; Passos et al., 2015, 2013; Wieczorek et al., 2015).

Tabla 2.2 BMP values reported for microalgal biomass and the impact of pretreatments.

Main microalgal species (Sources, location)	**Type of biomass growth**	**Operating conditions during microalgae cultivation**	**BPM without pretreatment (mL CH_4 g SV^{-1})**	**Pretreatment**	**BPM after pretreatment (mL CH_4 g SV^{-1})**	**Reference**
Acutudesmus obliquus, *Oocystis* (Anaerobic digestion effluent, biomass was harvested from the bottom of settler, Valladolid, Spain)	Dispersed cells	180 L open photobioreactor. Indoor operation under continuous mode at HRT of 36 days and artificially illuminated.	198 ± 2	Thermal treatment: 110 -170ºC for 15 min Ultrasound: applied power of 400W, corresponding to specific energies of 10-57 MJ per kg of total solids	262 ± 44 217 ± 7	(Alzate et al., 2012)
Microspora (Anaerobic digestion effluent, biomass was harvested from the surface of HRAP, Valladolid, Spain)	Dispersed cells		255 ± 2	Thermal treatment: 110 -170ºC for 15 min Ultrasound: applied power of 400W, corresponding to specific energies of 10 to 57 MJ per kg of total solids	384 ± 27 305 ± 7	(Alzate et al., 2012)
Scenedesmus obliquus *Chlorella vulgaris* *Chlamydomonas reindharditii* (Swine manure, Alicante, Spain)	Dispersed cells	1 L closed photobioreactor. Indoor operation in semi-continuous mode at HRT of 8 days at 23°C and 14 h of artificially illuminated (77 µmol m^{-2} s^{-1})	307 ± 10	None	-	(Molinuevo-Salces et al., 2016)
Scenedesmus sp., *Coelastrum* sp. (Municipal wastewater, Tampere, Finland)	Dispersed cells	0.5 L closed photobioreactor. Indoor operation in batch mode for 8 days, artificially	222 ± 10	Thermal treatment: 80º C for 3h	259 ± 13	(Kinnunen and Rintala, 2016)

Scenedesmus sp., **(**Digestate from anaerobic digester fed with biosludge from pulp and paper industry, Tampere, Finland**)**	Dispersed cells	illuminated (90- 200 μmol m^{-2} s^{-1})	154 ± 5	Thermal treatment: 80º C for 3h	173 ± 10	(Kinnunen and Rintala, 2016)
Stigeoclonium sp. , *Monoraphidum* sp. *Nitzschia* sp., *Navicual* sp. (Muncipal wastewater, Barcelona, Spain)	MABAs	470 L HRAP. Outdoor operation in continuous mode at HRT of 8 days under solar irradiance conditions.	105 ± 2	Thermal treatment: 95°C for 10h	181 ± 5	(Passos et al., 2015)
				Hydrothermal treatment: 130ºC for 15 min	127 ± 5	
				Microwave treatment: applied power of 900W, corresponding to 34 MJ per kg of total solids	135 ± 2	
				Ultrasound: Applied power of 70W for 30min, corresponding to 27 MJ per kg of total solids	113 ± 2	
Stigeoclonium sp. , *Monoraphidum* sp. *Nitzschia* sp., *Navicual* sp. (Muncipal wastewater, Barcelona, Spain)	MABAs	470 L HRAP. Outdoor operation in continuous mode at HRT of 8 days under solar irradiance conditions.	188 ± 3	Enzymatic: Addition of cellulase 1% (w/w), 6h	203 ± 1	(Passos et al., 2016)
				Enzymatic: Addition of 1% (w/w) of enzyme mix (cellulose, glucohydrolase and xylanase), 6h at 37 ºC and continuous mixing.	217 ± 7	(Passos et al., 2016)
Oocystis sp, Diatoms (Urban wastewater, Barcelona, spain)	MABAs	470 L HRAP. Outdoor operation in continuous mode at HRT of 8 days under solar irradiance conditions.	83 ± 1	Enzymatic: Addition of laccase from *T. versicolor* (100 UL^{-1}) for 20 min at 25°C and agitation of 100 rpm	144 ± 2	(Hom-Diaz et al., 2016)

Ulothrix sp., *Klebsormidium* sp. (Aquaculture wastewater, Belgium)	MABAs	12,000 L HRAP. Outdoor operation in sequencing batch mode at HRTs of 4-8 days and 10-20°C under solar irradiance conditions.	178 ± 13	Microwave treatment: applied power of 700W for 14 min, corresponding to 2.35 MJ per kg of total solids	193 ± 9	(Van Den Hende et al., 2015)
Chlorella sp., *Acutodesmus* sp., *Chlamydomonas* sp. (Wastewater from paper industry, Munich, Germany)	MABAs	1,200 L open photobioreactor. Outdoor operation in continuous mode at HRT of 8.4 days, 10 to 30°C under solar irradiance conditions.	219 ± 4	Enzymatic: Addition of Onozuka R-10, Macerozyme R-10 commercial enzymes (both enzymes at a 1:1 $^{w}/_{w}$ ratio) at final concentrations of 25-200 mg L^{-1}.	271 ± 4	(Wieczorek et al., 2015)
				Frech press: 500- 2000 psi for biomass concentrations from 4 to 12 gVS L^{-1}.	250 ± 3	
				Ultrasound: 50-500 J mL^{-1} for biomass concentrations from 4 to 12 gVS L^{-1}.	249 ± 4	
Stigeoclonium sp. (Municipal wastewater, Querétaro, Mexico)	MABAs	50 L HRAP. Indoor operation in continuous mode at HRT of 10 days, 20-25°C, 12 h of artificial illumination (200 μmol m^{-2} s^{-1})	347 ± 3	None	-	(Arcila and Buitrón, 2016)

Thermal pretreatments may increase up to 60% the BMP values of dispersed microalgal biomass (Alzate et al., 2012). This pretreatment was also effective for MABAs with BMP increases of up to 72% (Passos et al., 2015). Enzymatic pretreatments have been also reported to boost the BMP values of MABAs by over 20% (Passos et al., 2015; Wieczorek et al., 2015). The potential of enzymatic pretreatments were also corroborated by other authors, who observed that enzymes produced by *Trametes versicolor* and *Raoultella ornithinolytica* MA5 increased 75% and 158% the BMP of MABAs and dispersed microalgal biomass, respectively (Hom-Diaz et al., 2016; Muñoz et al., 2014). Recently, the use of ozone was evaluated to disrupt the microalgae cell wall (Cardeña et al., 2017). An increase on the BMP production from 253 up to 433 mL CH4 g VS^{-1} was observed after ozonation (382 mg O_3 g VS^{-1}). These results confirmed that several pretreatments could effectively be used to improve the resource recovery from MABAs. Nevertheless, further studies on the costs associated with these pretreatments must be done to determine if such costs are covered by the additional CH_4 obtained.

2.5 Conclusions

In this review, the potential advantages of MABAs-based treatments over technologies using dispersed microalgal-bacterial biomass have been highlighted and critically discussed. Nevertheless, research is still required to fully understand the fundamentals underlying the formation of MABAs and their wastewater treatment performance. Specifically, we identified the following research niches

- Evaluation of MABAs formation and their characteristics (e.g. settleability) under outdoor conditions for each season.
- Long term evaluation of MABAs formation and their performance at several solid retention times in continuous and SBR operation.
- Evaluation of MABAs formation in full-scale HRAPs, which are characterized by a plug flow. These studies will be useful to determine if the flow regime constitutes a hydrodynamic barrier for MABAs formation.

- Systematic studies on the effect of microbial population dynamics (microalgae, bacteria and protozoa) and EPS composition on MABAs formation under outdoor conditions.
- Determination of the BMP of MABAs generated in different wastewater sources (e.g. wastewater from several industries) and its impact on the economic feasibility of the treatment.

Acknowledgments

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3

Microalgae – bacteria aggregates: effect of the hydraulic retention time on the municipal wastewater treatment, biomass settleability and methane potential

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Abstract

BACKGROUND: The use of microalgae – bacteria systems is particularly attractive for wastewater treatment, and the generated biomass can be further used for methane production. The aim of this study was to evaluate the influence of the hydraulic retention time (HRT) on the organic matter, nutrient removal, settling properties and the biochemical methane potential using a granular microalgae – bacteria system in a high rate algal pond.

RESULTS: The primary microorganisms present in the system were constituted by diatoms, green filamentous microalgae and bacteria. At 2 d of HRT the system showed the lowest performance while high chemical oxygen demand (COD) (>92%), ammonium (>85%) and phosphorus (up to 30%) removal was observed at 6 and 10 d of HRT. High settling velocities (up to 8 m h^{-1}) were observed due to agglomerates and granules as dominant structures. The highest methane yield and production rates (348 mL CH_4 g^{-1} VS and 56 mL CH_4 g^{-1} VS d^{-1}) were observed with the biomass obtained at 10 d of HRT.

CONCLUSION: Flocs and granules were the dominant structures in the system. High settling velocities and low effluent total suspended solids concentrations were obtained at higher HRT. An inverse relationship between HRT and the biochemical methane potential was observed.

Keywords: microalgae; wastewater; high rate algal pond; settling velocity; biogas; granules

3.1 Introduction

The need for a sustainable wastewater treatment process makes it necessary to develop technologies to remove organic matter and nutrients and to generate biomass that can be valorized in biofuel production. Aerobic wastewater treatment processes, such as activated sludge, are high cost systems because of the high energy inputs associated with the O_2 supply, the large amount of sludge that must be disposed of and the environmental impact resulting from the emission of greenhouse gases such as CO_2. Currently, the use of microalgae – bacteria systems for wastewater treatment is particularly attractive because of their ability to produce inexpensive O_2, to remove nutrients, pathogens, and heavy metals, and to fix CO_2 during a photoautotrophic process, which represents a considerable gain in the carbon available for CH_4 production compared with classic aerobic processes (Abdel-Rauof et al., 2012; Avagyan et al., 2013; Muñoz and Guieysse, 2006). Evaluation of the efficiency of microalgae – bacteria systems using wastewater in municipal facilities (Kim et al., 2014; Woertz et al., 2010), piggery effluent (de Godos et al., 2009; Gonzales-Fernandez et al., 2011), agro-industrial (Hernández et al., 2012) and industrial wastewater (Avagyan, 2011), have revealed good results in the removal of chemical oxygen demand (COD) and nutrients (phosphorous and nitrogen) and using high rate algal ponds (HRAP).

Recently, studies of microalgae – bacteria systems for the treatment of domestic wastewater have received great attention due to their particular properties, such as high settling and lipid accumulations of the biomass. Sequencing batch reactors (SBR) have been mostly used to conduct such studies (Medina et al., 2007). Strategies used with SBR, such as short settling and discharge times, have provided a selective process to form microalgae – bacteria bioflocs (Valigore et al., 2012). Su et al. (2011) showed how filamentous cyanobacteria used to treat municipal wastewater in a SBR had good settleability properties, reaching a total suspended solid (TSS) concentration of 16 mg L^{-1} within a 20 min sedimentation. On the other hand, Gutzeit et al. (2005), observed the formation of an agglomerate of microalgae and bacteria when the system was inoculated with *Chlorella vulgaris* and activated sludge from a municipal wastewater treatment plant (WWTP), reaching a TSS of 18 mg TSS L^{-1} in the supernatant. According to Van den Hende et al. (2014a, 2014b, 2015), aggregates of microalgae – bacteria (MAB-Flocs) formed in SBR are suitable candidates for growth using

industrial wastewater because of the relative easy of harvesting. However, there is scarce information on the growth and behavior of microalgae – bacteria aggregates in continuous systems.

In addition to their efficient performance in removing pollutants from wastewater and improvement of their settleability abilities, it is important to biochemically analyze the microalgae – bacterial biomass in order to determine whether the system is producing high value-added products. Kim et al. (2014) evaluated the lipid content of a microalgae – bacteria agglomerate formed mainly from green microalgae of the *Stigeoclonium* genus. They tested different hydraulic retention times (HRTs) and found a maximum lipid content at an HRT of 8 d. However, the real energetic content of this agglomerate structure of microalgae and bacteria and their application in productive processes such as anaerobic digestion remains unknown. Consistent with the results of Sialve et al. (2009), the integration of wastewater treatment coupled with anaerobic digestion is a promising method for the production of bioenergy. Evaluation of the energetic potential of the microalgae – bacteria biomass produced when treating domestic wastewater is necessary to identify the sustainability of the process, mainly because it has been observed that biochemical methane potential depends on the type of microalgae and its composition (carbohydrate, proteins and lipids) (Van Den Hende et al., 2015; Mendez et al., 2015) as well as the biomass pretreatment (Wieczorek et al., 2015).

Several control parameters in a HRAP may influence the selection of microalgal biomass with high settling properties. One of these parameters is the hydraulic retention time that will affect the pre- dominance of microalgae in the microalgae – bacteria aggregates grown in wastewater. The hydraulic retention time will also affect the organic matter and nutrients removal as well the digestibility of the biomass for biogas production. The aim of this study was to evaluate the influence of the HRT on the organic matter, nutrient removal, settling properties and the biochemical methane potential using a granular microalgae – bacteria system, also known as MABAs in a continuous HRAP.

3.2 Materials and Methods

3.2.1 Experimental design

The experimental set-up consisted of a continuous HRAP constructed in fiberglass with a total capacity of 80 L, a working volume of 50 L and a surface area of 0.26 m^2. The water level in the reactor was 15 cm. The HRAP system was operated in continuous mode from August 2014 to December 2014 using LED lamps as the light source under the following laboratory conditions: light intensity of 200 μmol m^{-2} s^{-1} measured on the water surface with light – dark photoperiods of 12 h (light):12 h (dark). The components were mixed by a six-blade paddle wheel driven by a motor engine at 10 rpm (Cole Parmer, USA), which generates a liquid velocity of 0.2 m s^{-1}. The effluent was settled using a 7 L settler. There was no external diffusion of carbon dioxide through mechanical systems during all experimental phases. The HRAP system was initially fed with a secondary effluent and inoculated with 5 L of a mixture of microalgae and activated sludge in a 1:1 (w/w) ratio. The HRAP was operated for a period of 10 d in a batch mode to acclimate the microalgae – bacteria biomass. Afterwards, the reactor was continuously fed with a primary effluent of municipal wastewater (Table 3.1). Three different HRTs (10, 6 and 2 d) were studied (at least during three HRT times). The 2 days condition was followed until the system collapsed (6 days of operation). Biomass was not recycled; then, the solids retention time was equal to the HRT for each case. 200 mL samples of HRAP influent and effluent were collected twice a week for analysis. The temperature, dissolved oxygen (DO), pH and oxidation-reduction potential (ORP) were periodically monitored on line Samples for microbiological characterization and chemical profiling were taken at the end of each HRT. The biomass used to analyze the chemical profile was dried in an oven at 60 ∘C for 2 d.

3.2.2 Microorganisms

The HRAP was inoculated with a mixture of 833 mg volatile suspended solids (VSS) L^{-1} microalgal biomass and 2740 mg VSS L^{-1} of activated sludge obtained from the aeration tank of a municipal wastewater treatment plant. The microalgal biomass was composed of a mixed microalgae collected from an aquatic environment in Queretaro State, Mexico, and

propagated in Bold medium (Cea-Barcia et al., 2014),*Scenedesmus* sp. was microscopically identified as the dominant genera.

Tabla 3.1. Municipal wastewater characterization

Parameter	**Mean ± SD**
CODs (mg/L)	593 ± 58
VSS (mg/L)	149 ± 26
Ph	7.4 **± 0.3**
N-NH_4^+ (mg/L)	71.2 ± 4.5
N-NO_3^- (mg/L)	2.2 ± 0.5
N-NO_2^- (mg/L)	2 ± 0.4
P-PO_4^{3-} (mg/L)	15.3 ± 1.3

3.2.3 Wastewater source

The municipal wastewater was collected from a wastewater treatment plant located in Santa Rosa Jauregui, Queretaro, Mexico. The sampling point was chosen after primary treatment (coarse and fine screening and primary sedimentation). The wastewater was passed through a sieve (Tyler No 65) and stored in a cold room at 4 ∘C for seven days maximum. The average influent of municipal wastewater quality is shown in Table 3.1.

3.2.4 Biochemical methane potential tests

The biochemical methane potential (BMP) tests were conducted in serum bottles of 160 mL filled with 80 mL of a mixture of anaerobic granular inoculum and a microalgae bacterial consortium. All the tests were performed under a volatile solid (VS) substrate/inoculum (VSalgae:VSinoculum) ratio of 0.5, with a microalgae concentration of 3 g VS kg^{-1} (Alzate et al., 2012). The bottles were closed with butyl septa, sealed with aluminum caps, purged with nitrogen for 1 min and incubated at 35 ∘C. Control tests were carried out in order to determine the CH_4 production potential of the anaerobic inoculum. The methane production of each biomass at a particular HRT was determined. The tests were run in triplicate. The

biogas generated was measured by saline water (pH 2) displacement. The results were expressed at a standard temperature and pressure (STP) of 0 ∘C and 1 atm, respectively. Carbon dioxide and methane were analyzed with a gas chromatograph (SRI 8610C) equipped with a thermal conductivity detector and two packed columns (6 ft × 1/8 in, silica gel packed column and 6 ft × 1/8 in, molecular sieve 13 × packed column). The injector and detector temperatures were 90 ∘C and 150 ∘C, respectively. The initial column temperature was 40 ∘C, which was held for 4 min and then gradually increased to 110 ∘C at a rate of 20 ∘C min^{-1}. The final column temperature was held for 3 min. Nitrogen was used as a carrier gas at a flow rate of 20 mL min^{-1}. Finally, the microalgae methane production was obtained through subtraction of the CH_4 total production and the methane produce by the inoculum (blank tests). The productivity was calculated as mL CH_4 g^{-1}VS algae.

3.2.5 Analytical procedures

The parameters of total suspended solid (TSS), volatile suspended solids (VSS) and volatile solids (VS) concentration and the settling velocity (S_v) of the microalgae – bacteria system was analyzed according to standard APHA methods. The settleability percentage was calculated conforming to Equation (1). All samples were filtered through 1.6 μm pore size membranes (Whatman glass microfiber filters, Grade GF / A). Ammonium ($N\text{-}NH^{4+}$) and chemical oxygen demand (COD) were determined through the 8000 Hach and 10031 Hach colorimetric methods. Nitrate ($N\text{-}NO^{3-}$) and nitrite ($N\text{-}NO^{2-}$), were analyzed following the 10237 and 10206 (diazotization method and dimethylphenol method) Hach colorimetric methods. For phosphate ($P\text{-}PO_4^{3-}$), the 10127 (molybdovanadate with acid persulfate digestion method) Hach method was used. The OD, pH and ORP were measured on line (Oxymax COS61, Sensorex pH 5450C and Atlas scientific, respectively).

For the identification of microalgae, a Leica DM500 microscope with an image acquisition system (Leica ICC50 HD) and a stereoscopic lens (Zeiss Stemi DV4) was used. Proteins were measured in the dry matter using bovine serum albumin as the standard (Lowry et al., 1951) Carbohydrates were determined using a phenol – acid method using D-glucose as the standard (Dubois et al.,1951). Before protein and carbohydrate assay, the samples were treated ultrasonically, as follows. 20 mg aliquots of dried biomass (at 60 ∘C for 2 d) were suspended for 20 min in 10 mL of lysis buffer in a Falcon tube. Then, the ultrasound

pretreatment (high intensity ultrasonic processor, 500 W model) was applied at an amplitude of 30% for 10 min and On/Off pulses of 30/10 to avoid overheating the samples. The lipid content was calculated by the method of Bligh and Dyer (Bligh y Dyer, 1959).

$$\text{Settleability percentage (\%)} = 100\left(1 - \frac{X_e}{X}\right) \quad (3.1)$$

Where X_e is the TSS in the effluent and X is the TSS in the HRAP.

3.2.6 Statistical analysis

Analysis of variance (ANOVA) for a single factor was used to analyze the effect of the HRT on the removal efficiency (% COD, % $N\text{-}NH_4^+$, % $P\text{-}PO_4^{3-}$) and biochemical profile (carbohydrate, proteins and lipids) from the microalgae – bacteria biomass. To identify the best operating condition for the HRT, the examination of differences between pairs of groups after the global analysis was conducted with a post hoc analysis and using the Fisher's least significant difference (LSD). All the tests were made under a significance level (α= 0.05) and a minimum number of replicates (n = 3) for each analyzed variable.

3.3 Results and Discussion

3.3.1 Organic load and nutrient removal

During the experimental process, the three HRT were measured in evaluation periods defined as: HRT 10 d, HRT 6 d and HRT 2 d. For the HRT 10 d period, the highest average values were (for both light and dark photoperiod, respectively) DO (10.2 ± 2.7 to 3 ± 0.5 mg O_2 L^{-1}), pH (8.2 ± 0.3 to 7.4 ± 0.2) and ORP (284 ± 14 to 175 ± 8 mV). This period showed good aerobic conditions (Table 3.2). At HRT of 10 d, the average concentration values in the effluent of the reactor for COD and $N\text{-}NH_4^+$ were 65 ± 39 mg L^{-1} and 0.5 ± 0.2 mg L^{-1}, respectively. That corresponds to COD and $N\text{-}NH_4^+$ removal efficiencies of 91 ± 4% and 99 ± 1%, respectively (Table 3.3, Fig. 3.1(a) and (b)). For phosphorous ($P\text{-}PO_4^{3-}$ efficiencies were as much as 49 ± 11% (Table 3.3, Fig. 3.1(c)).

