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Shabana Bhatti, Robert Richards and Patrick McGinn

ABSTRACT

In recent years, the use of microalgae as feedstock for many marketable products, such as animal/aqua feeds, bioplastics and fertilizers, has gained renewed interest due to their fast growth potential coupled with relatively high lipid, carbohydrate and nutrient content. An algal biorefinery at an industrial site has the potential to sustainably and profitably convert carbon dioxide emissions into microalgal biomass and concomitantly reduce nitrogen and phosphorus from wastewaters. Industrial wastewaters are a potential alternative to traditional media used for large-scale microalgal cultivation. Pulp and paper mills are major consumers of water resources and discharge a huge amount of water to nearby lakes or rivers. This study investigated whether pulp and paper mill waste water is suitable for microalgal cultivation with the aim of achieving significant biomass production. Six different process waters from one Canadian pulp and paper mill were tested with two freshwater green microalgae. All of these waters were unable to support growth of microalgae due to inadequate nutrient concentrations, colour, turbidity and possible toxicity issues.

Key words | effluents, green algae, microalgae, pulp and paper industry, remediation, wastewater

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HIGHLIGHTS

- Two freshwater microalgae strains were screened for potential growth on pulp and paper effluents.
- Pulp and paper wastewater effluents were unable to support growth of the microalgae.
- Chemical residues from the pulping process, such as peroxide and tannin, may inhibit algal growth.

INTRODUCTION

Microalgae are a large and diverse group of photosynthetic microorganisms. With little more than photons, water, and a few essential nutrients, microalgae reduce CO_2 to carbohydrates. Large-scale microalgal cultivation has several advantages over traditional agricultural production of cash crops. These include greater rates of areal productivity, the ability to use marginal lands for cultivation and the potential to use nutrient-rich wastewater streams (McGinn *et al.* 2011). In recent years, research has started to focus on exploiting wastewater rather than freshwater as a growth medium for microalgal biomass production. Municipal and agricultural wastewaters contain high levels of nitrogen and phosphorus, essential nutrients for algal growth. Typically, nitrogen and phosphorus concentrations can be 10–100 $mg{\cdot}L^{-1}$ in municipal wastewaters and >1,000 $mg{\cdot}L^{-1}$ in agricultural wastewaters. These high levels of nitrogen and phosphorus support robust growth of many different species of freshwater microalgae and thereby represent a very effective remediation strategy at the same time (Woertz *et al.* 2009; Mohsenpour *et al.* 2021).

A range of products can be produced from microalgae. Commercial production of microalgae includes human health food and functional food, feed additives, aquaculture, colouring substances, antioxidants and polyunsaturated

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fatty acids (Bajpai 2001), although the algal biomass cultivated in wastewater is unsuitable for any food or feed uses.

Various components can then be extracted from the algae and refined into several different products, such as biodiesel, bioplastics and lubricating oil. The anaerobic digestion of algae to produce methane/renewable natural gas or hydrothermal processing to a hydrothermal liquefaction (HTL) oil are other approaches that are garnering increased attention world-wide (Milledge *et al.* 2019). There has been a large interest in using microalgae for biofuel production, with the extraction of energy-dense triacylglycerols and their subsequent conversion to diesel or jet fuels being the most commonly discussed pathway. In the context of biofuel production, growth of microalgae on wastewater could be acceptable as the product would not enter the human or animal food chain. In addition to biodiesel, other fuels that could potentially be produced from microalgal biomass are ethanol through fermentation of the carbohydrate, methane production by anaerobic digestion or thermal gasification, or direct combustion to produce heat and electricity.

Canada's forest sector is a major contributor to its growing bioeconomy. While traditional forest products have experienced declining markets and global competition in the past decade, market demand for liquid biofuels and green chemicals is expanding (Hurmekoski *et al.* 2018). Renewable hydrocarbons can displace fossil fuels and petrochemical feedstocks, thereby reducing the carbon intensity of extracting and refining fossil resources. The production of energy, chemicals and materials integrated within an existing pulp mill offers additional revenue streams and potential economic returns. This biorefinery approach is a way to expand the product portfolio and to open new business opportunities. A number of options are being explored for the pulp and paper industry, and one that has not yet received much attention but has interesting potential is the integration of algal biomass production into mill operations. This kind of integration could be facilitated by the mill's existing infrastructure, expertise and marketing experience for bio-based products. Underused process streams, such as low-grade heat, or underused capital equipment, including dyers and tanks, could be brought in to service with minor investment costs. This concept is not new: in Sweden, there is a project underway to develop a process that effectively allows algae to treat nutrients in mill wastewater and fix carbon dioxide from flue gases (Ekendahl *et al.* 2018).

The development of an algal biorefinery would provide a number of benefits to a pulp and paper mill: (1) low-cost treatment of wastewaters and other processing streams, thereby reducing outflow; (2) a second product stream largely derived from the wastes (nutrients, heat, CO_2) from the pulp and paper mill core business. Algal biomass could be sold by the mill, converted into electricity and sold, or used as a fuel to reduce mill operating costs; (3) the ability to capture and recycle carbon, which, in the future, may provide additional income through credits that can be sold while marketing paper products as less CO_2-intensive than those of competitors; (4) production of algal biomass that could be used for the production of paper, animal feed, polymers, and other biomaterials.

Conventional paper and pulp wastewater treatment includes physicochemical and biological methods (Pokhrel & Viraraghavan 2004; Buyukkamaci & Koken 2010). Biological treatment methods use living microorganisms such as bacteria, fungi and algae for wastewater treatment, and are known to be more environmentally friendly and cost effective in comparison to physicochemical treatments, which require high energy input. The treatment of pulp and paper mill wastewater effluents and recycling of water from pulp and paper mills by microalgae is particularly interesting from the point of view of environmental sustainability.

Integrating microalgal cultivation with pulp and paper mill and industrial CO_2 emissions represents an opportunity to develop, test and optimize the necessary technologies to make microalgal biofuels and bioproducts more cost effective and efficient (Kouhia *et al.* 2015).

Basic overview of the pulp and manufacturing process at Port Hawkesbury Paper Mill, Nova Scotia

The pulp and paper mill chosen for this study is owned and operated by Port Hawkesbury Paper and is located on the shores of the Strait of Canso near the town of Port Hawkesbury in Nova Scotia, Canada (45.61603°N, −61.35513°E). At this mill, raw softwood logs are debarked, chipped and pulped using a thermo-mechanical process (TMP), which is extremely energy intensive. During TMP, steam is injected to soften the wood chips, loosening the fibres to encourage the formation of pulp. The pulp is subsequently dewatered by multiple pressings and then bleached and repressed. Virgin pulp is then mixed with clay and other additives as it enters the paper machine (PM2).

The pulp and paper industry is considered to be a major consumer of natural resources, energy and water. The most significant environmental impacts result from the pulping and leaching processes: some pollutants are emitted to the air, others are discharged to the wastewaters, and solid wastes are generated (De la Noüe *et al.* 1992; Hubbe *et al.*

2016). In general, pulp and paper mill effluents contain a mixture of various organic compounds, such as degradation products of carbohydrates, lignin, and extractives (Uğurlu *et al.* 2008). Polluting effluents are formed especially in bleaching operations, but also in wood preparation, pulping, pulp washing, screening, the paper machine, and coating operations (Ali & Sreekrishnan 2001; Pokhrel & Viraraghavan 2004).

Prior to discharge, wastewater from the mill operations is treated for biochemical oxygen demand (BOD), suspended fibrous wood solids, and toxicity reduction. A flow diagram showing the basic elements of the wastewater treatment system used by Port Hawkesbury Paper is shown in Figure 1. Briefly, bar-screened effluent from TMP and PM2 containing fibrous suspended solids are sent to primary clarifiers. The primary clarified effluent is sent to a series of bioreactors for secondary treatment by an activated sludge process, which is an aerobic biological treatment method that uses the metabolic process of a suite of microorganisms to remove dissolved organic matter (DOM) from the effluent, allowing complete reduction of BOD. This outflow empties into the secondary clarifiers, a series of four large outdoor concrete tanks, for further settling before finally flowing into the Strait of Canso. Settled sludge collected from the primary and secondary clarifiers is de-watered and burnt in the biomass furnace to produce steam.

Wastewaters from pulp and paper mills may offer an inexpensive source of nitrogen and phosphorus. By reducing the levels of nitrogen and phosphorus in the wastewater, paper mills are aided to meet environmental standards. Efficient combination of a CO_2 source (flue gas), if available at the site, and appropriate wastewater is one of the challenges to make algae production cost effective. In this sense, pulp and paper mills may serve as a suitable site for microalgal cultivation, since the CO_2 source and the wastewater can easily be combined at one location. In addition, the waste heat from the process water can be used, reducing heating costs.