Tabla 3.2. Average of control parameters in the HRAP reactor during the light and dark photoperiod under the three HRTs evaluated

Variables	**HRT 10d**		**HRT 6 d**		**HRT 2 d**	
	Dark	**Light**	**Dark**	**Light**	**Dark**	**Light**
Temperature (ºC)	17 ± 1	23 ± 2	14 ± 3	22 ± 2	14 ± 2	21 ± 3
pH	7.4 ± 0.2	8.2 ± 0.3	7.4 ± 0.3	8 ± 0.5	7.3 ± 0.1	7.5 ± 0.1
DO (mg O_2 L^{-1})	3.0 ± 0.5	10.2 ± 2.7	1.1 ± 0.6	3.2 ± 0.3	0.0	0.0
ORP (mV)	175 ± 8	284 ± 14	164 ± 13	198 ± 23	-216 ± 100	-198 ± 98

The concentrations of $N\text{-}NO_3^-$ and $N\text{-}NO_2^-$ were monitored in order to evaluate the photosynthetic oxygenation capacity to support nitrifying bacteria. $N\text{-}NO_3^-$ was detected in the effluent at an average concentration of 32.4 ± 4.1 mg L^{-1}, and $N\text{-}NO_2^-$ of 2.08 ± 1.5 mg L^{-1}, corresponding to a nitrification percentage of 48.8 ± 18% based on the concentration of total inorganic nitrogen measured in the municipal domestic wastewater (Table 3.1).

During the HRT 6 d period, the average values of DO were 3.2 ± 0.3 to 1.1 ± 0.6 mg O_2 L^{-1} under light and dark cycles, respectively. This value clearly shows that the oxygen levels in the light period are above the minimum required for the proper development of an aerobic biomass (>2 mg O_2 L^{-1}), including heterotrophic microorganisms to oxidize the organic matter and autotrophic bacteria to carry on the nitrification processes (Metcalf and Eddy, 2003). However, the removal efficiencies for COD (92 ± 1%) and $N\text{-}NH_4^+$ (96 ± 3%) remained relatively high compared with the first period (HRT 10 d). The removal efficiency for $P\text{-}PO_4^{3-}$ (29 ± 4%) was lower than the value reached in the HRT 10 d period. The nitrification processes decreased as a consequence of oxygen limitation; the concentration in the effluent was 10.0 ± 1.0 mg $N\text{-}NO_3^-$ L^{-1} and 6.8 ± 4.9 mg $N\text{-}NO_2^-$ L^{-1} with a nitrification percentage of 11 ± 3%.

That behavior was related to the increase in the organic load rate (OLR) from HRT 10 d (54 ± 19 mg COD L^{-1} d^{-1}) to HRT 6 d (119 ± 19 mg COD L^{-1} d^{-1}), leading to incomplete nitrification or no nitrification. This behavior is related to the competition between autotrophic (nitrifying) and heterotrophic bacteria for the limited DO. Because heterotrophic

bacteria have a maximum growth rate of five times and yields two to three times higher than autotrophic nitrifying bacteria, the organic matter removal occurs before nitrification under limited conditions (Ward et al., 2011). The COD and N-NH_4^+ removal observed for 6 and 10 d of HRT is consistent with the data reported in other studies (Park et al., 2011; Posadas et al., 2014; Kim et al., 2014). These authors observed high removal values of COD (>80%) and N-NH_4^+ (>90%) using different types of wastewater. The P-PO_4^{3-} removal observed in our study was lower than the removals reported because of the low N /P ratio (4.5) present in the wastewater used. Wang et al. (2010) found that the optimal ratio for maximum nitrogen and phosphorus uptake by microalgae bacteria biomass is between 6 and 10.

On the other hand, a decrease in the MABAs biomass concentration from 654 ± 104 to 409 ± 89 mg VSS L^{-1} was observed when the HRT passed from 10 to 6 d. Nevertheless, no differences in the biomass productivity were observed between these two HRTs, reaching an average biomass productivity of 12.7 ± 2.3 g m^{-2} d^{-1}. This value agrees with the productivity reported for microalgae growth (5–27 g m^{-2} d^{-1}) in outdoor HRAP using wastewater (de Godos et al., 2009). In the last experimental period (HRT 2 d), the increase in the OLR from 119 ± 20 mg COD L^{-1} d^{-1} to 361 ± 29 mg COD L^{-1} d^{-1} rapidly slowed the removal of COD and N-NH_4^+, producing removal efficiencies of 12 ± 8% and 15 ± 1%, respectively.

Tabla 3.3. Average concentration in the effluent and removal percentage (%) of COD, N-NH_4^+ and P-PO_4^{3-} in the HRAP reactor.

	COD mg L^{-1}, (%)	**N-NH_4^+mg L^{-1}, (%)**	**P-PO_4^3 mg L^{-1}, (%)**
HRT 10d	65.0 ± 39(91 ± 4)	0.5 ± 0.2 (99 ± 1)	6.8 ± 1.6 (49 ± 11)
HRT 6d	53.6 ± 13 (92 ± 1)	2.6 ± 1.2 (96 ± 3)	12.3 ± 1.3 (29 ± 4)
HRT 2d	562.0 ± 119 (12 ± 8)	50.0 ± 7 (15 ± 1)	14.1 ± 0.5 (9 ± 7)

This result was associated with the absence of dissolved oxygen and negative ORP (< −216 ± 100 mV), which are evidence of anaerobic processes. Reports of HRAP operated under low HRT are found in the literature. For example, an HRT of 2 d was used to treat municipal wastewater, obtaining high COD (85%) and nitrogen (92%) removals (Kim et al., 2014). However, that study used a significantly lower OLR (60 mg COD L^{-1} d^{-1}) than our study (6 times lower). Posadas et al. (2014), studied fish farm and domestic wastewaters, and obtained

the maximum COD and nitrogen removal with an OLR of 75 mg COD L^{-1} d^{-1} (10 d HRT). Thus, it seems that the OLR is a more important design parameter than the HRT.

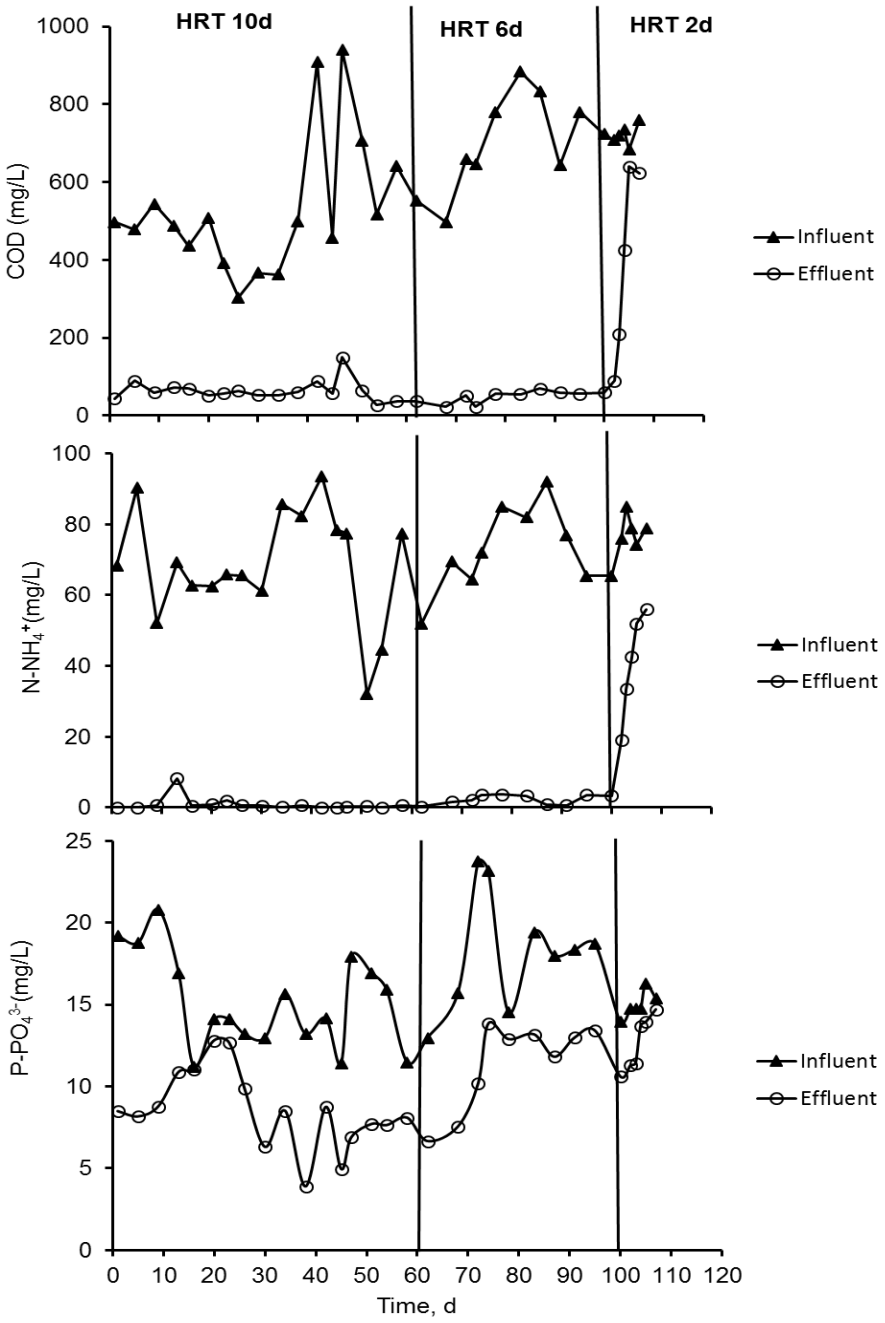

Figura 3.1. Time course of the influent and effluent (a) COD, (b) $N\text{-}NH_4^+$, (c) $P\text{-}PO_4^{3-}$ concentration in the HRAP reactor during the three HRT periods (HRT 10 d, HRT 6 d and HRT 2 d).

When the total inorganic nitrogen removal was evaluated (Fig. 3.2(a)), the highest removal percentage was reached when the system was operated under HRT 6 d, achieving values as high as 80%. Theoretically, nitrogen as ammonium can be removed from wastewater in three different ways: nitrification (carried out by nitrifying autotrophic bacteria), assimilation (conversion of ammonium to organic nitrogen) and stripping (when the pH is higher than 9). Figure 3.2(b) shows a decrease in the nitrifying processes coupled with an increase in the total inorganic nitrogen removal. This behavior was noted by Park and Craggs (2011a) where a decrease in the HRT causes a drop in the concentration of the biomass microalgae – bacteria into the HRAP reactor, producing a low self-shade effect, which increases the photosynthetic efficiency and the ammonia nitrogen assimilation of the microalgae – bacterial system.

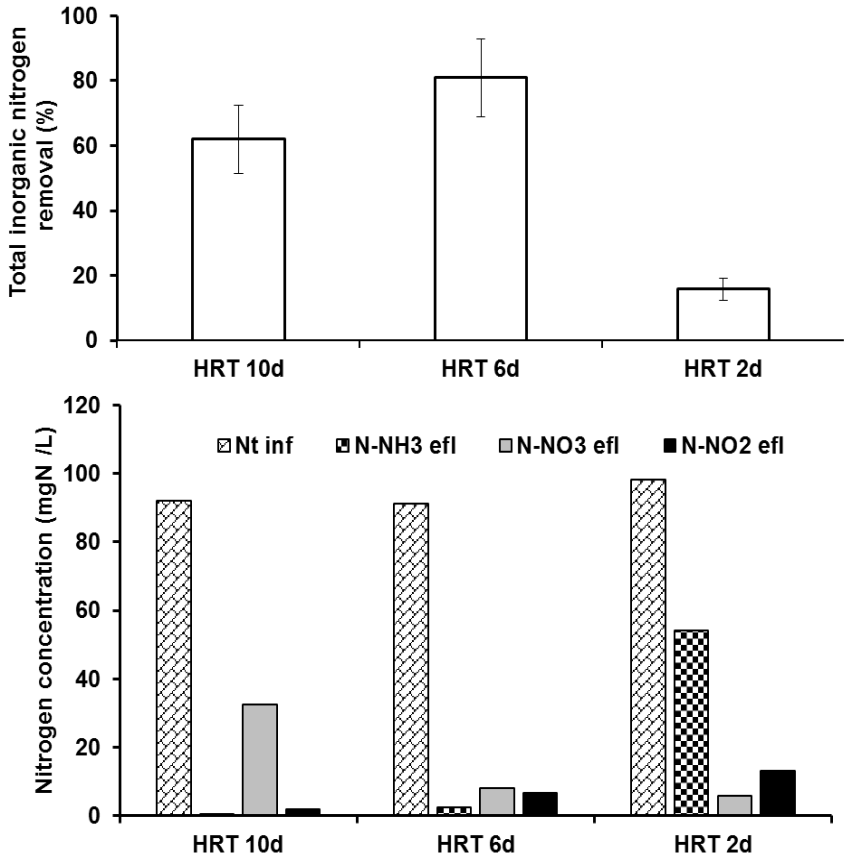

Figura 3.2. (a) Average removal percentage of total inorganic nitrogen; (b) total inorganic nitrogen balance in HRAP during the periods of HRT 10 d, HRT 6 d, and HRT 2 d

3.3.2 Microalgae – bacteria consortium and settleability

The harvesting of the microalgae – bacterial biomass is the bottle- neck in terms of energy input. During the experimental process, we observed an increase in the settling velocity (S_v) when decreasing the HRT from 10 d to 6 d, which is associated with the morphologic and dominant species in the microalgae – bacteria flocs. An excellent effluent quality, indicated by low suspended solids concentration (13.5 mg ± 5.1 to 40.1 ± 12.8 TSS L^{-1}), was observed (Table 3.4). Comparisons with the 2 d HRT could not be made because the biomass was washed out of the HRAP system.

Tabla 3.4. Settling velocity (S_v) total suspended solids in the effluent (X_e) and settleability percentage (%) for HRT 10 d, 6 d, and 2 d.

HRT (d)	**S_v (m h^{-1})**	**X_e (mg TSS L^{-1})**	**Settleability (%)**
10	1.4 ± 0.2	13.5 ± 5.1	98.3
6	8.3 ± 0.8	40.1 ± 12.8	92.7
2	--	238.3 ± 25.2	0

During the periods to HRT 10 d and HRT 6 d, microalgae agglomerates were formed through biomass growth. Green filamentous microalgae of the *Stigeoclonium* genus attached to a central aggregate of diatoms, specifically *Navicula* and *Nitzschia*. Microscopic observations confirm the presence of this genera in the granular microalgae bacteria structure (Fig. 3.3). (Fig~~ure~~ 3.4(a) and (b)), corresponding to HRT 10 d, shows a central agglomeration of diatoms and bacteria supporting the radial growth of filamentous microalgae. In this granular structure, the central aggregation can be associated with the production of extracellular polymeric substances (EPS) (Bahulikar et al., 2007)

The hydrodynamic flow regimen of mixing prevalent in the HRAP (completely mixed promotes) promotes a flagellar radial structure of *Stigeoclonium* around the diatom agglomerate, increasing the area of influence of the algal cell and hence its probability of contact with other cells (Hondzo et al., 1998). The morphologic structure found under HRT 10 d revealed that the increase of a central aggregate leads to the formation of a granular morphology in the absence of filamentous microalgae, which suggested a final stage in floc

formation (Fig. 3.4(c)). With a decrease in the HRT from 10 to 6 d, a substantial decrease in granular morphology was observed.

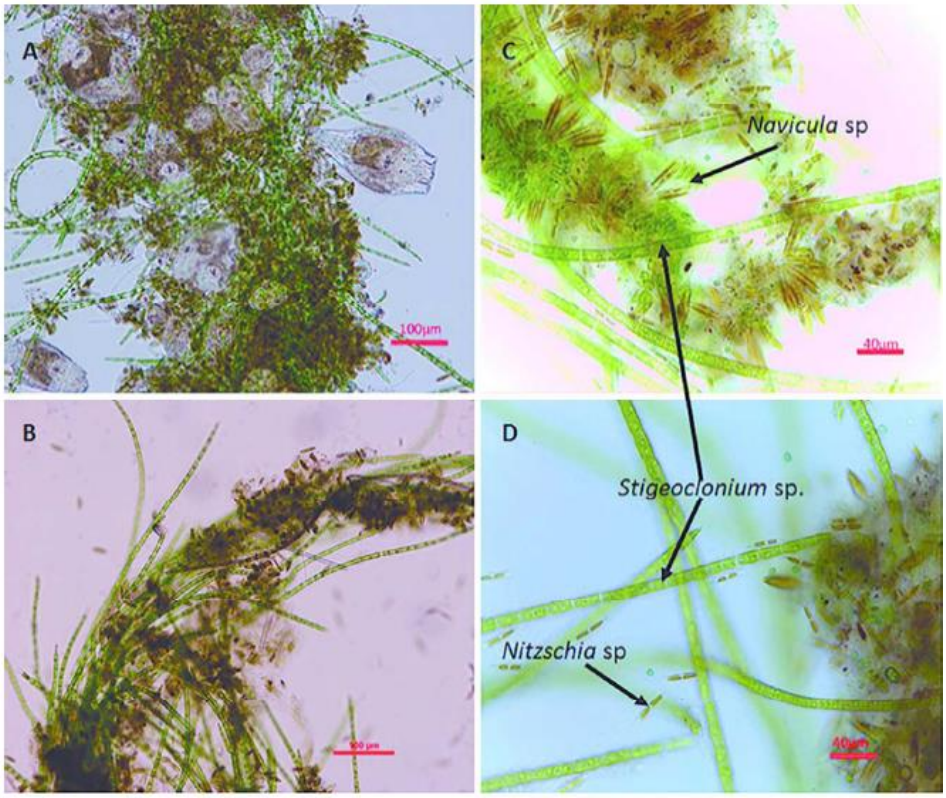

Figura 3.3. (a) – (b) Structure of the microalgae – bacteria aggregate (MABAs). (c) – (d) Dominant microalgae within the aggregates.

Figure 3.4(d) – (f) shows the agglomerates with a high density of filamentous microalgae similar to that shown in Fig. 3.4(a) and (b)). However, there were differences in color, from green to a brownish green, because the diatom colonization was not only in the central zone but also in the apical region of the filamentous microalgae. This behavior is related to the increase in OLR, which caused the bloom of the diatom population in the system. According to Logan and Alldredge (1989) the increase in diatom aggregation causes an increased nutrient uptake, nitrogen removal and S_v.

The S_v was evaluated and the results are presented in Table 3.4. The S_v increased almost 6 times when the HRT was decreased from 10 to 6 d. This result is attributed to the diatom population bloom, which because of their heavy cell wall (formed of silica), increases the settling velocity of the biomass. However, this increase also results in the emergence of free

floating diatoms in the system, decreasing the effluent quality (Table 3.4) measured as total suspended solids (TSS).

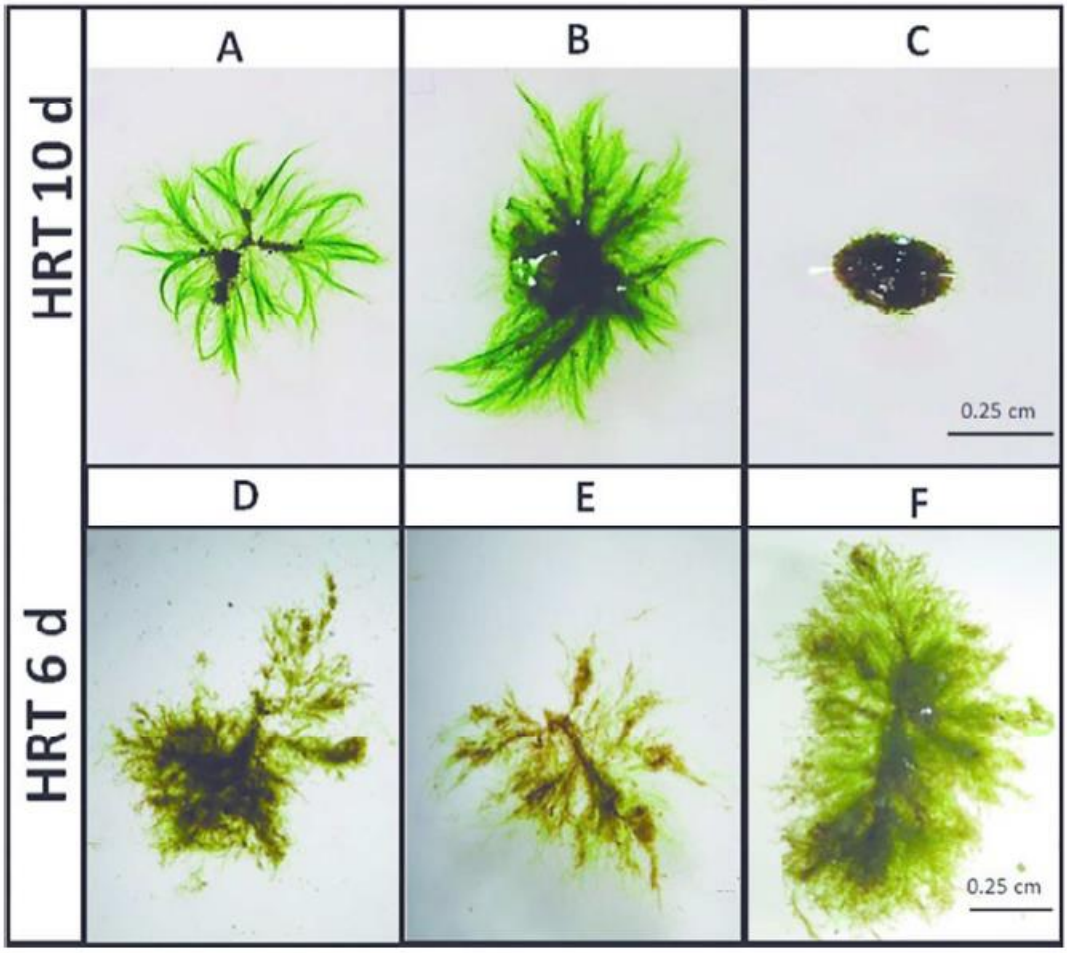

Figura 3.4. Agglomeration of microalgae – bacteria during the experimental process. The image corresponds to the predominant structures generated during operation of the HRAP. Each condition (a to f) is described in the text.