The purpose of this study is to investigate if the different pulp and paper mill wastewaters collected from different processing steps at Port Hawkesbury Paper mill can be used as a suitable medium for microalgal growth.

MATERIALS AND METHODS

Microalgal strains and cultivation conditions

Two axenic freshwater green microalgae strains were selected for this study. *Chlorella vulgaris* (UTEX 26) was obtained from the UTEX Culture Collection of Algae at the University of Texas at Austin (UTEX, Texas, USA). *Dictyosphaerium* sp. (MSWW S7) was retrieved from the algal library maintained at the National Research Council of Canada (NRC). The two strains were chosen because they are frequently isolated from wastewater ponds and are readily adaptable to such wastewaters. *C. vulgaris* has been studied extensively in wastewater treatment and biofuel production. This freshwater species grows in a range of pH and in media varying from swine slurry to secondary clarifier effluent. *Dictyosphaerium* sp. (MSWW S7) is an in-house strain that was originally isolated from the Mill Cove wastewater treatment plant in Bedford, Nova Scotia, and has been shown to grow very well on wastewater effluents (Park *et al.* 2015).

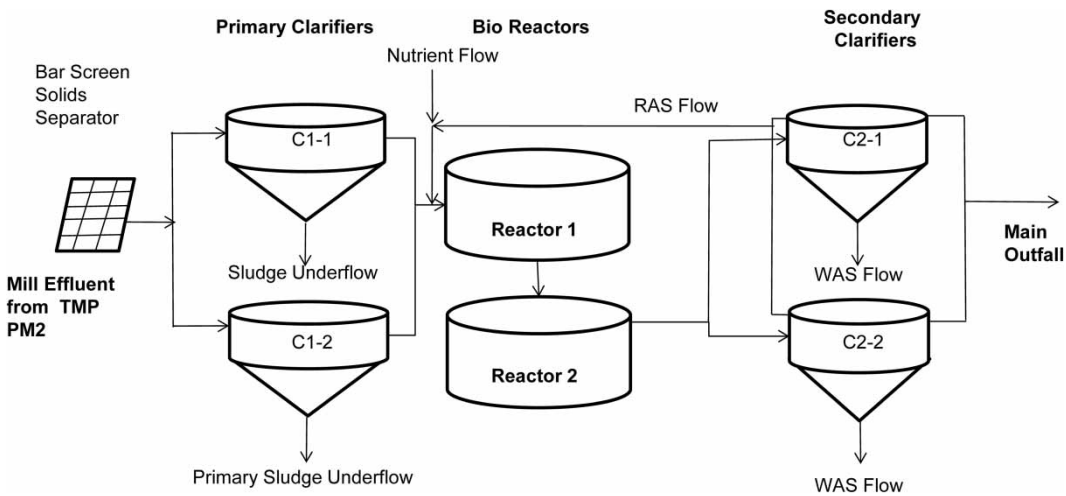

Figure 1 | A flow diagram of Port Hawkesbury Paper secondary treatment plant.

The two strains were cultivated photoautotrophically in Bold's 3NV (B3NV) growth medium in 250 mL flasks (125 mL of culture) and shaken at 120 rpm (Innova 2100, New Brunswick Scientific, USA) at 22 °C under continuous illumination of 100 μmoles·m^{-2}·s^{-1} of photosynthetically active radiation (PAR) in an environmentally controlled growth chamber (Conviron, Canada). B3NV is a modified version of Bold's basal medium (BBM) with three times the nitrate. The composition of B3NV is as follows: $NaNO_3$, 30 mL·L^{-1}, 8.82 mM; $CaCl_2{\cdot}2H_2O$, 10 mL·L^{-1}, 0.17 mM; $MgSO_4{\cdot}7H_2O$, 10 mL·L^{-1}, 0.3 mM; K_2HPO_4, 10 mL·L^{-1}, 0.43 mM; KH_2PO_4, 10 mL·L^{-1}, 1.29 mM; NaCl, 10 mL·L^{-1}, 0.43 mM; vitamin B12, 1 mL·L^{-1}; and a trace metal solution: $Na_2EDTA{\cdot}2H_2O$, 2 mM; $FeCl_3{\cdot}6H_2O$, 0.36 mM; $MnCl_2{\cdot}4H_2O$, 0.21 mM; $ZnCl_2$, 0.037 mM; $CoCl_2{\cdot}6H_2O$, 0.0084 mM; $Na_2MoO_4{\cdot}2H_2O$, 0.017 mM. B3NV provides a broad range of nutrients for a wide variety of freshwater microalgae, which in turn makes this medium suitable for the screening.