On the other hand, it is interesting to note that the S_v for the microalgae – bacteria system is comparable with the values obtained for activated sludge (5 – 13 m h^{-1}). Nevertheless, even if the Sv for the HRT of 10 d is lower than for activated sludge, this value is still high compared with the values reported for microalgae systems ranging between 1×10^{-3} and 1.26 m h^{-1}(Choi et al., 2006). The settleability percentage (Equation (3.1)) was 98 and 92% for HRT of 10 and 6 d (Table 3.4). When the reactor was operated with 2 d HRT, the settleability percentage was near to zero, since washout of the reactor occurred. Valigore et al., (2012) reported settleability percentages near to 95% for aggregates obtained in SBR and under high

solid retention time (12 d) and low HRT (1.4d). The results of the present study evidence that the formation of microalgae – bacteria aggregates in continuous reactors, exhibit similar settling capacities to the aggregates obtained with SBR systems reported in the literature (Van Den Hende et al., 2014a). The association of bacteria and microalgae formed in this type of system is promising to reduce the cost of harvesting and to overcome the operational economic gap present in microalgal systems for energy generation.

3.3.3 Chemical profile of the biomass

The biomass obtained at the end of each of the periods (HRT 10 d, HRT 6 d and HRT 2 d) was analyzed, obtaining a composition profile of proteins, carbohydrates and lipids (Table 3.5). The period HRT 2 d presented a similar chemical profile to the period HRT 6 d; for that reason, and because of the short duration this period will not be discussed. After determining the one-factor ANOVA value at a significance level of 0.05, it was concluded that the operating factor HRT does not influence the lipid content in the biomass. A high percentage of the initial nitrogen as ammonia was assimilated (Fig. 3.2). However, some nitrification was observed; therefore, nitrogen as nitrates and nitrites remain available in the liquid media, and no nitrogen limitation was present. Besides, the relatively high assimilation of nitrogen by the cells (up to 75%) increased the cellular nitrogen content. Richardson et al. (1969) found that the lipid content of cells is dependent on the cellular nitrogen content. The lipid content increases when the cellular nitrogen content falls to approximately 3% of dry weight. Hence, it is plausible that the nitrogen present in the cell content in this study exceeded 3%, and thus the HRT had no influence on lipids content. González-Fernández et al. (2010) observed that the lipids content remained constant when the microalgae cultures were cultivated under different operational conditions. In that case, the initial nitrogen was highly assimilated, and the nitrogen cell content was 10%. An increase in the protein content was observed as the HRT was decreased because of the microalgae nitrogen assimilation (Fig. 3.2(b)). Carbohydrates are the second constituent of the algae – bacterial biomass, and increase with HRT. According to the results obtained at different HRT, it is possible to establish operating and process conditions in microalgae – bacteria systems operated in HRAP reactors to obtain biomass with characteristics suited to biofuel production.

Tabla 3.5. Chemical composition profile of microalgae – bacteria systems during the periods HRT 10 d, 6 d and 2 d.

HRT (d)	Lipids (%)	Carbohydrates (%)	Proteins (%)
10	**9 ± 2**	**22 ± 1**	**58 ± 3**
6	**9 ± 1**	**16 ± 2**	**69 ± 3**
2	**9 ± 3**	**14 ± 1**	**65 ± 1**

3.3.4 Biochemical methane potential

Biochemical methane potential (BMP) tests were carried out to follow the biogas production over 20 d periods. A substrate/inoculum (S/I) ratio of 0.5 g VS algae g^{-1} VS inoculum was used. The results revealed that HRT influenced methane production. A direct relationship between the HRT and the BMP was observed. The biomass changes that occurred when decreasing the HRT in the wastewater treatment process from 10 to 6 d produced a drop in methane yield (MY) from 347.9 ± 3.2 mL CH_4 g^{-1} VS to 290.4 ± 12.1 mL CH_4 g^{-1} VS, as shown in Fig. 3.5 and Table 3.6. Mendez et al. (2015) observed that cyanobacteria *Aphanizomenon ovalisporum* and *Anabaena planctonica* presented a 1.6-fold higher methane yield (287 to 380 mL CH_4 g^{-1} VS) than the most widely used chlorophyta *Chorella vulgaris*. Van den Hende et al. (2015) reported a methane yield of 226 mL CH_4 g^{-1} VS using microalgae – bacteria flocs grown in wastewater using HRAP systems. To enhance methane production the use of enzymatic pretreatment of the biomass has been tested reaching a maximum methane yield of 271.3 ± 6.6 mL CH_4 g^{-1} VS (Wieczorek et al., 2015).

Tabla 3.6. Maximum methane yield and methane production rate for the waste biomass obtained under HRT conditions of 10 d, 6 d and 2 d in the HRAP

Period	Methane yield (mL CH_4 g^{-1}VS)	CH_4 production rate (mL CH_4 g^{-1}VS d^{-1})
HRT 10 d	347.9 ± 3.2	55.7 ± 0.4
HRT 6 d	290.4 ± 12.1	27.5 ± 3.5
HRT 2 d	329.0 ± 5.5	25.8 ± 1.7

Likewise, the trend for methane production rate was to decrease, from 55.7 ± 0.4 mL CH_4 g^{-1} VS d^{-1} to 27.1 ± 3.5 mL CH_4 g^{-1} VS d^{-1} (Table 3.6). The anaerobic biodegradability was calculated dividing the actual methane yield by the theoretical methane production (350 mL CH4 g^{-1} COD) and the values ranged from 48.1 ± 0.5 to 40.7 ± 1.1% for 10 and 6 d of HRT, respectively (Table 3.6). This behavior evidences the difficulty in the biological breakdown of the cell walls in the microalgae species found in HRT 6 d, attributed to an increase in the population growth of diatoms, specifically the *Nitzschia* and *Navicula* genera.

A direct relationship between biogas production and the type of microalgae fed to the system has been observed. Debowski et al. (2013), found that there was a decrease of approximately 12% in methane production when using Diatoms as substrate compared with Chlorophyta taxa, which could be associated to the silica content in Diatoms. Silica is a recalcitrant compound of low biodegradability (Gunnison et al., 1975). Another factor that could contribute to the difference in the methane yield is the lower C/N ratio in HRT 6 d samples, where a decrease in carbohydrates is coupled with an increase in proteins in the biomass profiles (Table 3.6). This low C/N ratio might be the cause of the reduction of the efficiency of the fermentation processes and the rise in total ammonia concentration that inhibits methanogenesis processes. Nevertheless, this last cause is not considered relevant because the experimental results show a total ammonia production of 1430 mg N-NH_4^+ L^{-1}, 970 mg N-NH_4^+ L^{-1}, and 1100 mg N-NH_4^+ L^{-1} for the biomass in the period HRT 10 d, HRT 6 d and HRT 2 d, respectively. All values are below the inhibition values of 1500 to 3000 mg N-NH_4^+ L^{-1} for anaerobic systems (Rajagopal et al., 2013).

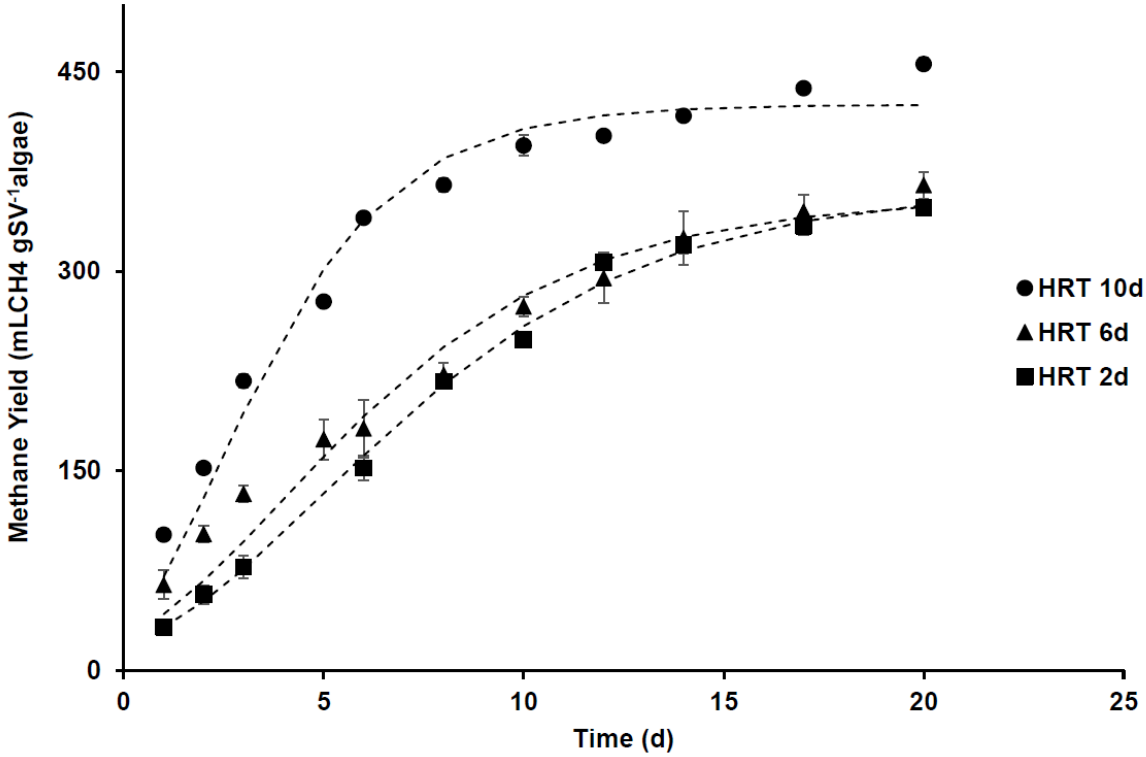

Figura 3.5.Methane yield for waste biomass. Dotted lines represent the Gomperzt model fitting.

Higher methane yields were obtained in this study 347.9 ± 3.2 mL CH4 g^{-1} VS) compared with other works that varied from 227 to 271 mL CH_4 g^{-1} VS mL CH_4 g^{-1} VS (Gutzeit et al., 2005; Van Den Hende et al., 2014a). The differences might be related to the particular microalgae developed and the type of anaerobic inoculum used for the tests. The methane yields obtained in this study are similar to those obtained for the digestion of activated sludge (150 to 300 mL CH_4 g^{-1} VS), lignocellulosic biomass (231 to 424 mL CH_4 g^{-1} VS) and cyanobacteria biomass (240 – 380 mL CH_4 g^{-1} VS) (Mendez et al., 2015; Mussgnug et al., 2010; Sawatdeenarunat et al., 2015).

3.4 Conclusions

The formation of flocs and granules as dominant structures of the microalgae – bacteria biomass was observed in the continuous HRAP. These morphologic structures are mainly constituted of diatoms, green filamentous microalgae and bacteria. The microalgae – bacteria

biomass showed high COD (92%) and nutrient removal (85% and 30% for $N\text{-}NH_4^+$ and $P\text{-}PO_4^{3-}$, respectively) when wastewater was treated for 10 and 6 d of HRT. High settling velocities and low effluent TSS concentrations were obtained. A poor performance was observed for 2 d of HRT, where the high OLR was identified as the limiting factor. An inverse relationship between HRT and BMP was observed because of the dominance of diatoms in the agglomerates. Under the conditions studied, the results demonstrated that municipal wastewater can be adequately treated using a HRAP for carbon, ammonia and phosphorous removal. To promote efficient separation of the microalgae – bacteria aggregates a HRT longer than 6 d must be utilized.

3.5 Acknowledgements

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4

Influence of solar irradiance levels on the formation of microalgae-bacteria aggregates for municipal wastewater treatment

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Abstract

Light intensity is considered an important factor in the production of extracellular polymeric substances and the formation of microalgae-bacteria aggregates. The influence of the solar irradiance level on the formation of microalgae-bacteria aggregates was studied, considering the settling properties and the removal of organic matter and nutrients. Three different solar average irradiance levels of 6213, 2741 and 3799 Wh m^{-2} d^{-1} were studied in an 80 L outdoor high rate algae pond (July to November, 2015) operating at a hydraulic retention time of 10 days and treating municipal wastewater. The highest irradiance level (6213 ± 1186 Wh m^{-2} d^{-1}) showed a poor wastewater treatment performance related to low removal efficiencies of TN (36 ± 12 %), total COD (50 ± 8 %). However, the removal efficiency of P-PO_4^{3-} evidenced the highest values (92 ± 1 %). Furthermore, low settling velocity (S_v) and settleability ($4x10^{-3}$ m h^{-1} and 26 ± 11 %, respectively) were associated with a poor aggregation formation in the system. In contrast, low irradiance levels (< 3800 Wh m^{-2} d^{-1}) promoted the formation of microalgae-bacteria flocs and granules with high settling velocity and settleability (18 m h^{-1} and 85 %, respectively). Moreover, under low irradiance levels, high removal efficiencies for TN (60 ± 5 %), total COD (89 ± 3 %) and P-PO_4^{3-} (28 ± 7 %) were observed. Nitrification mechanism was only detected at low irradiance levels, which contributed, on average, to 30% of the TN removal from the influent. A relevant factor in the overall good performance of the microalgae-bacteria systems was EPS formation.

Keywords: granulation; EPS; wastewater treatment; microalga-bacteria; sedimentation

4.1 Introduction

Aerobic processes driven by microalgae-bacteria associations can be seen as a feasible alternative for wastewater treatment. Microalgae produce the required oxygen that allows bacteria to remove pollutants, generating the CO_2 needed by bacteria (Abdel-Raouf et al., 2012; Muñoz and Guieysse, 2006). Furthermore, the mixotrophic characteristic of the microalgae growth associated with their ability to use both organic and inorganic carbon sources, it has been considered as another mechanism for the removal of organic matter in the systems of wastewater treatment (Lowrey et al., 2015). This conglomerate of photosynthetic and heterotrophic organisms has made the microalga-bacteria system efficient in high rate algae pond systems (HRAP) used to treat different types of wastewater, including municipal (Kim et al., 2014; Posadas et al., 2014), piggery (de Godos et al., 2014; Gonzáles et al., 2008) and industrial effluent (Van den Hende et al., 2014). An important issue in HRAP systems is the dominance of microalgae, such as *Scenedesmus* sp. and *Chlorella* sp., with low settling properties (usually less than $3.6x10^{-3}$ m h^{-1}), which make their separation from water difficult (Assemany et al., 2015; Cho et al., 2015). The low settling velocity observed in microalgae is an obstacle for biomass harvesting and therefore can limit the cost-effectiveness of biomass recovery and the expansion of microalgae-bacteria to commercial scale (Milledge et al., 2013). The formation of microalgae-bacteria flocs and aggregates has been studied to overcome settling problems (Arcila and Buitrón, 2016; Medina and Neis, 2007; Valigore et al., 2012; Van den Hende et al., 2014a). To induce the formation of microalgae-bacteria aggregates, and reach high settleability, different factors such as hydraulic and solid retention times (HRT and SRT, respectively) have been evaluated (Guitzeit et al., 2005; Medina y Neis, 2007). Aside from the microalgae-bacteria floc system, the generation of granular structures in batch systems has been another strategy to promote highly efficient recovery of biomass (Tiron et al., 2015). However, it has been observed that solar irradiance has an adverse impact on granular stability because of decreases in both extracellular polymeric substance (EPS) production and nutrient removal (Huang et al., 2015).

Studies of microalgae-bacteria aggregates, for both flocs and granules, have emphasized the relevance of the production of EPS by microalgae and bacteria as the key factor for forming

the aggregates. Nevertheless, the presence of EPS is affected not only by operative factors, such as hydraulic retention time (HRT) and solids retention time (SRT) but also by environmental factors such as temperature and light intensity, which affect the algae growth kinetics of both planktonic and biofilm structures.

Species such as *Microcystis aeruginosa*, *Arthrospira platensis*, and the cyanobacterium *Nostoc* sp. show a positive correlation between EPS production and light intensity in temperature conditions at approximately 30 °C. These species achieved the maximum content of EPS at a high light intensity between 100 and 180 µmol m^{-2} s^{-1} (Ge et al., 2014; Trabelsi et al., 2013; Zhen and Fanxiang, 2013). On the other hand, *Graesiella* genus presents different behavior, where the largest production of EPS is related to low light intensity (40 µmol m^{-2} s^{-1}) (Mezhoud et al., 2014). Nonetheless, when high irradiance levels are present (environmental solar irradiance ≥ 1000 µmol m^{-2} s^{-1}), the relation with EPS still needs to be assessed. Currently, the control parameters for microalgae-bacteria flocs and granules are related to operative factors (HRT and SRT), and most of the experiments have been carried out in SBR-mode. However, the experiments in continuous operative mode have also shown the formation of these microalgae-bacteria flocs, using anoxic-aerobic algal-bacterial photobioreactor (AA-ABPh) and HRAP under laboratory conditions (Arcila et al., 2016; García et al., 2017). Environmental parameters such as light intensity and its influence on EPS production need to be assessed to determine how much they affect microalgae-bacteria aggregates formation in continuous flow systems treating wastewater. The aim of this study was to evaluate the influence of solar irradiance on the formation of microalgae-bacteria aggregates, considering the settling properties and the organic matter and nutrient removal in a continuous HRAP treating municipal wastewater. The reactor under the different solar irradiance levels was operated until steady state was reached and the steady state was maintained for at least two HRTs.

4.2 Materials and Methods

4.2.1 Experimental design

The experimental setup consisted of a continuous HRAP made of fiberglass with a total capacity of 80 L (50 L of working volume) and surface area of 0.26 m^2. The HRAP system

was operated outdoors from July to November, 2015 with an HRT of 10 days. The measurements and the experiment were carried out in Queretaro, Mexico (20° 42 N, 100° 26 W), situated at 1900 meters above the sea level. HRAP mixing was assured by a six-blade paddle wheel driven by a motor engine at 10 rpm (Cole Parmer, USA), resulting in a liquid velocity of 0.2 m s^{-1}. The effluent was settled using a 3 L gravity settler. The start-up, acclimatization, and stability of the microalgae-bacteria system were accomplished as previously reported by Arcila and Buitron (2016). Three different solar irradiance levels were evaluated: 6213 ± 1186, 2741 ± 667 and 3799 ± 373 Wh m^{-2} d^{-1}, namely, high irradiance level (HIL), low irradiance level (LIL) and medium irradiance level (MIL), respectively. To provide different solar irradiance levels, the HRAP was covered with several layers of greenhouse nylon screen that provided different shade levels. The nylon material not evidenced a selective wavelength absorption, maintaining the spectral composition of sunlight invariant during all experiment stages (Figure 4.1).

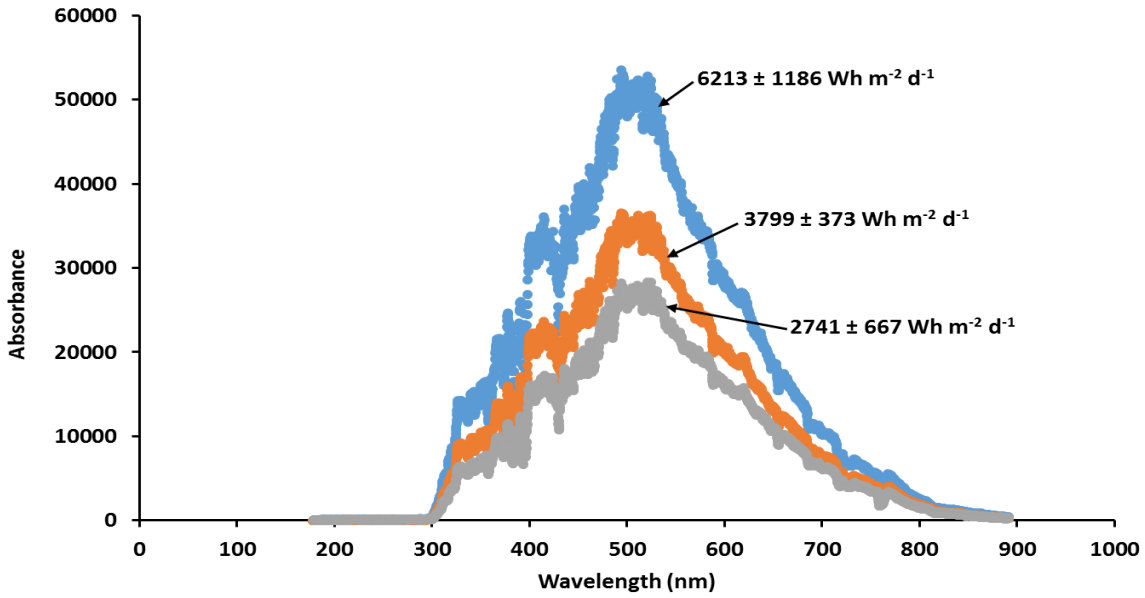

Figura 4.1. Spectral composition of the sunlight with different layers of greenhouse nylon screen, HIL: 6213 ± 1186 Wh m^{-2} d^{-1}, MIL: 3799 ± 373 Wh m^{-2} d^{-1}, LIL: 2741 ± 667 Wh m^{-2} d^{-1}

The HRAP under the different solar irradiance levels were operated until steady state was reached and the steady state was maintained for at least two HRTs.

Samples of 300 mL were taken from two points, namely at the HARP reactor and at the settler. Parameters such as total and soluble chemical oxygen demand (COD), total and volatile suspended solid (TSS and VSS), nitrogen sources as ammonium ($N\text{-}NH_4^+$), nitrate ($N\text{-}NO_3^-$) and nitrite ($N\text{-}NO_2^-$), as well as phosphorus ($P\text{-}PO_4^{3-}$), the settling velocity (S_v) were taken twice a week, while the proteins and carbohydrate in the extracellular polymeric substance (EPS) and the total Kjeldahl nitrogen (TKN) was evaluated by triplicate once a week. The solar irradiance in the HRAP reactor was measured on the liquid surface. Moreover, the environmental solar irradiance and temperature data were obtained from the Geosciences Center meteorological station at the UNAM, nearby the location of the HRAPs. There was no external addition of carbon dioxide to the HRAP except for that naturally introduced by the paddles. There was no biomass recycling, and hence, the SRT equals the HRT during all experimental phases.

4.2.2 Microorganisms

The HRAP was inoculated with a mixture of 833 mg volatile suspended solids (VSS) L^{-1} of microalgae biomass and 2740 mg VSS L^{-1} of activated sludge obtained from the aeration tank of a municipal wastewater treatment plant. The microalgae biomass was composed of mixed microalgae collected from an aquatic environment in Queretaro State, Mexico and propagated in Bold medium (Cea-Barcia et al., 2014). *Scenedesmus* sp. was microscopically identified as the dominant genus

4.2.3 Wastewater source

The municipal wastewater was collected from a wastewater treatment plant located in Santa Rosa Jauregui, Queretaro, Mexico. Samples were taken after primary treatment (coarse and fine screening and primary sedimentation). Before feeding the HRAP, the wastewater was passed through a sieve (Tyler No 65) and stored in a cold room at 4 °C for a maximum of seven days. The average quality of the municipal wastewater influent is shown in Table 4.1**.**

Tabla 4.1. Municipal wastewater characterization. Values of means ± standard deviation have been calculated using weekly data obtained for all the three periods studied (n: 17).