Collection and processing of process waters

In order to investigate whether wastewater effluents from a pulp and paper mill could be used as an inexpensive means of cultivating freshwater microalgae, six process waters were collected from the Port Hawkesbury Paper mill operation. They were collected from different processing steps and varied in colour, nutrient levels, and pH, as shown in Figure 2 and Table 1. The process waters collected were: primary clarifier effluent (1°), secondary clarifier effluent (2°), TMP effluent, PM2 effluent and samples from three nearby bodies of water (Goose and Grant Lakes and Landrie Lake), which are drawn and used for mill operations. The waters were collected and placed in 1 L plastic jugs into an insulated cooler where the temperature was maintained at 4–6 °C at all times by lining the bottom of the cooler with ice. Samples were transported back to the NRC's Marine Research Station (MRS) and stored at 4 °C until further processing.

Samples of the process waters were immediately sent to Maxxam Analytics Ltd (Bedford, Nova Scotia) and assayed for total nitrogen (TN), total phosphorus (TP) and total organic carbon (TOC) and trace metal element composition; these results are summarized in Table 2. In the table, there are two TP values provided by Maxxam Analytics Ltd The TP done by block digestion involves a more rigorous digestion in a sulphuric acid matrix at a higher temperature (~125 °C). The TP from the metal scan involves a gentle digestion with mixture of nitric and hydrochloric acids and is done at a lower temperature (~95 °C). The following