Parameter	**Mean ± SD**
CODt (mg L^{-1})	816 ± 129
CODs (mg L^{-1})	591 ± 92
VSS (mg L^{-1})	135 ± 23
pH	7.4
N-NH_4^+ (mg L^{-1})	64 ± 10
N-NO_3^- (mg L^{-1})	3 ± 2
N-NO_2^- (mg L^{-1})	2 ± 1
TKN (mg L^{-1})	105 ± 30
TN (mg L^{-1})	110 ± 16
P-PO_4^{3-} (mg L^{-1})	15.3 ± 1.3

4.2.4 Analytical procedures

TSS and VSS concentrations, TKN and sludge index volume (SIV) were analyzed according to APHA standard methods (APHA, 2005). N-NH_4^+ and COD, both total and soluble, were determined through the 8000 HACH and 10031 HACH colorimetric methods. Nitrate and nitrite were measured following the 10237 and 10206 HACH colorimetric methods. Likewise, the 10127 HACH colorimetric method was used P-PO_4^{3-}. OD and pH were measured on line (YSI Model 50B and Sensorex pH 5450C, respectively). All the soluble

parameters were filtered through 0.45 µm pore size membranes (Whatman glass microfiber filters, Grade GF / A). For the identification and quantification of microalgae, a LEICA DM500 microscope with an image acquisition system (LEICA ICC50 HD) and a stereoscopic lens (Zeiss Stemi DV4) was used. The microalgae genera were identified according to Wehr y Sheath (2003). The quantification was assessed according to microscopic methods for quantitative phytoplankton analysis using the counter chamber method in a Sedgewick-Rafert counting slide (UNESCO, 2010). Five samples were collected along the steady state period for each irradiance level for microbiological analysis and granular structure determination. Samples were homogenized by sonication (500 watts) for 60 s until the granular structures was disaggregated and then counted. Six random fields composed of sixteen squares were counted per slide. Chlorophyll (a) was determined according to Strickland et al. (1972). The settling velocity (S_v) of the sludge and granules were measured according to the methodologies of Zhaowei et al. (2010) and Yu et al. (2008), respectively. As for the granular structures, it was extracted from the broth culture and the S_v of each granule was measured separately. The settleability percentage was calculated based on Eq. 4.1. The diameter of the granules was determined by using a stereomicroscope (Zeiss Stemi DV4, Germany) and the software analyzer tools.

$$Settleability\ percentage\ (\%) = 100 * \left(1 - \frac{Xe}{X}\right) \quad (4.1)$$

Here, Xe is the TSS in the effluent of the secondary clarifier, and X is the TSS in the HRAP.

EPS extraction

EPS values were calculated as the sum of the protein and polysaccharide contents. Two types of EPS were evaluated, namely, bound EPS (B-EPS) and free EPS (F-EPS). B-EPS is related to the EPS firmly bound to the cells, while F-EPS is established as the EPS weakly bound to the cells or present in solution (Sheng et al., 2010). The B-EPS and F-EPS were extracted according to the procedure described by Arellano-Badillo et al. (2014). The F-EPS was determined by taking 50 mL of mixed liquor and centrifuging at 4000 rpm for 20 min. The supernatant was filtered through a GF/A glass with a 1.6 µm pore size. The filtrate was used

to measure free proteins and polysaccharides. The pellets obtained from centrifugation were diluted in an isotonic saline solution (0.9 % NaCl) and heated for 1 h at 80 °C. After that, the sample was centrifuged at 4000 rpm for 20 min; then, it was filtered through a GF/A glass filter, and the protein and polysaccharides of B-EPS were measured. Proteins and polysaccharides were measured in accordance with the Lowry et al. (1951) and Dubois et al. (1956) methods, respectively.

Scanning electron microscopy (SEM)

The structure of microalgae-bacteria granules was analyzed by SEM (Zeiss, model EVO 50). The granular samples were previously fixed and dehydrated using a glutaraldehyde protocol (Talbot y White, 2013). Subsequently, each sample was covered by gold using physical sputtering in a low vacuum coater (Leica, model EM ACE200).

4.2.5 Analytical procedures

The results are expressed as the mean values with error bars representing the standard deviation for each set of conditions. For the analysis of the influence of the factors, one-way ANOVA followed by Fisher's Least Significant Difference (LSD) post-hoc tests were used. For statistical hypothesis testing, the significance level was 0.05. Statgraphics centurion was used. To evaluate the granular diameter distribution, the samples size for infinite population and variance unknown was calculated according to Spiegel et al. (1198). Sample size was 75, under a significance level of 0.05.

4.3 Results

In the course of the experiments, three irradiance levels, 6213 ± 1186, 2741 ± 667 and 3799 ± 373 Wh m^{-2} d^{-1}, were applied in consecutive order and denoted HIL, LIL, and MIL, respectively. The lowest solar irradiance level evaluated is even higher than observed in another research using HRAP under outdoor conditions (Assemany et al., 2015; Cho et al., 2015; Kim et al., 2014). Results of the ANOVA analysis and comparative tests are presented in the (Table 4.2). To assess the microalgae-bacteria performance, besides the typical parameters for the characterization of wastewater and sludge, the B-EPS content and F-EPS content were assessed to evaluate the granule and *floc* formation (Fig 4.2).

During all experimental processes, a low concentration of F-EPS was observed compared to that of B-EPS. Additionally, the polysaccharide (PS) content of the B-EPS presented no significant variation. That led us to use the protein (PN) content of B-EPS as the relevant factor to monitor at all the irradiance levels evaluated.

Tabla 4.2. The p-values of parametric and non-parametric test for all experimental comparative variables in the system. HIL: high solar irradiance levels, MIL: media solar irradiance levels, LIL: Low solar irradiance levels, Hypothesis, Ho: p-values=0, Ha= p-values<0.05.

Parameter	**Levene's test**	**Shapiro Wilks**	**One-way ANOVA**	**LSD Post-hoc***
DO (mg L^{-1})	0.965	0.335	0.0000	HIL≠ LIL, HIL≠ MIL LIL = MIL
pH	0.243	0.168	0.0000	HIL≠ LIL, HIL≠ MIL LIL = MIL
Temperature (ºC)	0.419	0.092	0.0000	HIL≠ LIL, HIL≠ MIL LIL = MIL
Productivity (g m^{-2} d^{-1})	0.194	0.725	0.001	HIL≠ LIL, HIL≠ MIL LIL ≠ MIL
CODt (mg L^{-1})	0.522	0.851	0.0048	HIL≠ LIL, HIL≠ MIL LIL = MIL
CODs (mg L^{-1})	0.913	0.706	0.128	HIL= LIL HIL= MIL LIL = MIL
N-NH_4^+ (mg L^{-1})	0.761	0.019	0.1189	HIL= LIL, HIL= MIL LIL = MIL
N-NO_3^- (mg L^{-1})	0.753	0.771	0.0039	HIL≠ LIL, HIL≠ MIL LIL = MIL
TKN (mg L^{-1})	0.413	0.521	0.0000	HIL≠ LIL, HIL≠ MIL LIL = MIL

P-PO_4^{3-} (mg L^{-1})	0.8103	0.979	0.0368	HIL≠ LIL, HIL≠ MIL LIL = MIL
SVI (mL $gSST^{-1}$)	0.194	0.1098	0.0001	HIL≠ LIL, HIL≠ MIL LIL ≠ MIL
Sv (m h^{-1})	0.295	0.203	0.0001	HIL≠ LIL, HIL≠ MIL LIL ≠ MIL
Settling percentage (%)	0.319	0.983	0.0000	HIL≠ LIL, HIL≠ MIL LIL ≠ MIL
Chlorophyll (%)	0.083	0.862	0.014	HIL≠ LIL, HIL≠ MIL LIL = MIL
Ash (%)	0.032	0.966	0.01	HIL≠ LIL, HIL≠ MIL LIL = MIL
Proteins B-EPS (mg $gSSV^{-1}$)	0.143	0.532	0.0001	HIL≠ LIL, HIL≠ MIL LIL = MIL
Carbohydrate B-EPS (mg $gSSV^{-1}$)	0.254	0.632	0.123	HIL= LIL, HIL=MIL LIL = MIL

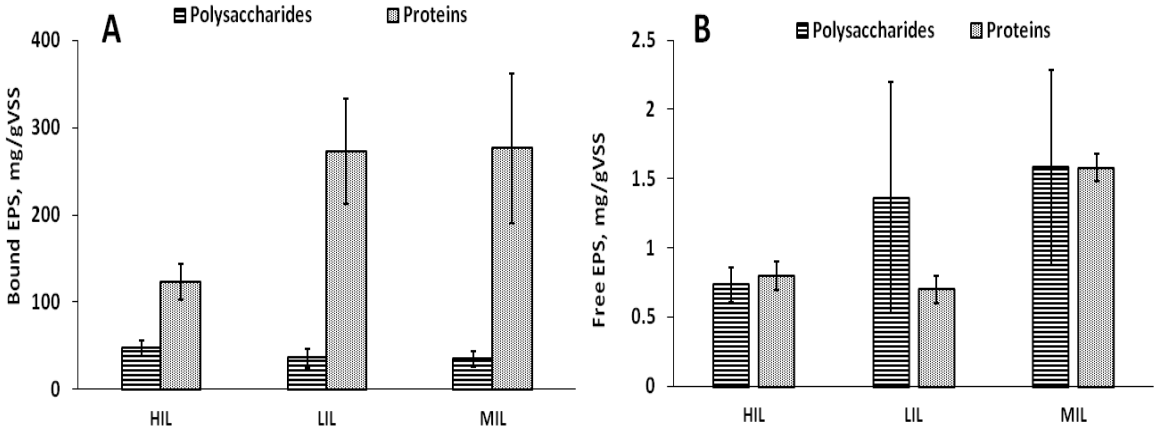

Figura 4.2. Content of polysaccharides and proteins in the extracellular polymeric substances (EPS) under three irradiance levels, HIL (6213 ± 1186 Wh m^{-2} s^{-1}), LIL (2741 ± 667 Wh m^{-2} s^{-1}) and MIL (3799 ± 373 Wh m^{-2} s^{-1}). (A) Bound EPS, (B) Free- EPS. Error bars represent standard deviations. Sample size HIL (n: 8), LIL (n: 6), MIL (n: 5). LSD Post-hoc test, were performed, $p<0.05$. MIL=LIL, HIL≠LIL, HIL≠MIL.

Irradiance condition of 6213 ± 1186 Wh m^2 d^{-1} (HIL), concomitantly with a high-temperature level of 26 ± 2 °C, presented the highest values of biomass productivity, OD, and pH reported for all experimental periods (Table 4.3). Opposite trends were observed for the settling properties and EPS production. A disperse growth with small flocs and free microalgae were observed and no granules were formed under that condition. Parameters such as S_v, the percentage of settleability and PN content of the B-EPS reached their lowest values of the study (4 ± 1x10^{-3} m h^{-1}, 26 ± 11 % and 122.8 ± 35.8 mg PN $gVSS^{-1}$, respectively, Table 4.4). These characteristics caused high total solid content in the effluent (Xe) of the secondary settler (526 ±78 mg SST L^{-1}, Table 4.5). Linked to this behavior, the TN and CODt showed the lowest removal of 36 ± 12 % and 50 ± 8 %, respectively. For soluble parameters, such as CODs, N-NH_4^+ and P-PO_4^{3-}, removal efficiencies of 84 %, 98 % and 92 %, respectively were observed (Table 4.5).

The decrease in the solar irradiance to LIL (2741 ± 667 Wh m^{-2} d^{-1}) caused a decrease in photosynthetic activity, indicated by the low DO and pH values of 6.8 ± 1.1 mg L^{-1} and 8.3 ± 0.3, respectively. As a consequence, the biomass productivity reached its lowest value of the study (7.0 ± 0.7 g m^{-2} d^{-1}, Table 4.3). Under the LIL condition, the B-EPS presented a two-fold increase in PN content (272.8 ± 34.6 mgP $gVSS^{-1}$) compared to that experienced under the HIL condition. The decrease in irradiance level resulted in a higher PN to PS ratio, from 2.6 ± 0.7 to 7.6 ± 1.2 mgPN $mgPS^{-1}$. Under this irradiance level, the formation of aggregates (flocs and granules) with high settleability percentage (85 ± 5 %) and low TSS in the effluent (60 ± 24 mgTSS L^{-1}) was favored. At the same time, a significant increment ($p < 0.05$) in the removal efficiencies of TN and CODt (an average of 60 and 89 %, respectively) was observed. Under this condition, nitrification was significant (31 mgN-$NO_3^-L^{-1}$), at the secondary settler effluent. This high N-NO_3^- concentration affected the C/N ratio in the system, decreasing this ratio from 41.4 ± 14.1 to 2.9 ± 0.6 mg COD/ mg N. The phosphorus removal exhibited a significant decrease (Table 4.5).

The increase in the irradiance level (MIL) from 2741 ± 667 to 3799 ± 373 Wh m^{-2} d^{-1} did not significantly influence the PN / PS ratio or the content of B-EPS, which maintained values of 7.9 ± 1.9 and 276.2 ± 85 mg g VSS^{-1}, respectively, similar to those recorded under the LIL

condition. Similarly, the removal efficiencies of nutrients and organic matter and the $N\text{-}NO_3^-$ concentration in the effluent also remained statistically invariant (Table 4.5).

Tabla 4.3. Physicochemical and environmental characteristics of the microalgae-bacteria systems in HRAP under different irradiance levels. Values represent the mean ± standard deviation calculated using daily data for HIL (n:50), LIL (n:40), MIL (n:25), except productivity, with HIL (n: 12), LIL (n: 12) and MIL (n: 10).

	Solar irradiance ($Wh\ m^{-2}\ d^{-1}$)	**Maximum irradiance (W/m^2)**	**Productivity ($g\ m^{-2}\ d^{-1}$)**	**DO ($mg\ L^{-1}$)**	**pH**	**Liquid T (ºC)**
HIL	6213 ± 1186	1065 ± 72	12.7 ± 1.6	10.7 ± 1.1	10.2 ± 0.3	26.0 ± 2.0
LIL	2741 ± 667	479 ± 84	7.1 ± 0.7	6.8 ± 1.1	8.3 ± 0.3	21.0± 0.0
MIL	3799 ± 373	665 ± 47	9.1 ± 0.9	6.4 ± 0.8	8.5 ± 0.2	22.0 ± 1.0

Tabla 4.4. Characteristics of microalgae-bacteria aggregates. Values represent the mean ± standard deviation. Data were calculated during the steady state period. HIL (n: 12), LIL (n: 12) and MIL (n: 10).

	HIL	**LIL**	**MIL**
SVI ($mL\ gSST^{-1}$)	40 ± 9	741 ± 79	163 ± 50
Sv ($m\ h^{-1}$)	0.004 ± 0.001	0.240 ± 0.040	0.784 ± 0.080
Settling percentage (%)	26 ± 11	85 ± 5	88 ± 2
Chlorophyll (%)	0.6 ± 0.1	0.4 ± 0.1	0.8 ± 0.1
Ash (%)	15.1 ± 5.7	12.7 ± 2.5	27.2 ± 2.3

Tabla 4.5. Water quality in the effluent obtained under each irradiance level. Parenthesis indicates the removal efficiency respect to the average value of the influent of each period. Values represent the mean ± standard deviation. Data were calculated during the steady state period. HIL (n: 8), LIL (n: 6) and MIL (n: 5).

	Secondary settler effluent		
Parameters	**HIL**	**LIL**	**MIL**
CODs (mg L^{-1})	84 ± 11 (84 ± 4)	64± 18 (91 ± 4)	68 ± 15 (89 ± 5)
CODt (mg L^{-1})	539 ±175 (50± 8)	103 ± 25 (89 ± 3)	113 ± 10 (89 ± 5)
N-NH_4^+ (mg L^{-1})	1.3± 0.1 (98 ± 1)	0.5 ± 0.4 (99 ± 1)	1.4± 0.4 (98 ± 1)
N-NO_3^- (mg L^{-1})	2 ± 3	31 ± 9	32 ± 11
N-NO_2^- (mg L^{-1})	2 ± 1	3 ± 1	3 ± 2
TKN (mg L^{-1})	52 ± 7 (45 ± 12)	11 ± 4 (89 ± 5)	8 ± 2 (92 ± 2)
TN (mg L^{-1})	62 ± 11 (36 ± 12)	47 ± 8 (60 ± 5)	44 ± 8 (62 ± 7)
P-PO_4^{3-} (mg L^{-1})	2.1 ± 0.4 (92 ± 1)	9.8 ± 2.0 (28 ± 7)	10.3 ± 2.1 (25 ± 3)
Xe (mgTSS L^{-1})	526 ± 78.3	60 ± 24	68 ± 12

Under both LIL and MIL irradiance conditions the formation of well-defined granules was observed in combination with floc structures (Figure 4.3 B and D). A significant increase in the Sv and a decrease in the SVI ($p < 0.05$) of microalgae-bacteria sludge (flocs-granules) from 0.24 to 0.78 m h^{-1} and 741 ± 79 to 163 ± 50 mL $gTSS^{-1}$, respectively, were noted. The granular structures observed at both the LIL and MIL irradiance levels were separated and characterized. For both conditions, similar values for Sv (18.1 ± 2.1 m h^{-1}) and average diameters (1.3 mm) were observed % (Figure 4.3 A-D).

The assessment of the microalgae-bacteria composition was conducted by classifying the periphyton systems as described by Lakatos and Birö (1991) and microscopic observations. The abundance of non-filamentous microalgae (green microalgae and diatoms) was evaluated quantitatively (Figure 4.3 E-F), while filamentous microorganisms, such as

cyanobacteria and fungi, were characterized qualitatively, regarding the relative abundance of each microorganism observed through the microscope. The formation of granular structures and flocs was related to the presence of green microalgae, diatoms, and filamentous cyanobacteria. Figure 4.3 (E and F) displayed a higher content of green algae (≥ 90 %) than diatoms (≤ 10 %) in both cases (granules and flocs).

During the highest irradiance condition (HIL), green microalgae belonging to *Scenedesmus* sp. were the dominant genera in the system (>95 %), growing both as unicells and coenobia of two or four cells. The formation of large colonies and aggregates under this condition was not detected (Figures 4.4 A and B).

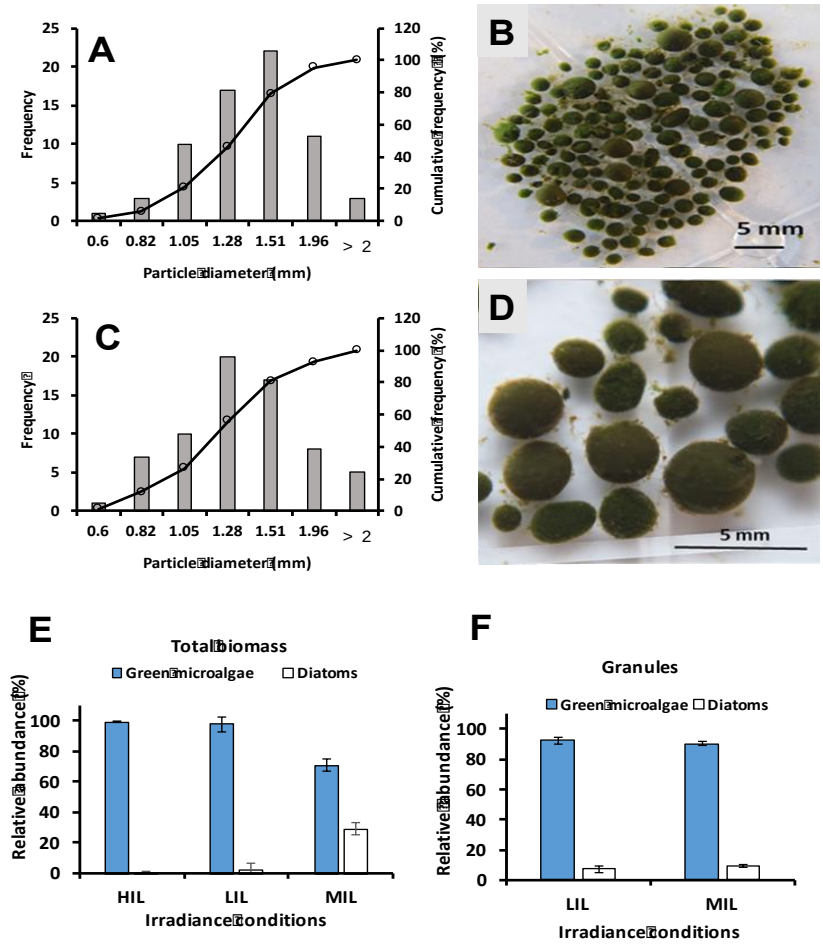

Figura 4.3. Histogram of particle diameter distribution and granular structures obtained under LIL (A, B) and MIL (C, D) conditions. The granular structures were separated from the bulk culture media to determine their particular properties. Sample size (n: 75); (E) Abundance of green microalgae and diatoms in the whole biomass and (F) in the granule structure. Error bars represent standard deviations, both total biomass, and granules for sampling size HIL=LIL=MIL (n: 30).