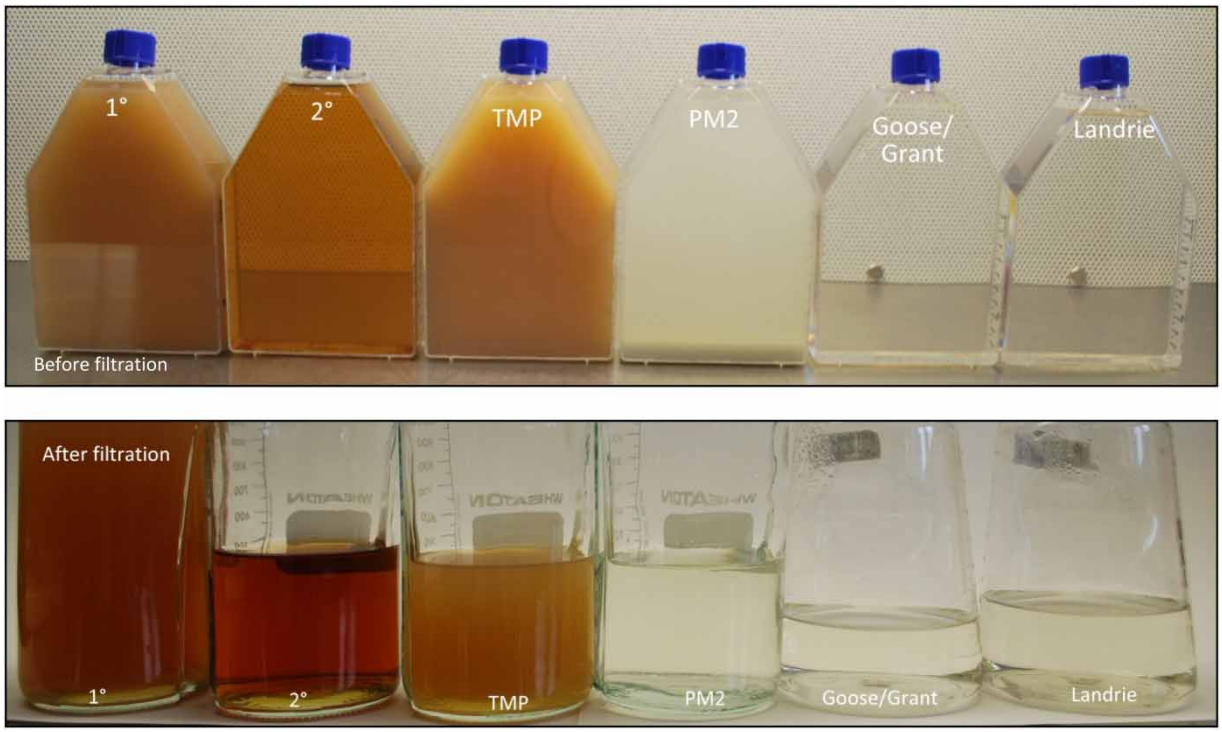

Figure 2 | Six different processed water samples collected from the Port Hawkesbury Paper mill before and after filtration: primary clarifier waste effluent (1°); secondary clarifier waste effluent (2°); TMP effluent; PM2 effluent; raw water from Goose/Grant Lakes; and raw water from Landrie Lake.

Table 1 | Description of raw unfertilized process waters collected from the Port Hawkesbury Paper mill (PHP) and nearby lakes ($n = 1$)

Sample ID	Process water	pH			Total nitrogen mg·L^{-1}	Total phosphorus mg·L^{-1}	Total organic carbon mg·L^{-1}	NaCl ppm	EC mS·cm^{-1}	TDS ppm	Temp °C
		In field	In lab	In cultures							
PHP-1	Primary clarifier effluent (1°)	6.11	6.83	7.95	8.80	0.80[a]	1,100[a]	963	1,909	1,130	24.8
PHP-2	Secondary clarifier effluent (2°)	7.80	8.38	7.95	5.93	4.20	150[b]	927	1,825	1,310	23.0
PHP-8	TMP effluent	8.03	7.95	6.98	33.5	0.58	2,000[b]	2,090	3,880	2,840	70.0
PHP-9	PM2 effluent	5.94	7.52	7.59	<1.00	0.16	190[b]	423	850	613	26.5
PHP-10	Goose/Grant Lakes	6.78	7.45	7.72	0.19	0.03	5.70	17.2	40.8	29.0	4.10
PHP-11	Landrie Lake	6.75	7.26	7.59	0.30	0.03	5.40	29.6	69.4	49.3	4.50
B3NV	Bold's basal medium (3N)	–	6.80	–	123	53.3	–	–	–	–	–

[a]Analysis performed on decanted sample due to sediment content.
[b]Elevated reporting limit due to sample matrix.

parameters were measured and recorded for each sampling site: water temperature (°C), salinity (NaCl, ppm), total dissolved solids (TDS), pH, and electrical conductivity (EC). The pH was measured at the site of each collection site (in the field), in the laboratory and in microalgal cultures after completion of growth experiments. The sampling sites' latitude and longitude coordinates were recorded using a Garmin Colorado 300 global positioning system (GPS) receiver (Garmin, Olathe, KS, USA).

Preliminary screening of microalgal strains on pulp and paper effluents in flasks

Prior to screening, the process waters were passed through a series of filtrations with the final filtration at 0.22 or 0.45 μm (depending on the process water used) to minimize any debris, particulates and microorganisms, and autoclaved. The process waters collected from the pulp and paper mill did not undergo test dilutions because of the possibility of low nutrient concentrations and that further dilutions into standard large-scale algal media would require potable water (freshwater), which can be quite expensive.

Prior to the start of the experiments, the two microalgal strains were pre-cultured in flasks containing B3NV growth medium in a Conviron on a shaker and allowed to acclimate to growth conditions as described below. For the flask-level screening, the microalgae were cultivated in 150 mL shake flasks for a period of 10–12 days in a replicated experimental block. Cultures of *C. vulgaris* and *Dictyosphaerium* sp. were grown in the six process waters with an initial cell count of approximately 60,000 cells·mL^{-1}. Three treatments were carried out for each process water: (1) process water with algae; (2) process water plus addition of nutrients (B3NV) with algae; and (3) process water plus nutrients with no algae added. The experiment was conducted on a shaker at a speed of 125 rpm in a growth chamber with a temperature of 22 °C under a continuous light regime of approximately 100 μmoles·m^{-2}·s^{-1} of PAR. Cultures were aerated with air on day 0 and switched to air enriched to 0.5% CO_2 on day 3, followed by 2% CO_2 on day 7 for the remainder of the experiment.

Growth was monitored daily by enumeration of algal cells in a Coulter Counter particle analyzer (Beckman Multisizer 3). Microalgal biomass was harvested at the end of the experiment and freeze-dried for long-term storage. The specific growth rate and total dry weight were determined for each culture grown. The flask-level experiments were performed in duplicates and results are presented as the mean values with the corresponding standard error of the mean (SEM).

Screening of freshwater green microalgae on pulp and paper effluents in microtitre plates

Screening of strains on pulp and paper effluents was also investigated using a high-throughput method based on the use of 96-well microtitre plates to determine if growth on different process waters was similar to flask-level screening. This high-throughput method provides uniform growth rates from well to well over the entire plate and can be used to screen a large number of strains on many wastewater streams. It incorporates CO_2 delivery to the entire plate in

Table 2 | Total nitrogen, total organic carbon, total phosphorus and trace metal analyses of processed waters from Port Hawkesbury Paper (PHP) mill (Maxxam Analytics Ltd)

	Water sample ID							
Analyses	Element	Units	PHP-1 Primary	PHP-2 Secondary	PHP-8 TMP	PHP-9 PM2	PHP-10 Goose/Grant	PHP-11 Landrie
Total nitrogen	N	$mg \cdot L^{-1}$	8.80	5.93	33.5	<1.00	0.189	0.30
Total organic carbon	C	$mg \cdot L^{-1}$	1,100[a]	150[b]	2,000[b]	190[b]	5.70	5.40
Total phosphorus	P	$mg \cdot L^{-1}$	0.80[a]	4.20	0.58	0.16	0.025	0.032
Total aluminum	Al	$\mu g \cdot L^{-1}$	1,400	330	1,000	1,900	120	110
Total antimony	Sb	$\mu g \cdot L^{-1}$	ND	ND	ND	ND	ND	ND
Total arsenic	As	$\mu g \cdot L^{-1}$	1.50	ND	ND	1.20	ND	ND
Total barium	Ba	$\mu g \cdot L^{-1}$	340	92.0	450	16.0	3.10	9.30
Total beryllium	Be	$\mu g \cdot L^{-1}$	ND	ND	ND	ND	ND	ND
Total bismuth	Bi	$\mu g \cdot L^{-1}$	ND	ND	ND	ND	ND	ND
Total boron	B	$\mu g \cdot L^{-1}$	71.0	ND	89.0	0.12	ND	ND
Total cadmium	Cd	$\mu g \cdot L^{-1}$	2.70	2.50	2.80	2,200	0.011	ND
Total calcium	Ca	$\mu g \cdot L^{-1}$	26,000	16,000	31,000	3.20	1,900	3,700
Total chromium	Cr	$\mu g \cdot L^{-1}$	3.40	3.20	4.50	0.48	1.30	12.0
Total cobalt	Co	$\mu g \cdot L^{-1}$	1.30	0.97	1.20	6.6	ND	ND
Total copper	Cu	$\mu g \cdot L^{-1}$	30.0	21.0	39.0	670	ND	ND
Total iron	Fe	$\mu g \cdot L^{-1}$	1,100	950	660	3.10	270	370
Total lead	Pb	$\mu g \cdot L^{-1}$	7.60	5.70	8.00	700	ND	ND
Total magnesium	Mg	$\mu g \cdot L^{-1}$	7,600	7,000	8,200	67.0	780	950
Total manganese	Mn	$\mu g \cdot L^{-1}$	6,000	3,300	7,700	ND	35.0	48.0
Total molybdenum	Mo	$\mu g \cdot L^{-1}$	ND	ND	ND	2.6	ND	ND
Total nickel	Ni	$\mu g \cdot L^{-1}$	4.50	4.00	4.70	440	6.60	11.0
Total phosphorus	P	$\mu g \cdot L^{-1}$	2,200	3,700	2,700	1,800	ND	ND
Total potassium	K	$\mu g \cdot L^{-1}$	26,000	23,000	24,000	ND	250	380
Total selenium	Se	$\mu g \cdot L^{-1}$	ND	ND	ND	ND	ND	ND
Total silver	Ag	$\mu g \cdot L^{-1}$	0.60	ND	1.4	ND	ND	ND
Total sodium	Na	$\mu g \cdot L^{-1}$	500,000	460,000	1,100,000	200,000	3,300	7,300
Total strontium	Sr	$\mu g \cdot L^{-1}$	140	100	180	19.0	11.0	19.0
Total thallium	Tl	$\mu g \cdot L^{-1}$	0.24	0.13	0.24	ND	ND	ND
Total tin	Sn	$\mu g \cdot L^{-1}$	30.0	3.00	110	2.5	ND	ND
Total titanium	Ti	$\mu g \cdot L^{-1}$	19.0	5.80	11.0	30.0	2.30	3.00
Total uranium	U	$\mu g \cdot L^{-1}$	0.16	2.30	0.22	0.13	ND	ND
Total vanadium	V	$\mu g \cdot L^{-1}$	9.20	11.0	13.0	11.0	ND	ND
Total zinc	Zn	$\mu g \cdot L^{-1}$	450	380	470	61.0	ND	5.40