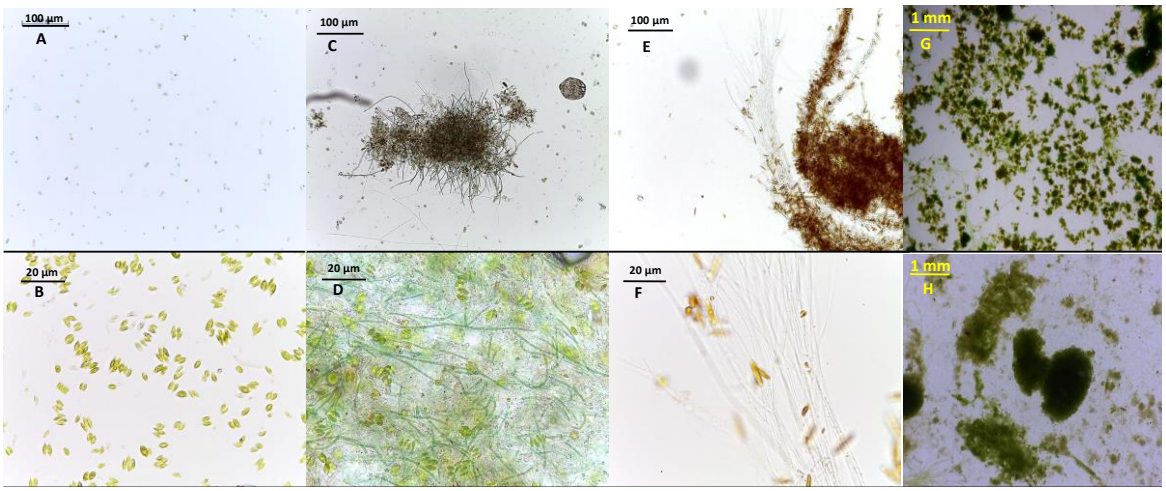

Figura 4.4. Microscopic images of the liquid culture. Micrographs A to F represent the community observed at 20 and 100 µm. A and B: microalgae growth under HIL condition, when granular structures were not observed. C and D: LIL condition and D and E: MIL condition. Under LIL and MIL conditions the growth of microalgae-bacteria aggregates was observed. Figures G and H are the stereoscopic images of the liquid culture for LIL and MIL conditions, respectively.

Based on the classification of Lakatos and Birö (1991), the values of chlorophyll percentage (0.67 ± 0.09 %) and ash (15.1 ± 5.7 %) suggested the presence of biomass with autotrophic characteristic and notable organic matter content. Under LIL, the chlorophyll and ash percentages were 0.4 ± 0.1 % and 12.7 ± 2.5 %, respectively, the microalgae-bacteria community properties did not evidence relevant changes, remaining the dominance of autotrophic microorganisms in the biomass (Table 4.4). Microscopic observation demonstrated a bloom of filamentous cyanobacteria on the floc structures under the LIL condition (Figures 4.4 C to D).

When the irradiance was raised from 2741 ± 667 (LIL) to 3799 ± 373 (MIL) Wh $m^{-2}d^{-1}$, an increase in the chlorophyll percentage (0.8 ± 0.1 %) indicated that the autotrophic characteristic of the microalgae-bacteria system was recovered. However, the ash percentage of 27.2 ± 2.3 % indicated an increment of the inorganic content in the biomass. Likewise, shifts from filamentous cyanobacteria to fungi were observed (Figures 4.4 E to F). Quantitative analysis of the floc structure revealed changes with respect to green microalgae and diatom abundance. A decrease in green microalgae (*Scenedesmus* sp.) from 97 to 70 % and an increase in diatoms from 2 to 29 % were observed. Nevertheless, the microalgae abundance in the granular structures remained unchanged for both green microalgae and

diatoms at 90 % and 9 %, respectively. (Figure 4.3 D to E). Image analysis of the granular structures obtained under LIL and MIL conditions was carried out by scanning electron microscopy (SEM), focusing on two well-distinguishable regions: (i) the periphery and (ii) the core (Figure 4.5). Interactions among green microalgae (*Scenedesmus* sp.), diatoms (probably associated with *Navicula* sp-, *Nitszchia* sp.) and accompanying rod-shaped bacteria were observed at the surface of the aggregates. The growth of filamentous cyanobacteria, fungi, and stalked ciliates was also noticed (Figures 4.4 A and B).

Nonetheless, the diversity of microorganisms in the core region was found to be completely different, resulting eventually in the dominance of green microalgae (Figures 4.5 C and D).

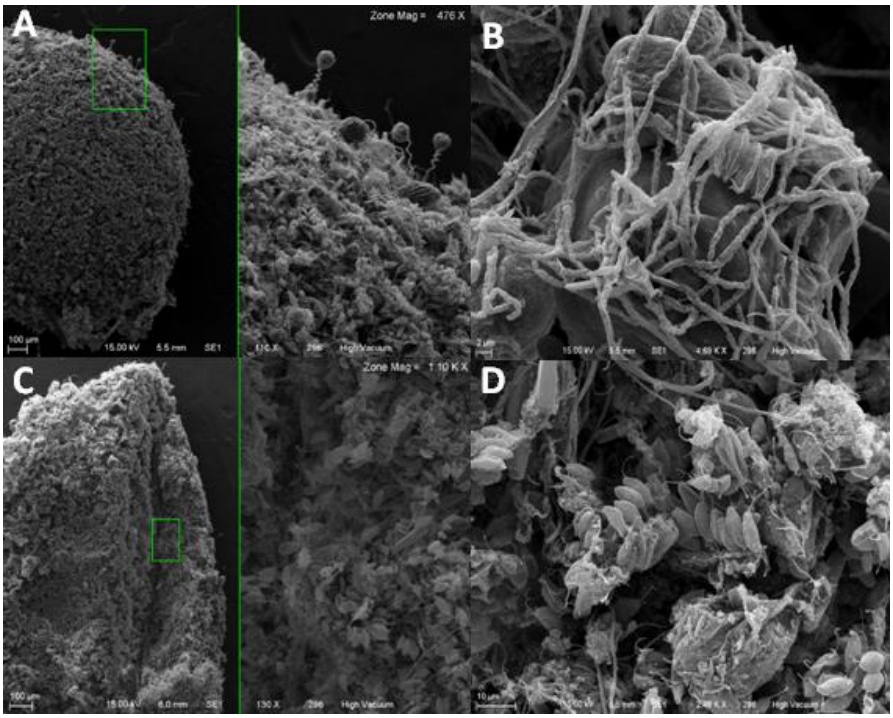

Figura 4.5. Image analysis of the granular structure by scanning electron microscopy (SEM). (A-B) Microorganisms diversity in the periphery of a granule; (C-D) Microorganisms diversity in the core of a granule.

4.4 Discussion

The variation in the irradiance level (from 6213 to 2741 Wh m^{-2} d^{-1}) affected the settling properties of the microalgae-bacteria as well as the percentage of nutrients and organic matter

removed by the system. Granulation was not obtained when the reactor received direct sun light or high light intensity (1065 ± 72 W m^{-2}). The concentration of extracellular polymeric substances (EPS) was the key factor in the increase in the settling properties. The high hydrophobicity and positive surface charge promoted self-aggregation. This phenomenon has already been observed in the formation of bacterial aggregates (flocs and granule structures) under anaerobic and aerobic conditions (Arellano-Badillo et al., 2014; Guo et al., 2011; Hulshoff et al., 2004). During the first experimental period (HIL), the HRAP was exposed to the highest irradiance levels and temperatures, which favored the activity of microalgae and were associated with high biomass productivity and DO in the system. In addition, under this irradiance condition, green microalgae (*Scenedesmus* sp.) were dominant, presenting disperse growth without flocculant properties. *Scenedesmus* is also characterized by its inefficient mechanisms for concentration and utilization of CO_2, due to the decrease in the half-saturation rate constant for dissolved inorganic carbon ($K_{0.5[DIC]}$) at high pH of 10, affecting the HCO_3^- transport on the plasma membrane (Li et al., 2016; Thielmann et al., 1990). These limitations were associated with the low availability of inorganic carbon sources because of the alkaline conditions observed in the reactor (pH values > 10). Under such conditions, the solar energy absorbed and stored by the microalgae as NADPH2 and ATP could be principally used to maintain cellular density, leading to a negative impact on the production of EPS. A similar trend was reported (Cordoba-Castro et al., 2012) using *Scenedesmus* sp. under low CO_2 availability and high light intensity of 180 µE m^{-2} s^{-1}. In this sense, the control of the solar irradiance levels should be considered a key process variable to maintain the CO_2 requirements that favor the release of the EPS by *Scenedesmus* sp. Medium to low irradiance levels (479 ± 84 to 665 ± 47 W m^{-2}) and average temperatures of 21-22 °C produced a decrease in biomass productivity; however, the PN content of the B-EPS increased, favoring the formation of aggregates and granules. An increase in the protein content of *Scenedesmus* sp. as a consequence of the irradiance reduction under nitrogen limiting conditions has been already documented by Liu et al. (2012). On the other hand, the nitrogen source present in the wastewater had a strong effect on the PN content and the microalgae abundance in biofilms. For activated sludge, a high PN content in the EPS can be produced by reducing the C/N ratio, to 5 (Durmaz et al., 2001). In this study, a five-fold increase in the N-NO_3^- concentration (observed under the LIL condition) produced a decrease

in C/N, which showed a direct relationship with the increase in the PN in the B-EPS and the formation of microalgae-bacteria aggregates. This PN increase positively affected the PN/PS ratio of the B-EPS. According to Xiong y Liu (2007) and Zhang et al. (2007), an increase in PN/PS (> 4.9) positively affects the formation of flocs and granular structures, as was observed in this investigation. At high PN/PS ratios, the negative surface charge of cells decreases, reducing the electrostatic repulsion between cells and allowing aggregates and granulation.

The presence of nitrates in the effluent of LIL and MIL (31 and 32 mg N L^{-1}, respectively) sustained the bloom of filamentous cyanobacteria and the growth of nude morphotypes, facilitating the attachment of small colonies and supporting the formation of flocs and granules (Çelekli et al., 2016; Otero et al., 2004). Conversely, under HIL a low nitrate concentration was observed (2 mg N L^{-1}). The increase in N-NO_3^- under LIL and MIL conditions is related to the presence of nitrifying bacteria, which have been found to play an important role in the stability of both microalgae and aerobic bacteria granule structure (Liu et al, 2015; Tiron et al., 2015; Zhou et al., 2015). Good settling performance (> 90 %) and floc formation are reported for microalgae-bacteria systems presenting nitrate concentrations higher than 30 mg N-NO_3^- L^{-1} and using aquaculture, domestic or dairy wastewater (Arcila et al., 2016; Tiron et al., 2015; Van Den Hende et al., 2014). Although, it has been reported that high light levels (> 500 μmol m^{-2} s^{-1}) caused a reduction in the nitrification process up to 71 % (Vergara et al., 2016), our results suggest that the formation of granular structures of microalga-bacteria probably decrease the light penetration efficiency, causing that the solar irradiance observed under LIL and MIL do not inhibit the nitrification mechanisms. However, the spatial distribution of nitrifying bacteria into the flocs should be explored. These findings suggest that the production of N-NO_3^- and pH conditions (7 to 9) that favor the presence of CO_2 and HCO_3^- sources in the system could be a triggering factor for the development of aggregates and granules.

Changes in the filamentous community of the flocs were characterized by a high abundance of cyanobacteria under the LIL condition, while fungi reached a maximum under the MIL condition (Figure 4.4). The suppression of filamentous cyanobacteria under conditions similar to the MIL condition has been observed for *Arthrospira platensis* (Wu et al., 2005). A filamentous disruption was reported when the culture was exposed to an irradiance of 341

W m^{-2}. These results confirm the sensitivity of filamentous cyanobacteria to the intensity of solar radiation. The irradiance intensity produced a shift in the dominance of filamentous biodiversity (Figures 4.4 E and F), but neither the granular structure nor the production of PN in the B-EPS was modified. That finding could be related to the ability of filamentous fungi to produce hydrophobic proteins favoring the adherence of bacteria and microalgae on their hyphae and contributing to the formation of cell aggregates (Zhang et al., 2012). The observed filamentous community coupled with the high content of EPS created a microenvironment that facilitated the retention and sharing of nutrients, promoting associations between heterotrophic and autotrophic microorganisms and protection against predators (Rossi et al., 2015; Schmidt et al., 2015; Schnurr et al., 2015; Snoeijs et al., 2004). It was observed that the filamentous species under LIL and MIL conditions promote the formation of granules in our microalgae-bacteria system. That is particularly important for the start-up of and operation of the ponds. That condition can be easily achieved in practice by decreasing the irradiance with methods as simple as the greenhouse screens. As for the anaerobic granules, in the microalgae aggregates the filamentous microorganisms contribute to the development of extracellular polymeric matrices, building bridges between the microflocs and creating a network of polymer (Arcila and Buitrón, 2016; Kim et al., 2015). In this way, the floc structures and filamentous microorganisms observed represent a transitory stage of maturation for the microalgae-bacteria granular structure formation. The dominance of green microalgae in the core of the granular structure (Figure 4.5) suggests that autoflocculation due to EPS may also be responsible for the granule formation. The positively charged polymer excreted by *Scenedesmus* sp. itself and patched to the cell surface creates an attraction field for other cells, as has been observed in the autoflocculation of *Ettliatexensis* (Salim et al., 2011, 2014), *Scenedesmus quadricauda* (Aljuboori et al., 2016) or microalga-bacteria consortia, cultivated with the algae (*Scenedesmus* sp and *Chlorella* sp.) and aerobic granules (Lin et al., 2017). Perturbation of the irradiance levels during the experimentation period revealed changes not only in the aggregates formation of flocs and granules but also in the effluent quality. HIL conditions were associated with an elevated pH (10) and low EPS content, revealing a positive relation with phosphorus removal (> 90 %) due to a precipitation mechanism. However, other factors such as S_v and the settleability percentage showed a negative effect, resulting in a low-quality effluent with a high content

of TN and COD. In contrast, lower irradiance levels, characterized by a lower pH ranging from 8 to 9, showed low phosphorus removal. The EPS formation is a relevant factor in the overall good performance of microalgae-bacteria systems for treating municipal wastewater is, which favors floc and granule formation and high removal percentages of organic matter and TN. More studies operating the system in long term periods considering all year outdoor conditions are needed. Particularly, considering that solar irradiance will vary and thus all the parameters evaluated could vary as well.

4.5 Conclusion

The development of flocs and granules of microalgae-bacteria was studied in high rate algal ponds treating municipal wastewater in continuous mode under outdoor conditions. The production of bound EPS plays a certain role in the formation of granular structures in the microalgae-bacteria system. Solar irradiance ranging from 3799 to 2741 Wh m^{-2} d^{-1} (low and medium levels) which are considered high in most parts of the world, supported the formation of granules and flocs composed mainly of green microalgae, diatoms, filamentous cyanobacteria, and fungi. The formed aggregates increased the settling velocity by a factor of 60 and the removal efficiency of TN and total COD by a factor nearby of 2 compared to the values obtained under the high irradiance level. The biological interactions among green microalgae, filaments and diatoms must be further studied to better understand the contribution of each one to the formation of granular structures.

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5

Microbial structure, dynamics and ecological interactions in microalgae-bacteria agglomerates (MABAs) treating municipal wastewater

La version original está en proceso de elaboración: Juan Sebastián Arcila, Idania Valdez-Vazquez, Germán Buitrón, Microbial structure, dynamics and ecological interactions in microalgae-bacteria agglomerates (MABAs) treating municipal wastewater, para someterse en Bioresource Technology.

Abstract

Microalgae-bacteria agglomerates (MABAs) have shown a promising performance to treat wastewater in comparison with activated sludge. Previous studies assumed positive ecological interactions between the photosynthetic community (PC) with the O_2-consuming bacteria but without a deeper understanding. Here, next-generation sequencing revealed the structure and dynamics of prokaryotic and eukaryotic communities in MABAs treating wastewater in a high rate algae pond (HRAP) operated at three hydraulic retention times (HRTs, 10, 6 and 2 d). Alfa- and beta- diversities explained the dynamics of both kind of communities and a multivariable statistical analysis correlated abundance of 16S and 18S amplicons with the performance observed at each HRT. Results indicated that at the two longer HRTs, the high removal efficiencies of organic matter and nutrients (> 90% for organic matter and ammonium and > 29% for phosphates) were well correlated with the abundance of both, the prokaryotic and eukaryotic PCs with a ratio of PC to bacteria (PC/B) > 2.2. Under such conditions, plant growth-promoting bacteria were observed as an indicator of mutualist relations with the eukaryotic PC. So on, the successful establishment of PC yielded enough dissolved oxygen (> 3.2 mg L^{-1}) to promote nitrification by *Nitrosomonas* sp. and *Nitrospira* sp., which in turn favored to the PC leaving nitrates available as nitrogen source. The HRT reduction to 2 d inverted the PC/B ratio to < 1. Worms, fungi and anaerobic bacteria become relevant in the MABAs. The eukaryotic PC suffered predation and parasitism exerted by worms and fungi, and possibly antibiosis amensalism occurred if the observed *Pseudomonas* species inhibited the cyanophyta growth. All these negative interactions contributed to the desegregation of the MABAs, reducing the treatment capacity of HRAP by 80 % for organic matter and ammonium, and by 68% for phosphates. This study demonstrates that the efficient wastewater treatment by the MABAs was a direct consequence of several positive interactions between their members. Finally, the HRT resulted in a successful control parameter to establishment positive or negative microbial interactions.

5.1 Introduction

The performance of microalgae-bacteria communities in different aquatic ecosystems has been associated with a wide spectrum of symbiotic interactions that cover from positive interaction such as mutualisms to negative interaction such as parasitism (Ramanan *et al*., 2016; Wang *et al*., 2017). In wastewater treatment systems, one of the most relevant mutualist interaction between microalga and bacteria is where microalgae provide O_2 to bacteria which it used as electron acceptor during the oxidation of organic matter, in turn bacteria provide CO_2 to microalgae for growth. This mutualist interaction avoids the cost of aeration as well as the reduction of CO_2 emissions to the atmosphere (Muñoz and Guieysse, 2006; Olguín, 2012; Wang *et al*., 2016). In recent years, the wastewater treatment processes have turned their attention to microalga-bacteria agglomerates (MABAs) due to their satisfactory performance for reducing the organic matter content, superior settling properties in comparison with activated sludge facilitating the processes of dewatering and biomass concentration. Thus, MABAs could reduce costs of the downstream processing making feasible to valorize it for producing a wide spectrum of products such as biofuels, fertilizer, fine chemicals, among others (Gerardo *et al*., 2015; Van Den Hende *et al*., 2014a; Vulskete *et al*., 2017). Under this context, the understanding of the ecological interactions established in MABAs could help us to enhance the biomass productivity and the flocculation process, but mainly to development robust resilient treatment processes that compete with conventional activated sludge processes.

The microbial diversity and morphology of MABAs is susceptible to changes in the operation conditions such as temperature, irradiance, pH, and hydraulic retention time (HRT), as has been demonstrated in previous studies. de Godos et al. (2009) and Ferrero et al. (2011) evaluated the microbial diversity of MABAs during the treatment of piggery wastewater in a high rate algal pond (HRAP) under different environmental conditions and organic loading rates (OLR, from 13 to 180 g m^{-2} d^{-1}). In these studies, denaturing gradient gel electrophoresis (DGGE) analysis and microscopic observations served to analyze the diversity of microalgae and bacteria, respectively. Temperature and the irradiance levels were the main factors

driving the diversity in MABAs. A microalgae community of Chlorophyta and Baccilliarophyta formed these MABAs. While that the phylum Verrucomicrobium was ubiquitous within prokaryotes being associated their presence with the formation of the microalgae-bacteria flocs. Additionally, the mechanisms of nitrification and denitrification observed during the experimental process were associated with the presence of members belonging to Gammaproteobacteria and Firmicutes. DGGE and microscopy were also used for evaluating the eukaryotic and prokaryotic diversities of MABAs in a novel anoxic-aerobic photobioreactor with internal recycling treating domestic wastewater (Garcia *et al*., 2017). The internal recycling promoted a monoalgal culture of *Scenedesmus* sp. with satisfactory settling properties. DGGE analysis revealed the dominance of Proteobacteria in the photobioreactor along with the presence of other phyla such as Acidobacteria, Verrucomicrobia, Firmicutes and Actinobacteria. Furthermore, *Pseudomonas* and *Litorilinea* were associated with the mechanism of denitrification while the nitrifying bacteria of family *Xanthomonadaceae*, and genera of *Aeromonas*, *Aquamicrobium* and *Luteliobacter* were associated with the mechanism of nitrification. Granular structures of microalgae-bacteria also have been generated in batch reactors using a BG11 modified medium under nutrient starvation stress (Zhou *et al*., 2015). The DGGE analysis detected the presence of the order Sphingobacteriales and *Sphingobacterium* sp. that authors associated as relevant in the flocculation process of biomass of *Chlorella regularis*. Positive ecological interactions were related with the presence of *Rhizobium* sp. as a promoter of algal growth, and once again, a nitrifying activity was observed although attributed to *Stenotrophomonas maltophilia*. Another type of morphology in MABAs are the microalgal-bacterial flocs (MaB-flocs) developed under a mode of sequential batch operation (Van den Hende *et al*., 2011). The microalgal diversity in MaB-flocs changed depending on the type carbon source fed to the synthetic wastewater (bicarbonate or glucose). Microscopic examinations evidenced that bicarbonate promoted the growth of the filamentous *Phormidium* in MaB-flocs, while glucose promoted the growth of non-filamentous *Chlorella* sp. without the formation of MaB-flocs. A further study with these MaB-flocs demonstrated that filamentous microalgae plays a relevant role in the formation of flocs, and depending on the type of industrial wastewater treated is the type of filamentous microalgae dominant (Van den Hende *et al*., 2014). By microscopy, authors determined that *Phormidium* sp. prevail in aquaculture

wastewater, the same for *Stigeoclonium* sp. in chemical industrial wastewater, in contrast, unidentified coccal microalgae species dominated the floc structure in food and manure wastewaters. In our recent study, microscopic visualizations revealed that the filamentous *Stigeoclonium* sp. contributed substantially to the formation of MABAs with high settling properties and excellent treatment performance of municipal wastewater in HRAP (Arcila *et al*., 2016). In spite of *Stigeoclonium* sp. was observed during all the experimental process, but changes in the HRT affected negatively the settling properties, removal efficiencies of organic matter and the chemical profile of MABAs structures. The microscopic analysis was useful to follow the morphologic changes of MABAs result of the decrease in the HRT, but failed in revealing deeper information on the microbial diversity. In this sense, our study focus on relevant aspects such as (1) to identify possible ecological interactions established between microalgal and bacterial communities present in MABAs under different HRTs by using high-throughput sequencing, and (2) to establish the impact of these ecological interactions on the effluent quality from a HRAP.

5.2 Materials and Methods

5.2.1 Source of inoculum and wastewater

The inoculum consisted of a mixture of microalgal biomass enriched in Bold medium dominated by *Scenedesmus* and activated sludge. The HRAP treated municipal wastewater from the primary settling of wastewater plant treatment located in Queretaro, Mexico. Details were previously described in Arcila and Buitrón (2016).

5.2.2 Operation of HRAP

The system consisted of a HRAP with a total volume of 80L and a working volume of 50L. The HRAP was operated under laboratory conditions using LED lamps to supply a total light intensity of 200 μmol m^{-2} s^{-1} with a light-dark photoperiod of 12 h (light): 12 h (dark) to treat domestic wastewater. The design parameters and set-up (inoculation and stabilization) of the microalgae-bacteria system were described in Arcila et al., (2016) Three different hydraulic retention times (HRTs) were evaluated in the following order 10d, 6d, and 2d. The

description of HRAP performances, microalgae-bacteria characterization and methane yield for all operative conditions were summarized in Table 5.1.

Tabla 5.1. Performance and algal biomass characteristics of high rate algae ponds operated at different hydraulic retention times for treatment of municipal wastewater.

	HRT 10 d	**HRT 6d**	**HRT 2d**
Physicochemical Parameters			
Temperature (ºC)	23 ± 2	22 ± 2	21 ± 3
pH	8.2 ± 0.3	8.0 ± 0.5	7.5 ± 0.1
Dissolved oxygen (mg L^{-1})	10.2 ± 2	3.2 ± 0.3	0
Removal of N-NH_4^+(%)	99 ± 1	96 ± 3	15 ± 1
Removal of COD (%)	91 ± 4	92 ± 1	12 ± 8
Removal of P-PO_4^{3-} (%)	49 ± 11	29 ± 4	9 ± 7
Settleability percentage (%)	98 ± 4	92 ± 3	0
NO_3^- (mg L^{-1})	32.4± 4	10.0± 1	2.2±0.5
Characteristics of algal biomass			
Protein content (%)	58 ± 3	69 ± 3	65 ± 1
Carbohydrate content (%)	22 ± 1	16 ± 2	14 ± 3
Lipid content (%)	9 ± 3	9 ± 1	9 ± 3
Ashes (%)	11 ± 2	6 ± 2	12 ± 3
CH_4 yield (mL CH_4 g VS^{-1})	348 ± 3	290 ± 12	329 ± 6

5.2.3 Molecular diversity of prokariotes and eukatyotes

Once the physicochemical parameters were stabilized at each HRT, a 50 mL-sample was stored at – 70ºC until further analysis. Genomic DNA was extracted from 0.5 mL-samples containing supernatant and biomass using the Power Soil DNA Extraction kit® (Mo Bio Laboratories Inc., Carlsbad, CA, USA) according to the manufacture's instructions. The DNA concentration was adjusted to 20 ng/uL and sent to the Research and Testing Laboratory, RTL (Lubbock, Texas, USA) for the amplification of 16S rRNA gene for prokaryotic diversity and 23S and 18S rRNA genes for eukaryotic diversity. A total of three sample libraries were prepared with the following primers for bacterial 16S rRNA (28F 5'-GAGTTTGATCNTGGCTCAG-3', 519R 5'-GTNTTACNGCGGCKGCTG-3'), eukaryal/fungal 18S rRNA (EukA7F 5'-AACCTGGTTGATCCTGCCAGT-3', EUK555R 5'-GCTGCTGGCACCAGACT-3'), and chloroplast 23S rRNA (AlgaeF 5'-GGACAGAAAGACCCTATGAA, AlgaeR 5'-TCAGCCTGTTATCCCTAGAG-3') genes). Then, sequencing protocol was performed by RTL using a Roche 454 FLX Genome Sequencer system (Dowd *et al*., 2008). Quality filtering included a denoising protocol to remove short sequences, singleton sequences, noisy reads, and chimeric sequences using the USEARCH and UCHIME algorithms (Edgar *et al*., 2010, 2011). Only sequences larger than 250 pb and with a quality score larger than 30 were kept for the following analyses. Operational taxonomic units (OTUs) were selected with the UPARSE algorithm (Egdar *et al*., 2013). Filtered quality sequences were aligned using the RDP for the bacterial 16S libraries and the Silva SSU and LSU reference alignments for the 18S and algal 23S, respectively (Pruesse *et al*., 2012). OTUs were assigned using complete linkage clustering at 97% similarity. OTUs were taxonomically classified using a derived NCBI non-redundant nucleotide database. These sequence data were deposited in the GenBank database. Species richness was estimated using the number of OTUs found in each sample while Shannon diversity index (H) was estimated using the relative abundance of each OTU in the total sum. The similarity index -β, was calculated from Jaccob and Sørensen coefficient using the relative abundance of each OTU in the total sum. A ratio of the photosynthetic community (PC) to bacteria was calculated (PC/B ratio) as the sum of reads of photosynthetic eukaryotes (Chlorophyta and Bacillariophyta) and photosynthetic prokaryotes (Cyanobacteria) divided between the reads of prokaryotes (excluding Cyanobacteria).

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5.2.4 Analytical methods

All the parameters for characterization of wastewater, characteristics of algal biomass (carbohydrate, proteins and lipids) and biochemical potential methane (BPM) were analyzed as given by Arcila and Buitron (2016).

5.2.5 Statistical analysis

A multivariable method of principal component analysis (PCA) based on the Pearson's correlation matrix assessed the association between abundance of photosynthetic microorganisms (prokaryotic and eukaryotic ones) and bacteria with the environmental variables at each HRT. In order to narrow down the number of OTUs for further interpretation of their correlation with environmental variables, only the genus with average abundance over 5% across all samples were taken account in the multivariate analysis (Xu *et al*., 2015). On the other hand, the environmental parameters were grouped into two categories: the first one regarded parameters of wastewater treatment performance such as removal of ammonium (RE-N-NH_4^+), phosphorus (RE-P-PO_4^{3-}), chemical oxygen demand (RE-COD), settleability (% Sett), and nitrate production (N-NO_3); and the second one regarded biomass characteristics such as content of carbohydrate (%Carb) and proteins (%Prot). All data were converted into common scale trough Z-transformation so that mean and variance corresponded to 0 and 1. The statistical analysis was carried out using the statistical software XLSTAT for the Microsoft EXCEL®.

5.3 Results

5.3.1 HRAP performance

The longer HRTs of 10 d and 6 d favored the photosynthetic aeration by microalgae achieving dissolved oxygen concentrations above 3.2 mg O_2 L^{-1} during the light periods. The available oxygen concentrations were sufficient to support the removal activity of COD (> 91 %), N-NH_4^+ (> 96 %) and P-PO_4^{3-} (> 29 %) by heterotrophic bacteria (Table 5.1). During these HRTs (10 and 6 d) the presence of nitrate (N-NO_3^-) was recorded, identified the highest nitrate concentration of 32.4 ± 4 mg L^{-1} at HRT of 10 days. At these HRTs, microalga and

bacteria formed MABAs with settleability percentages > 90 % with noticeable morphological differences depending on the HRT (Arcila and Buitrón, 2016).

When the HRT decreased from 6 d to 2 d reaching the highest OLR of 361 ± 29 mg COD L^{-1} d^{-1}, the HRAP performance collapsed. At an HRT of 2 d, the photosynthetic aeration was lost, mainly affecting the removal activities of COD and N-NH_4^+ which decreased more than 80 % whereas the P-PO_4^{3-} removal decreased 68 %. Very importantly, the settling capability was lost completely (Table 1).

The microalgal biomass was characterized in terms of protein, carbohydrate and lipid contents. The highest carbohydrate content of 22 ± 1 % was observed at a HRT of 10 d, whilst the higher protein content of 69 ± 3 % was detected at a HRT of 6 d. The lipid contents remained unchanged at different HRTs.

5.3.2 Diversity analysis at different HRT

Microbial diversity was analyzed by pyrosequencing in the course of the HRAP operation at HRT of 10, 6 and 2 days. Following, prokaryotic and eukaryotic diversities are discussed separately.

Prokaryotic diversity

The reads after the filter quality were 21932, 14327 and 31933 for HRAP operated at HRT of 10 d, 6 d and 2 d, respectively. From them, 85 %, 96 % and 94% of OTUs were assigned into known bacterial taxa for the HRAPs operated at HRT of 10 d, 6 d and 2 d, respectively. Figure. 5.1A shows the changes in abundance for the eight phyla that composed the bacterial community. The major changes were observed for the Cyanobacteria and Proteobacteria phyla. First, the phylum of photosynthetic bacteria belonging to Cyanobacteria accounted for 30 % and 45 % of total reads at HRTs of 10 d and 6 d, respectively. However, the HRAP operation at an HRT of 2 d almost eliminated the presence of the Cyanobacteria phylum. Then, the Proteobacteria phylum represented between one half to one third of bacterial diversity for those HRAPs at HRT of 10 d and 6 d, respectively. But, the establishment of anaerobic conditions at an HRT of 2 d makes possible that facultative members belonging to Proteobacteria increased in number. The Nitrospirae phylum had a similar behavior to the

Cyanobacteria phylum, being more abundant at longer HRTs of 10 and 6 d than at 2 d. Opposite to this, the Bacteroidetes and Firmicutes phyla that comprise strict anaerobic bacteria were hardly observed at long HRTs when the aerobic conditions were well established (abundance less than 4 %), but together they reached up 21 % of abundance at an HRT of 2 d. The *Choloflexi* and *Acidobacteria* phyla were always scarce at all the tested HRTs. At species level (Fig. 5.1B), the Cyanobacteria phylum was mainly represented by two species, *Cyanobacterium* sp. and *Microcystis* sp. Changes in the HRT from 10 d to 6 d did not affect to *Cyanobacterium* sp. while *Microcystis* sp. increased their abundance from 7 % to 25 %. Nevertheless, both species were equally washed out at an HRT of 2 d reaching together <2% of abundance. The Proteobacteria phylum was mainly represented by 13 species belonging to the α, β and γ divisions averaged 31 % of total reads, and 54 species with < 1 % in abundance.

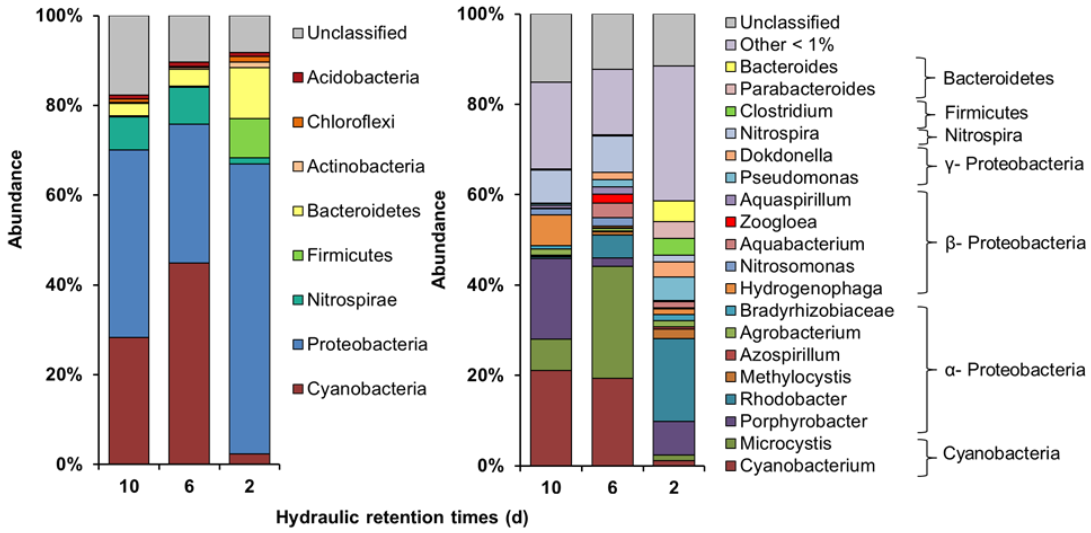

Figura 5.1. A) Main phyla belonging to Prokaryota present at different hydraulic retention times in raceway treating municipal wastewater. B) Main species belonging to Prokaryota present at different hydraulic retention times in raceway treating municipal wastewater

At an HRT of 10 d, the α-Proteobacteria comprised more than 57 % of Proteobacteria where *Porphyrobacter colymbi* was the dominant species. Then, β-Proteobacteria represented 32% of the Proteobacteria phylum, integrating members of *Hydrogenophaga* sp. and *Nitrosomonas* sp. which accounted for 51% and 11% of β-Proteobacteria abundance,

respectively. At an HRT of 6 d, the three divisions of Proteobacteria remained with minor changes with species equally represented, although, *Rhodobacter* sp. in the division of α-Proteobacteria gained relevance. At an HRT of 2 d, α-proteobacteria was the dominant division with 62% of OTUs in the Proteobacteria phylum, now with *Rhodobacter* sp. and *Porphyrobacter colymbi* as the dominant species. The divisions of β- and γ-proteobacteria were equally represented. As pointed out earlier, the Firmicutes and Bacteroidetes phyla were mainly observed in the HRAP operated at 2 d. The bacterial taxa found at this HRT were equally distributed where their abundances ranged from < 1% to a maximum value of 8 %. *Bacteroides* sp. and *Clostridium* sp. were representative taxa for these two phyla.

Eukaryotic diversity

The primer pairs AlgaeF/AlgaeR and EukA7F/Euk555R revealed different eukaryotic structure in the microalgae-bacteria agglomerates. For the primer pair AlgaeF/AlgaeR, 12462, 13077 and 14663 reads were recovered after the filter quality for HRT of 10, 6 and 2 d, respectively. From them, 99 % of sequences assigned into known microalgae tax a except for an HRT of 2 d where only 80 % of OTUs were taxonomically identified. The primer pair AlgaeF/AlgaeR revealed an algal diversity dominated by the Baccillariophyta phylum represented by unclassified species, *Nitzschia* and *Sellaphora* (Fig. 5.2A). However, the results obtained with the primer pair AlgaeF/AlgaeR did not reflect well the microalgal diversity observed under the microscope where filamentous microorganisms dominated the agglomerates.

For the primer pair EukA7F/ EUK555R, the reads after the filter quality were 21748, 28637 and 9302 for HRT of 10, 6 and 2 d, respectively. From them, 99% of sequences were assigned into known taxa for the HRAP in all HRT conditions (Fig.5.2C), being the system dominated by the Chlorophyta phylum (> 80 %), followed by Bacillarophyta (< 17 %) at HRTs of 10 d and 6 d. The HRT changes to 2 d stimulated the proliferation of other eukaryotic microorganisms belonging to the kingdoms Fungi, Animalia and Protists. The dynamic of the species belonging to the Chlorophyta phylum shows that HRTs of 10 and 6 days was dominated by *Chaetophora elegans* accounting for 79 – 89 %. However, when the HRT changed to 2 days, *Chaetophora elegans* decreased their abundance almost half, while that *Acutodesmus deserticola* and *Desmodesmus armatus* gained relevance from < 1% to 31 and

6 %, respectively. The Bacillarophyta phylum composed mainly by *Gomphonema affine* and *Cylindrotheca closterium* ranged from 10 % to 17 %, reaching their highest abundances at an HRT of 6 days. This primer pair more accurately displayed the eukaryotic diversity during each one of the reactor stage.

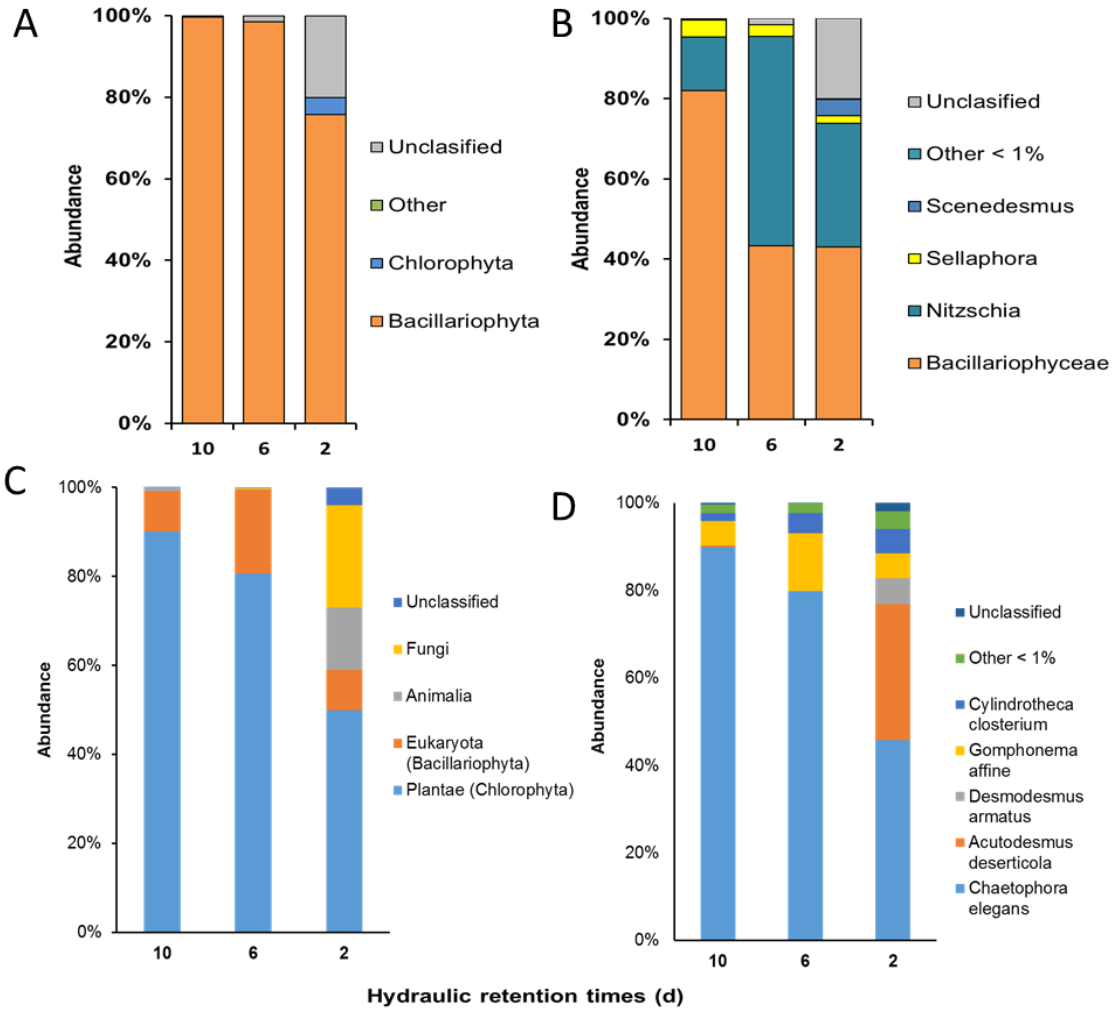

Figura 5.2. Main phyla belonging to Eukaryote present at different hydraulic retention times in raceway treating municipal wastewater with different pair of primers A) AlgaeF/AlgaeR and C) EukA7F/ EUK555R, B-D) Main species belonging to Eukaryote present at different hydraulic retention times in raceway treating municipal wastewater B) Primer AlgaeF/AlgaeR, D) Primer EukA7F/ EUK555R

Photosynthetic microorganisms / bacteria ratio

One of the most important factors determining the performance of microalgae-bacteria systems is the photosynthetic microorganisms/bacteria ratio (PM/B). In this study, we

determined this ratio using the total number of reads for photosynthetic microorganisms (prokaryotes and eukaryotes) divided by the total number of reads for bacteria. An HRT of 6 days stimulated the highest growth of photosynthetic microorganisms at the same time limited the bacteria growth yielding the highest PM/B ratio. Opposite to this, HRT of 2 days, stimulated the highest growth of bacteria at the same time limited the photosynthetic microorganism growth yielding the lowest PM/B ratio (Table 5.2).

Tabla 5.2. Microalgae-Bacteria ratio under three different operative conditions of hydraulic retention time (HRT).

Community characteristics	**Hydraulic retention time**		
	10d	**6d**	**2d**
Photosynthetic microorganism (PM)[1]	27,394	34,249	8,611
Heterotrophs	12,201	4,442	20,437
Ratio PM/B	2.2	7.7	0.45

1.PM composed by Chlorophyta and Cyanobacteria

α- and β- diversities

The observed richnesses (number of OTUs) for prokaryote was always 10 times higher than eukaryotes (Table 5.3). The Shannon index evidenced that the highest diversity for both type of microorganisms corresponded at HRT of 2 days. As for the β- diversities of Jaccards and Sorense, both index display that the prokaryotes showed structural variation at HRT of 2 days (β- index < 0.7), while the Eukaryotes maintained a similar structure during all the reactor stages (Table 5.2).

Tabla 5.3. Estimates of α- and β- diversities (between-group) for Prokaryotes and Eukaryotes.

	Prokaryotes			**Eukaryotes (algae)**		
HRT	**10 d**	**6 d**	**2 d**	**10 d**	**6 d**	**2 d**
Number of OTUs (observed richness)	78	68	123	8	9	11
Shannon-Wiener index	1.8	1.5	2.7	0.44	0.55	0.94
β- diversity measured as Jaccard index (similarity)						
HRT	**10 d**	**6 d**	**2 d**	**10 d**	**6 d**	**2 d**
10 d	1	0.56	0.46	1	0.70	0.72
6 d		1	0.44		1	0.81
2 d			1			1
β- diversity measured as Sorensen index (similarity)						
HRT	**10 d**	**6 d**	**2 d**	**10 d**	**6 d**	**2 d**
10 d	1	0.65	0.63	1	0.82	0.84
6 d		1	0.61		1	0.81
2 d			1			1

5.3.3 Relationships between microbial species and environmental parameters

A PCA analysis assessed the correlation between environmental factors and the microbial community for both eukaryotes and prokaryotes. According to the spread of the environmental variables along the principal component (Fig.5.3(A-B)), for both microbial communities, the PC1 contributed with more than 67 % of the total variance of the system, whilst the remaining variance was represented with the PC2. In regard to the eukaryotes and prokaryotes correlation and their association with the environmental variables, species such as *Cyanobacterium* sp., *Nitrospira* sp. and *Chaetophora elegans* evidences a positive strong

correlation with the good performance of wastewater treatment(% Set, RE- N-NH4+, RE-COD,RE-PO_4^{3-}), while species such as *Pseudomonas* sp., *Rhodobacter* sp., *Clostridium* sp, *Acutodesmus deserticola* and *Desmodesmus* aramatus associated to low HRT of 2 days showed a negative correlation between the same environmental parameters. Concerning to the principal component PC2, this was separated into two groups: i) Carbohydrate (%Carb) and Methane Yield (Y_{CH4}) ii) Proteins (%Prot). the first group of variables (% Carb, Y_{CH4}) increases at HRT of 10 days, whilst the second group (% Prot) reached the highest values at HRT of 6 days. The Fig.5.3(A-B) displayed that the % Carb and Y_{CH4} are positive correlation to *Phorphyrobacter* sp. and *Hydrogenophaga* sp. while the high production of proteins is correlated positively to *Microcystis* sp. and two types of Bacilliarophytas called *Gomphonemaaffine* and *Cylindrothecaclosterium*. On the other hand, chlorophyte species such as *Chaetophora elegans*, *Desmodesmus aramtus* and *Acutodesmus deserticola* not seems to have an influence on the changes in the biochemical profile %Cab, % Prot, and the Y_{CH4}.

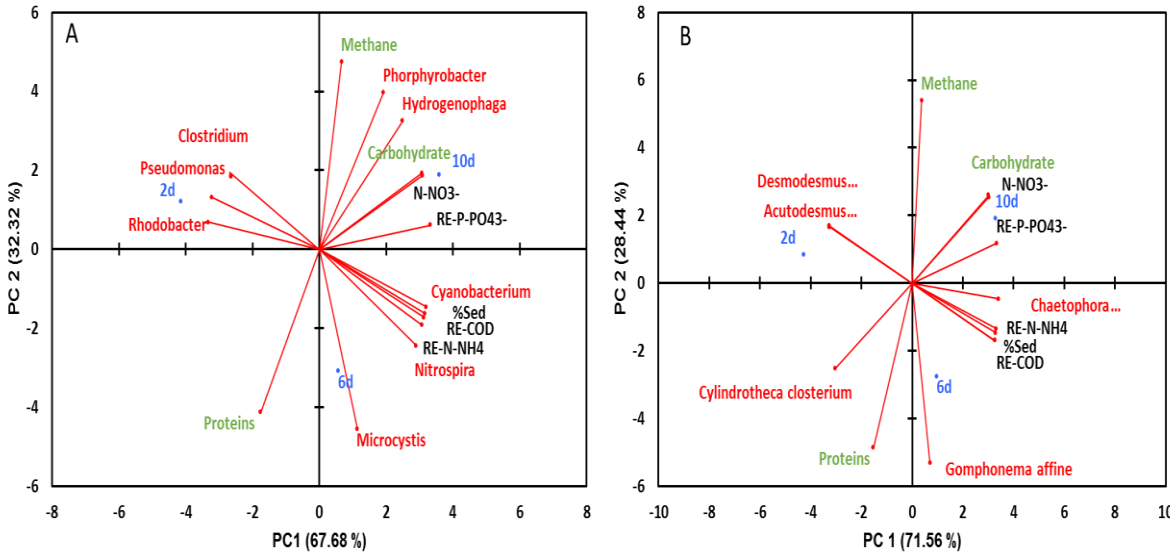

Figura 5.3. Statistical multivariable principal component analysis (PCA) to evaluate the correlation between A) environmental parameters and microalgae community, B) environmental parameters and bacteria community.

5.4 Discussion

During the course of HRAP operation, the variation in the HRT played an important role in the oxygen levels into the reactor establishing aerobic or anaerobic conditions, which in turn affected the removal efficiencies of organic matter and nutrients as well as the microbial community structures and ecological interactions established in the MABAs. The long HRT of 10 d promoted the establishment of genera such as *C. elegans* and *Cyanobacterium* sp. with enough photosynthetic activity to generate aerobic conditions under which the HRAP showed their best performance. The dominance of these genera in wastewater treatment systems is unusual since they typically thrive in oligotrophic environments with high levels of dissolved oxygen (García and Aboal, 2014). Additionally, their proliferation requires a quantity important of nitrates (9-36 mg L^{-1}), low concentrations of ammonium (0.04 - 0.81 mg L^{-1}) and N:P ratios less than 10 (Pastich *et al*., 2016 , Pelechata *et al*., 2016). All these conditions were satisfied in the HRAP operated at 10 d (N:P < 4.5, mg N-NO_3^- $L^{-1} \geq 32.4$). The available nitrates were a consequence of the nitrification activity by *Nitrosomonas* sp. (an ammonia-oxidizing bacterium, AOB) and *Nitrospira* sp. (a nitrite-oxidizing bacterium, NOB). The presence of nitrifying bacteria together with photosynthetic microorganisms suggest a mutualist interaction between them. The microalgae produced enough oxygen to promote the abundance of nitrifying bacteria, in return they convert ammonium into nitrates, benefiting the microalgae.

P. colymbi and *Hydrogenophaga* sp. were positively correlated at HRT of 10 d. The first one, *P. colymbi* is an obligate aerobe with photoheterotrophic growth and prevalence in cyanobacterial biofilms presumably by mutualistic cyclical exchanges of metabolites with autotrophs (Cole *et al*., 2014). *P. colymbi* succeed in autotrophic cultures by receiving fixed organic carbon released from cyanobacteria. In turn, this heterotrophic bacterium that belongs to α-proteobacteria (a class known as plant growth-promoting bacteria, Ramanan *et al*., 2015), benefit phototrophs when remineralizates organic carbon providing them inorganic and low molecular weight organic carbon (Cho *et al*., 2015; Ramanan *et al*., 2016). Regarding the genus *Hydrogenophaga* sp., is known as a plant growth-promoting bacterium

(Hou *et al*., 2015). Also, this genus could be involved in the high phosphorus removal in the HRAP operated at a HRT of 10 d, a fact reported previously (Chung *et al*., 2007).

A question of great interest about the MABAs formation, is which microorganisms are involved in the beginning of the formation of such structures? In our study, the MABAs with greater size and settling efficiency were observed at longer HRTs where *C. elegans* exert dominance in the HRAP (Figure 2). This branched filamentous Chaetophoraceae also was present in the formation of microalgae-bacteria agglomerates in early studies (Passos *et al*., 2015; Kim *et al*., 2016). According to Andrade et al. (2005), *C. elegans* had a great proton binding capacity in presence of high nitrate concentrations and ionic strength. The absorption of cations such as Ca^{2+}, Mg^{2+}, Na^{+} (commonly observed in domestic wastewater) on the exopolysaccharides structures of *C. elegans* had an effect of increasing the consistency and stability of extracellular polymeric substances (Xiao and Zheng, 2016), beside of reducing the repulsive electrostatic forces between other individual microalgae and bacteria. The branching morphology of *C. elegans* along with their ionisable groups, become it into a core with ability to trap microorganisms of slow settleability and small morphology. Unlike previous studies where the phyla Firmicutes and Bacteroidetes promoted the flocculation of microalgal systems (Cho *et al*., 2015; Ramanan *et al*., 2015), in our HRAP these two phyla were not representative when the MABAs reached their highest settleability. Instead, *P. colymbi* and *Hydrogenophaga* sp. were present in mature MABAs at a HRT of 10 d. *Porphyrobacter* spp. were present after 28 days of the biofilm formation being classified as secondary colonizers due to their ability to use the excreted metabolites coming from the mature biofilm (Douterelo *et al*., 2014). On the other hand, *Hydrogenophaga*, *Porphyrobacter* along with other bacteria accompanied the outbreak of *Microcystis aeruginosa* blooms (Zheng *et al*., 2008). In our MABAs, *P. colymbi* seems act as secondary colonizers hydrolyzing algal carbohydrates such as starch (Furuhata *et al*., 2013).

At a HRT of 6 d, the ratio PM/B increased 3-fold more than at HRT of 10 days. It was associated with proliferation of photosynthetic microorganisms such as *Microcystis sp*, *Gomphonema affine* and *Cylindrotheca closterium*. The bacterial reads decreased from 12201 to 4442 and the dissolved oxygen decreased from 10.2 mg L^{-1} to 3.2 mg L^{-1} during the light period, but the removal efficiencies of COD and $N\text{-}NH_4^{+}$ were unaffected (Table 1).

Unlike, the removal efficiencies of $P\text{-}PO_4^{3-}$ and $N\text{-}NO_3^-$ showed a decrease from 49 % to 29 % and from 32.4 to 10.0 mg L^{-1}, respectively. The increase of phosphorus in the systems could be related to absence of *Hydrogenophaga* sp. and the bloom of *Microcystis* sp., which has been reported to cause a massive release of phosphorus from the sediments in lakes (Xie *et al*., 2003). Similar to nitrates, phosphorus concentration seems to promote or inhibit of the *Microcystis* sp. blooms. At a HRT of 10 d, the absence of this genus might be related to the nitrate inhibition due to the accumulation of intracellular nitrite that affected its growth (Chen et al., 2009), whilst the decrease of nitrates observed at a HRT of 6 d favored its growth. The decrease in the nitrates could be caused by factors as the decrease of oxygen levels during the light periods (from 10.2 to 3.2 mg O_2 L^{-1} at HRT of 10 and 6 days, respectively), which caused a competition between heterotrophic and nitrifying bacteria for oxygen, reducing the conversion rate from $N\text{-}NH_4^+$ to $N\text{-}NO_3^-$. Moreover, another factor as the proliferation of *Rhodobacter* sp. (known as denitrifying bacteria) at a HRT of 6 d might be promoted a decrease in the nitrate concentration (Aqeel *et al*., 2016; Rosenberg *et al*., 2014a). In this case, a commensalism interaction seems be identified, where the commensal (*Microcystis* sp.) take advantage of the denitrify characteristics of the *Rhodobacter* sp. The highest protein content in the MABAs and its positive correlation with *Microcystis* sp. at a HRT of 6 d, evidenced the capability of this genus to maintain populations with high cell density and high content of proteins when the mainly source of nitrogen comes from $N\text{-}NO_3^-$, just as reported previously (Rückert *et al*., 2004). Nevertheless, the abundance of this species produced a negative impact on the quality of the treated wastewater because of the negative impact of the cyanotoxins such as microcystin, cyanopeptolin, cylondrospermopsin on the aquatic and soil ecosystems (Corbel *et al*., 2014).

Finally, the HRAP operated at a HRT of 2 d was characterized by the highest dominance of bacteria on the photosynthetic community (PC/B = 0.45). Furthermore, the system showed a poor performance in the wastewater treatment with removal efficiencies < 20 % (Table 1). Anaerobic conditions were stablished allowing that strictly anaerobic bacteria such as *Clostridium* sp., *Parabacteroidetes* sp. and *Bacteroidetes* proper, as well as facultative photobacteria such as *Rhodobacter* sp. (Figure 1). Besides that, the photosynthetic microorganisms showed a variation in their abundance, where *Desmodesmus aramtus* and *Acutodesmus deserticola* increased their abundance up to 30 % of total photosynthetic

microorganisms. The bloom of these species belonging to the family Scenedesmaceae seems to be related to their high half saturation constant (K_m) for ammonium uptake (Takeya *et al*., 2004), and their capability to adapt their internal N/P biomass to tolerate increase in the nitrogen concentration (Whitton *et al*., 2016). In order to detect the sharply decline to the species belonged to Cyanobacteria, the algicidal activity of *Pseudomonas* sp. could be associated to the inhibition growth of Cyanobacteria (Zheng *et al*., 2008; Zhou *et al*., 2016). Finally, the presence of Cryptomycota (Fungi), Platyhelminthes (Animalia) and Nematoda (Protist) observed at a HRT of 2 d, helps to understand the decline of the photosynthetic community that could suffer parasitism and predation by them.

5.5 Conclusions

This study described the microbial dynamics and ecologic interaction into the microalga-bacteria agglomerates (MABAs) to treat municipal wastewater using HRAP systems. The HRT variable was detected as an effective factor to control the photosynthetic microorganism growth (Chlorophyte and Cyanophyta), favoring mainly the positive ecology interaction as mutualisms, in turn to allow a satisfactory performance respect to the organic matter, nutrients removal and settling performance. Furthermore, the study of the metabolic ways and genes expression by means of metagenomics and metatranscriptomic would be an excellent approach to characterize microbial structure of the MABAs community.

6

A Dynamic Model for Microalgae-Bacteria Aggregates used for Wastewater Treatment

La version original está en proceso de elaboración: Alejandro Vargas, Juan Sebastián Arcila, Sebastián Escobar-Alonso, Germán Buitrón, Microbial structure, A Dynamic Model for Microalgae-Bacteria Aggregates used for Wastewater Treatment, para someterse en Water Research

6.1 Introduction

Microalgal-bacterial (MAB) systems used for wastewater treatment have been gaining attention in recent years because of their capability of simultaneous treatment and resource recovery from a waste stream. On one hand, the fact that microalgae produce oxygen and consume carbon dioxide (CO_2), while bacteria use the former and produce the latter, leads to a virtuous interaction that could avoid the need for external aeration, while mitigating CO_2 emissions (Muñoz and Guieysse, 2006). Additionally, the energy needed for operation is harvested from (sun)light, making it a low-energy solution to wastewater treatment. Recent research has shown that under adequate operating conditions, microalgae and bacteria can form aggregates that sediment easily (Arcila et al., 2016), thus opening the possibility for harvesting this biomass and using it as feedstock for other energy-producing (bio) processes. Thus, MAB systems have a promising future as zero- or even negative-energy systems.

Although several experimental studies have shown the potential of MAB systems to treat several types of wastewater without supplying any external oxygen (Manser et al., 2016), to form microalgal-bacterial aggregates (MABA) in a sustained manner, and to operate correctly for prolonged periods of time, the systems are not yet completely understood and may not be resilient enough to withstand the unavoidable variations in feed flow and composition, as well as changes in environmental conditions that could collapse the system. After all, they are biological (i.e. living) systems and are subject to population dynamics.

One way to counter the effects of changing environmental or external perturbations, as well as our ignorance of the biology involved, is to use feedback control strategies to maintain the proper operating conditions, or even to optimize the system operation with respect to some criteria, e.g. cost, biomass productivity, treatment efficiency, etc. However, feedback control design usually requires a mathematical model of the system dynamics, either to design the controller, or simply to have a proper numerical simulation platform. Of course, models are also useful for other analytical purposes, since they also help us understand better the system and sometimes even "discover" properties otherwise hidden by experimental data.

We propose here a mathematical model that describes the dynamics in a simplified MAB system and support it with experimental data. The model is based on the Activated Sludge Model 1 (ASM1) of the IWA (Henze et al., 2000), adding the effect of inorganic carbon production and consumption (modelled as dissolved CO_2) by also considering a generic microalgae population which consumes nitrogen as ammonia (NH_4^+) under aerobic conditions and nitrate (NO_3^-) under anoxic conditions, as originally proposed by Zambrano et al. (2016). In fact, the model proposed is a modification of the latter.

6.2 Materials and Methods

Three batch experiments were performed. For each experiment, MABA were collected from a 50 L laboratory high-rate algal pond (HRAP), described by Arcila and Buitrón (2016), operating with a HRT of 10 d. The volatile suspended solids (VSS) were measured according to Standard Methods. On the other hand, wastewater entering a nearby WWTP was also collected and supplemented with ammonia at different concentrations; its COD was also determined. The HRAP had been operated for more than one year so the MABA were stable with very good settling properties, e.g. a settling velocity of 1.4±0.2 m/h and an almost granular morphology.

Each experiment consisted of four batch cultivations in 0.5L bioreactors under the same initial COD concentration (same wastewater), but different initial biomass concentrations achieved through dilutions. This lead to four S0/X0 ratios of 0.25, 0.5, 1 and 2 gCOD g^{-1}VSS. The three experiments had fixed illumination for approximately 13 h, provided by LED lamps, alternating with approximately 11h of darkness, coinciding with the lighting conditions at the lab during each day. Temperature was not controlled, but remained at 20±3 ºC throughout the experiment, with slight decreases during the night.

For the first two experiments, the bioreactors were sprayed with CO_2–enriched air (35% CO_2 content) during the illumination periods, but not during darkness; anoxic conditions thus occurred during darkness and DO rose to mean saturation values of 7.53 mg/L during the day (the altitude at the lab is 1900 m). For the third experiment this enriched air was sparged continuously (no anoxic conditions). Samples for COD, ammonia, nitrite and nitrate were taken for off-line measurements whenever the illumination conditions were changed. The

data was afterwards analyzed using the Scilab numerical simulation platform, setting some parameters to values obtained from literature (mainly the stoichiometric coefficients) and fitting only the specific reaction rates using a Nelder-Mead numerical optimization algorithm to minimize the weighted squared sum of residuals.

6.3 Results and Discussion

The collected data are shown on Figure 6.1 for the three experiments, for soluble COD, ammonia (NH_4^+) and the sum of nitrate and nitrite (NOx in the figure); all units have been transformed to COD or nitrogen equivalents. It is interesting to notice that while clearly the NH_4^+ consumption rate is seemingly proportional to the biomass concentration; the COD consumption rate seems to be little affected by it. This clearly shows that the bacterial portion of biomass follows different kinetics than the algal counterpart. As expected, COD is consumed faster under aerobic conditions, while ammonia is not consumed during anoxia. There is nitrification and denitrification and when all the NH_4^+ has been consumed and COD is no longer present, the microalgae seem to consume nitrate at a slower rate.

With these observations we propose a simple model that considers only two populations, microalgae and bacteria, and at least five reactions: growth of bacteria by consumption of COD under aerobic conditions (heterotrophs), growth of bacteria by consumption of COD and nitrate under anoxic conditions (heterotrophic denitrifiers), growth of bacteria by consumption of nitrate under aerobic conditions (autotrophic nitrifiers), growth of microalgae under illumination by consumption of ammonia, and growth of microalgae by consumption of nitrate under illumination and ammonia limitation. Furthermore, we add decay/maintenance reactions for both bacteria and microalgae. The kinetic expressions are all Monod, expect for the growth of bacteria on COD, which is of Contois type to take into account the phenomenon observed. These are summarized in Table 6.1

As can be appreciated, the proposed model fits the data well, and the parameter variation is within the expectations. The rate of microalgae growth on NH_4 is much larger than the one on NO_3^-. Bacterial nitrification does occur, but at a slower rate than the uptake of NH_4^+ by microalgae, whereas there is a clear denitrification process during the anoxic phases. Figure

6.2, shows the result of the simulation for the first experiment after fitting some parameters, which are the ones shown on Table 6.2.

Tabla 6.1. matrix for the proposed model (the CO_2 and DO columns are omitted)

Process	**Variables**					**Rate equation**
	X_{alg}	X_{bac}	S_{COD}	S_{NH}	S_{NO}	
Microalgal growth on NH_4	1			$-\frac{1}{Y_{NH}}$		$\mu_{alg}^{NH} X_{alg} \frac{S_{NH}}{K_{NH}+S_{NH}} \frac{S_{CO2}}{K_{CO2}+S_{CO2}} \frac{I_{hv}}{K_{hv}+I_{hv}}$
Microalgal growth on NO_3	1				$-\frac{1}{Y_{NO}}$	$\mu_{alg}^{NO} X_{alg} \frac{S_{NO}}{K_{NO}+S_{NO}} \frac{K_{NH}}{K_{NH}+S_{NH}} \frac{S_{CO2}}{K_{CO2}+S_{CO2}} \frac{I_{hv}}{K_{hv}+I_{hv}}$
Microalgae decay	-1			f_N^{alg}		$b_{alg} X_{alg}$
Aerobic growth on bacteria		1	$-\frac{1}{Y_H}$	$-f_N^{bac}$		$\mu_{bac}^{COD} X_{bac} \frac{S_{COD}}{K_{COD} X_{bac}+S_{COD}} \frac{S_{O2}}{K_{O2}+S_{O2}}$
Denitrification		1	$-\frac{1}{Y_H}$	$-f_N^{bac}$	$-\frac{1-Y_H}{2.86 Y_H}$	$\mu_{bac}^{NO} X_{bac} \frac{S_{COD}}{K_{COD} X_{bac}+S_{COD}} \frac{S_{NO}}{K_{NO}+S_{NO}} \frac{K_{O2}}{K_{O2}+S_{O2}}$
Nitrification		1		$-\frac{1}{Y_A} - f_N^{bac}$	$\frac{1}{Y_A}$	$\mu_{bac}^{NH} X_{bac} \frac{S_{NH}}{K_{NH}+S_{NH}} \frac{S_{O2}}{K_{O2}+S_{O2}}$
Bacteria decay		-1		f_N^{bac}		$b_{bac} X_{bac}$

Tabla 6.2. Fitted parameters for the three experiments.

Parameter	μ_{alg}^{NH} **(d^{-1})**	μ_{alg}^{NO} **(d^{-1})**	μ_{bac}^{COD} **(d^{-1})**	μ_{bac}^{NO} **(d^{-1})**	μ_{bac}^{NH} **(d^{-1})**	K_{COD} **(gCOD/gCOD)**
Experiment 1	1.78	0.023	1.613	0.116	0.014	1.72
Experiment 2	3.57	0.011	1.686	0.042	0.018	3.87
Experiment 3	3.14	0.010	2.104	0.207	0.006	2.57

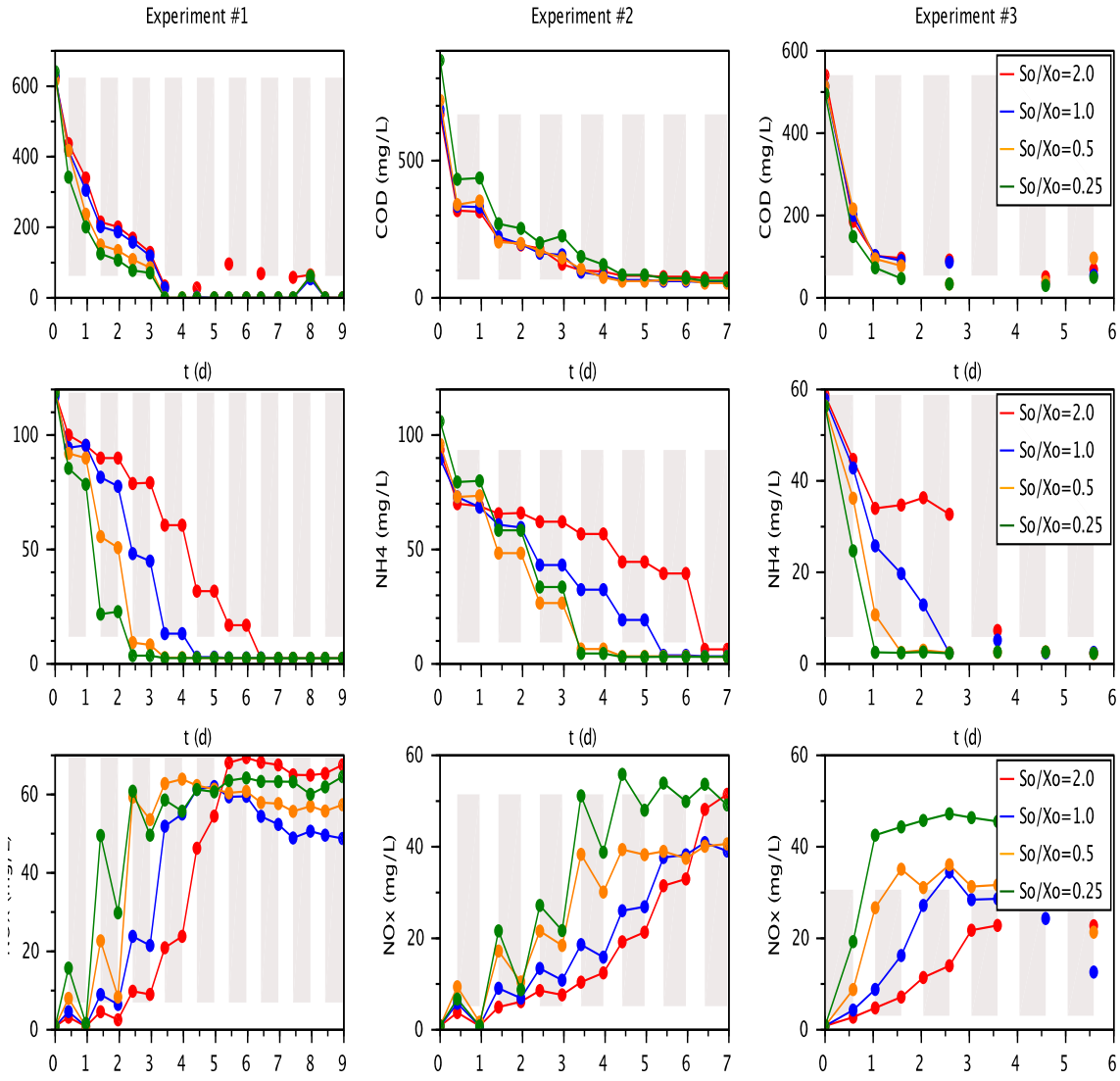

Figura 6.1. Experimental data to be fitted; gray bars indicate darkness periods; colors indicate different X_0.

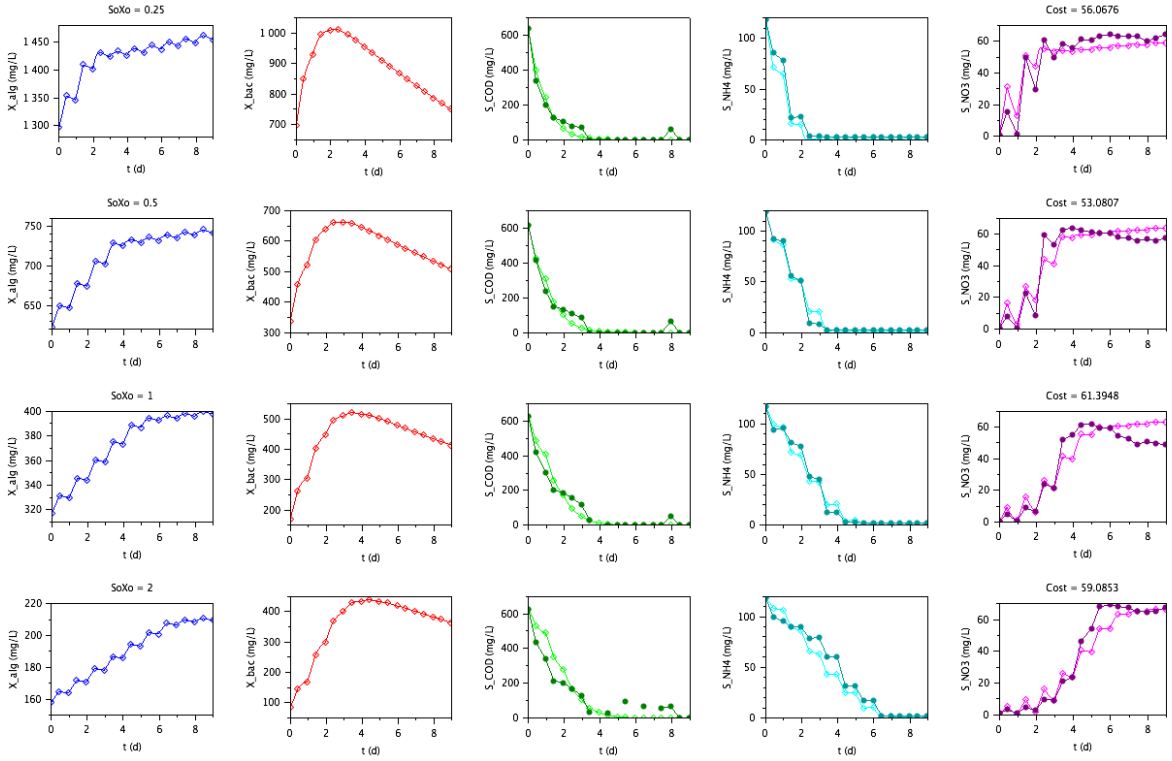

Figura 6.2. Simulation results for the first experiment showing a good fit with the data (dark colors).

6.4 Conclusion

A simple model for microalgae-bacterial aggregates dynamics for wastewater treatment has been proposed. It is a based on ASM1, adding the microalgal population and its processes. A novelty is the use of Contois kinetics for the COD uptake process rate. Further studies will test the model prediction capabilities for a continuous regime in an HRAP.

7

DISCUSIÓN Y CONCLUSIÓN GENERAL

7.1 Discusión general

La aplicación de los sistemas microalga-bacteria para el tratamiento de aguas residuales y el potencial uso de su biomasa para la generación de productos de valor agregado, ha sido limitada por el alto costo de la cosecha y deshidratación de este tipo de biomasa, llegando incluso a alcanzar valores hasta un 60 % del costo total de producción de la biomasa (Olguín, 2011; Van den Hende et al., 2011a), limitando la rentabilidad del proceso y su escalabilidad.

Entre las múltiples soluciones para disminuir los costos de cosecha, la generación de agregados microalga-bacteria conocidos como MABAs ("Microalgae bacteria aggregates") han surgido como una alternativa promisoria. Sin embargo, su formación hasta el momento ha sido posible bajo condiciones por lotes secuenciales, considerando factor clave los tiempos de sedimentación como una estrategia de presión de selección que favorece la proliferación de especies microalgales de mayor sedimentación (Gutzeit et al., 2005; Tiron et al., 2015; Van den Hende et al., 2011a).

En consecuencia, la investigación desarrollada ha permitido generar un avance científico orientado hacia el entendimiento de la formación de MABAs para el tratamiento de aguas residuales municipales, mediante el control de variables ambientales como la irradiancia solar y factores operativos como el tiempo de retención hidráulico (TRH), empleando reactores HRAP operados en modo continuo. La evaluación de estos parámetros fue llevada a cabo en estos reactores con un volumen de trabajo de 50 L, operados bajo condiciones de laboratorio (CL, intensidad lumínica de 3300 Wh m^{-2} d^{-1}, con fotoperiodos de oscuridad-luz (12h-12h) y TRH entre 2 y 10 días) y en condiciones al aire libre (AL, irradiancia solar entre 6213 y 2741 Wh m^{-2} d^{-1} y TRH de 10 días).

Los resultados experimentales bajo condiciones CL y AL evidencian que la formación de MABAs están directamente relacionadas con el aumento en la actividad nitrificante asociada a altas concentraciones de NO_3^- (entre 10 y 32 mg L^{-1}) y la proliferación de microalgas filamentosas, bajo pH cercanos a 8 (asociadas con alto contenido de CO_2 y HCO_3^-) y concentraciones de oxígeno disuelto (DO) mayores a 3 mg L^{-1}. Estas condiciones de proceso

fueron obtenidas en TRH de 10 y 6 días, bajo niveles de irradiancia solar menores a 3800 Wh m^{-2} d^{-1} o intensidades lumínicas constantes de 280 W/m^2 correspondientes a condiciones AL y CL, respectivamente. La inoculación en ambas condiciones experimentales fue realizada empleando un cultivo crecido en el laboratorio y dominado por el género *Scenedesmus* sp.

Durante la formación de MABAs en ambas condiciones experimentales (AL y CL) fueron identificadas dos etapas, la primera etapa llamada "agregado inicial" asociada a la formación de pequeños agregados (100 a 400 μm), caracterizados por la proliferación de microalgas filamentosas y la segunda etapa llamada "agregado maduro" compuesta por agregados de mayor tamaño (1000- 2600 μm), donde su morfología varía dependiendo de la condición experimental, siendo la formación de flocs mayoritaria bajo CL, mientras que en AL, las estructuras granulares fueron dominantes en el sistema.

La relevancia de las microalgas filamentosas en la formación de MABAs, está relacionada principalmente con características tales como, alta capacidad de absorción de cationes sobre la pared celular (Ca^{+2}, Mg^{+2}, Na^{+}, etc) (Andrade et al., 2005), crecimiento ramificado y formación de estructuras nodulares (Celekli et al., 2016; Otero et al., 2004), convirtiéndolas en una clase de biopolímero catiónico capaz de formar estructuras reticuladas que permitan la adhesión de microorganismos de vida libre que contribuye a la formación de agregados de mayor tamaño, tal y como fue observado en el capítulo 3 y 4. Con base en lo anteriormente mencionado, el mecanismo de puenteo planteado por Salim et al., (2011, 2014), el cual se fundamenta en la captura de células más pequeñas a través de la formación de redes de polímeros cargados positivamente, se observa como el mecanismo más apropiado para describir el proceso de agregación inicial para la formación de MABAs en nuestro sistema.

La presencia de microalgas filamentosas fue detectada en nuestro sistema como una respuesta al incremento en la actividad de las bacterias nitrificantes asociada a una alta concentración de nitratos (hasta 30 mg L^{-1}) y una baja concentración de amonio (menores a 1 mg L^{-1}) en el medio de cultivo, identificadas como condiciones propicias para su crecimiento (Pastich et al., 2016; Pelechata et al., 2016). Lo anterior deja en evidencia que la interacción mutualista

entre bacterias nitrificantes y microalgas filamentosas en nuestro caso *C. elegans* y Cianobacterias filamentosas es considerada clave en la etapa inicial de la formación de MABAs.

Por otra parte, en la etapa madura, la presencia o ausencia de microalgas filamentosas definen la morfología de los MABAs. En el caso de CL, la población microalgal de los MABAs presentó un crecimiento radial ramificado (Capítulo 3), dominado principalmente por microalgas filamentosas de la especie *C. elegans*, desplazando al género *Scenedesmus* sp. (usado como inóculo) como microalga dominante, llevando incluso a que su presencia fuese despreciable en el sistema (menores a 2 % de las lecturas totales para Eucariotas). La presencia de este microorganismo filamentoso puede estar asociado al inoculo microalgal o al cuerpo de agua residual domestica utilizada en nuestras pruebas experimentales. Caso contrario fue observado bajo condiciones AL, donde el género *Scenedesmus* sp. jugó un papel crucial en la formación de estructuras granulares de MABAs, presentando una abundancia relativa hasta un 95 % del total de microalgas presentes en el agregado.

Una de las principales causas por la que los gránulos de MABAs en su estado maduro (Etapa 2) no fue dominada por microalgas filamentosas, se asoció al efecto adverso de la exposición de este tipo de microalgas filamentosas (cianobacterias) a condiciones de irradiancia solar mayores a 341 W m^{-2}, cuyo espectro de onda incluye la irradiación UV (280 - 400 nm). Bajo está condición de irradiancia se ha demostrado que ocasiona el rompimiento en la estructura filamentosa de *Cianobacteria* como en el caso del género *Arthrospira platensis* (Wu et al., 2005). Este tipo de restricción de crecimiento dada por el espectro de onda que incide en el cultivo microalgal, debe ser considerado un factor relevante para la obtención de MABAs con morfología granular. Debido a esto, la optimización del espectro de luz absorbido por un sistema microalga-bacteria debe ser abordado con una mayor especificidad, con un interés principal en la actividad metabólica de los microorganismos participes en el proceso de formación de gránulos.

Adicionalmente, la formación de MABAs granulares evidenciaron una correlación positiva con el incremento en las sustancias exopoliméricas ligadas (SEP-L); compuestas

principalmente por proteínas. Estos resultados son coherentes con los observados por Xiong y Liu et al., (2007) y Zhang et al., (2015) donde el incremento de SEP como proteínas, disminuyen la carga superficial negativa, reduciendo la repulsión electroestática entre las células y favoreciendo la agregación y estabilidad de la estructura granular.

La producción de SEP como proteínas en los MABAs granulares no solo está asociada a bacterias. La alta densidad poblacional de la microalga perteneciente al género *Scenedesmus* sp acompañado de aglutinamiento de SEP observada en el núcleo del gránulo (Figure 4.5), muestran la relevancia de este género en la formación de SEP-L. Resultados observados por Liu et al., (2012), demostraron que el género *Scenedesmus* sp presenta un incremento en el contenido de proteínas debido a una disminución en los niveles de intensidad lumínica bajo condiciones limitantes de nitrógeno. Este antecedente, permite proponer para nuestro sistema que la disminución en la irradiancia solar de 6213 a 2741 Wh m^{-2} d^{-1}, bajos limitaciones en amonio como fuente de nitrógeno, promueven condiciones de estrés que propician la exudación de SPE como proteínas por parte del género *Scenedesmus* sp, contribuyendo a la estabilidad de la estructura granular en los MABAs.

En cuanto a las características de los sistemas MABAs con relación al tratamiento de aguas residuales, la contribución sustancial de este tipo de agregados está relacionada con el aumento en la velocidad de sedimentación del sistema microalga-bacteria, alcanzando una velocidad de sedimentación de 8.3 m h^{-1} en CL y 18 m h^{-1} bajo condiciones AL y remociones de sólidos suspendidos totales (SST) mayores a 90 % (menores a 70 mg L^{-1}), que cumple con los requerimientos establecidos en la NOM 001.

Adicionalmente, ambos tipos de MABAs (flocs y gránulos), presentan similares eficiencias de remoción de DQO (mayores a 95%), NT (entre 60 y 80 %) y P-PO_4^{-3} (hasta un 50 %), evidenciado que el tipo de agregado y la especia dominante que lo conforma no afecta la eficiencia de tratamiento del agua residual municipal. Sin embargo, la excelente eficiencia de remoción s se ve notoriamente afectada por altas irradiancias solares (6213 Wh m^{-2} d^{-1}) y TRH de 2 días, estas condiciones son caracterizadas por la ausencia de MABAs, donde el alto nivel de irradiancia solar presentó una alta densidad de *Scenedesmus* sp. de vida libre

que limitan la eficiencia de sedimentación (menores a 30 %), mientras que condiciones de TRH de 2 días, se identificó como una condición de operación que lleva al colapso del sistema de tratamiento (eficiencias de remoción de DQO, TN, $P\text{-}PO_4^{3-}$ menores al 15 %) y la expulsión de la biomasa microalgal del reactor (condiciones de lavado del reactor).

Retomando las excelentes características de tratamiento en presencia de MABAs, la variación en la remoción de NT observadas bajo CL, está relacionada con la actividad nitrificante en el sistema, donde la más alta remoción (del 80 %) fue obtenida bajo TRH de 6 días. Este comportamiento está relacionado con una disminución en el contenido de nitratos, causado por un incremento de casi el doble en la carga orgánica bajo TRH de 10 días (54 ± 19 mg COD L^{-1} d^{-1} a 119 ± 19 mg COD L^{-1} d^{-1}) y una disminución en el OD hasta 3 mg O_2 L^{-1}, ocasionando una competencia entre las bacterias nitrificantes (Autótrofos), heterótrofos y las microalgas por el oxígeno remanente y el $N\text{-}NH_4^+$ presente en el sistema.

En el caso de remoción de fósforo, los resultados obtenidos cuando el proceso fue operado con la presencia de MABAs (hasta 50 %) se encuentran dentro de los valores promedios reportados para el tratamiento de agua residual de origen porcícola (de Godos et al., 2009, 2011) y de efluente secundarios (Van den Hende et al., 2016) (Tabla 1.1), observándose alta presencia de nitratos (hasta 30 mg L^{-1}).

Aunque los parámetros de DQO, TN y $P\text{-}PO_4^{-3}$ cumplen con los lineamientos exigidos por la SEMARNAT (NOM 001) respecto a las descargas de aguas residuales en aguas y bienes nacionales, el uso de esto nutrientes ($N\text{-}NO_3^-$ y $P\text{-}PO_4^{3-}$) obtenido del efluente del procesos, podría ser utilizado en un proceso posterior que este enfocado en la optimización de la productividad de los MABAs, cuya fuente de carbón fuese el CO_2 proveniente de origen antropogénico, siendo este proceso de múltiple funcionalidad, incrementando la productividad de la biomasa obtenida de los MABAs y mitigando las emisiones de CO_2 al medio ambiente.

Otro de los factores de relevancia de los MABAs para el tratamiento de aguas residuales está relacionado con potencial energético de su biomasa. Los resultados obtenidos bajo CL

reflejan un alto potencial bioquímico de metano (PBM) de 347 ± 3 mL CH_4 g SV^{-1} bajo TRH de 10 días, siendo significativamente mayor a muchos de los sistemas microalga-bacteria que utilizan pretratamientos como se observa en la Tabla 2.2. Sin embargo, la disminución en el PBM de los MABAs en TRH de 6 días, deja como evidencia el efecto adverso de la diversidad microbiana presentes en los MABAs sobre el potencial energético, siendo identificadas las cianobacterias como *Microcystis* sp. y Diatomeas tales como *Nitzschia* sp., *Navicula* sp., *Gomphonema* sp. los prinicipales microorganismos que afectan las características energéticas de la biomasa, debido a su características de exudación de compuestos tóxicos como microtoxinas por parte de *Microcystis* y la presencias de estructura inorgánica de difícil biodegradación (silicatos) como en el caso de las diatomeas..

7.2 Conclusión general

Los resultados obtenidos en la investigación, evidencian la formación de agregados de microalga-bacteria a partir del tratamiento de agua residuales municipales, empleando HRAP operados en modo continuo. la operación de este tipo de reactor bajo tiempos de retención hidráulica (TRH) de 6 y 10 días, promueven el dominio de las comunidades fotosintéticas eucariotas en el sistema microalga-bacteria con altas eficiencias tanto de remoción de materia orgánica y nutrientes, así como la formación de agregados con altas velocidades de sedimentación (hasta 18 m h^{-1}). Sin embargo, TRH de 2 días, asociados a una alta carga orgánica de 290 mgCOD L^{-1} d^{-1} y un incremento en la comunidad de microorganismos anaerobias, se establece como una condición de operación inapropiadas, llevando a la desintegración de los agregados, la pobre eficiencia de remoción de materia orgánica y nutrientes (menores al 15 %), conduciendo a lavado de la biomasa microalga-bacteria en el reactor.

Condiciones de irradiancia (solar o artificial) inferiores a 3800 Wh m^{-2} d^{-1} y TRH de 10 días, caracterizados por niveles de pH cercanos a 8 y OD mayores a 3 mg L^{-1}, promovieron la interacción mutualista entre bacterias nitrificantes y microalgas filamentosas, donde la proliferación de estos microorganismos filamentosos perteneciente a clorófitas y

cianobacterias sirven como una matriz de soporte microbiano durante la etapa inicial de la formación de MABAs. Adicionalmente, la competencia por la fuente de $N\text{-}NH_4^+$ entre bacterias nitrificantes y la especie *Scenedesmus* sp. bajo estas condiciones operativas de baja irradiancia solar, inducen limitaciones en el consumo de $N\text{-}NH_4^+$ en el metabolismo de la microalga *Scendesmus* sp., propiciando un estado de estrés que promueve la exudación de EPS ligadas como proteínas, promoviendo la formación granular de los MABAs.

Finalmente, los cambios en la diversidad microbiana de los MABAs, propiciadas por variaciones en el TRH, afectaron significativamente el PBM de la biomasa obtenida del procesos de tratamiento, donde la proliferación del género *Microcystis* sp. bajo TRH de 6 días causó un decrecimiento en un 20 % del PBM, respecto a los valores más altos obtenidos a TRH de 10 días (347 ± 3 $mLCH_4$ gSV^{-1}) donde la presencia de este género es considerablemente baja (5 % del total de numero de secuencias leídas de procariotas en el sistema).

Los resultados obtenidos durante el proceso investigativo, dejan en evidencia factores claves para estudios futuros sobre la formación MABAs, entre los cuales se identifican los siguientes nichos de investigación:

- La optimización de las variables TRH e intensidad lumínica que conduzcan a la formación de morfologías granulares de MABAs con alto desempeño en el tratamiento de aguas residuales, excelentes condiciones de sedimentación y alto valor energético.
- La Identificación que rango de longitud de onda propicia la formación de agregados granulares.
- El estudio de la actividad metabólica de la población microbiana tanto de algas y bacterias durante la formación de MABAs, que permitan dilucidar de manera concreta los mecanismos de formación y los microorganismos claves en la génesis de los MABAs.
- La evaluación del efecto de la estacionalidad sobre eficiencia del tratamiento de aguas residuales, funcionalidad metabólica y la robustez de las estructuras granulares de los MABAs.

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