[a]Analysis performed on decanted sample due to sediment content.
[b]Elevated reporting limit due to sample matrix.
ND = not detected.

a uniform fashion and growth is monitored by determining chlorophyll a fluorescence increase over the duration of the experiment.

C. vulgaris and *Dictyosphaerium* sp. were screened for growth in 96-well microtitre plate cultures on shakers. Stock cultures for each selected strain were inoculated in 150 mL flasks in B3NV and grown under standard growth conditions of continuous light at 75–100 $\mu moles \cdot m^{-2} \cdot s^{-1}$ of PAR with shaking at approximately 100 rpm at 22 °C until the culture was dense (approximately 4 days). The cell

density was determined using a Coulter Counter particle analyzer. A suitable volume of growth medium was placed into a sterile conical tube and an aliquot of the stock culture was added to produce a final cell density of approximately 30,000 cells·mL^{-1}. Under aseptic conditions, a 200 µL volume of the prepared algal suspension was placed into each well of a flat-bottomed sterile 96-well microtitre plate (Corning). The plates were sealed using a gas-permeable Breathe-Easy adhesive membrane (Sigma-Aldrich) pressed into place over the wells with a brayer. The plates were then sealed into specially fitted, silicone O-ring sealed sandwich boxes (Sistema). The bottom of the boxes was covered with a layer of absorbent cloth (J-cloth) which was wetted with Milli-Q water to maintain a moist atmosphere inside the box. This prevented the edge evaporation effect often associated with the use of microtitre plates. Holes were drilled in the box lids and one edge to allow the mounting of a Luer fitting which accepted a Luer three-way valve. The edge valve, when required, was connected to a gas cylinder containing 5% CO_2 in air. The gas line was flushed for 1 min before being connected to the sealed box, the upper lid valve was opened and the box was flushed for 1 min at 0.5 L·min^{-1}. The valves were then closed and the boxes were secured to a rotary shaker inside the Conviron chamber. The boxes were kept in the aforementioned light and temperature conditions, but the plates were shaken at 300 rpm throughout the experiment.

The same process waters and treatments used for shake flasks were also used for the microtitre plate experiments. One microtitre plate per species with all the different process waters, blank and standard growth medium was sufficient for an entire experiment. There were quadruple wells or replicates per treatment (process water). To follow algal growth, boxes were removed from the shaker and opened to retrieve the plates. The plates were scanned using the PerkinElmer 2,030 multi-label plate reader Victor X5 in fluorescence mode. The scanner was fitted with an excitation filter of 430 nm and an emission filter of 670 nm, and each well was scanned for 0.5 seconds per well. Once the scanning was complete, the plates were returned to the sealed boxes, ensuring the absorbent cloth was still moist and flushing the box with additional CO_2/air as above. The plates were scanned at time zero and daily throughout the 5-day growth experiments, with the data collected and analyzed. The microtitre plate experiment for each species was conducted once; the experiments within a plate were performed in quadruples and all data were expressed as the means ± SEM.

Determination of microalgal dry weight in culture

The mean biomass concentration during growth in shake flask cultures was determined by gravimetric analysis of freeze-dried biomass samples. Duplicate, pre-weighed 25-mm Whatman GF/F glass microfiber filters were used to filter 4–10 mL of microalgal culture from flasks, followed by washing with same volume of dH_2O. Filtered and washed microalgal biomass was freeze-dried using a Freezone 4.5 L Benchtop Freeze Dryer (Labconco, USA). The filter weight was determined on a 0.001-mg resolution balance and reported in units of g·L^{-1}. Biomass data were measured in duplicate and expressed as the means ± SEM.

RESULTS AND DISCUSSION

Pretreatment and analysis of pulp and paper waste process waters

The TN levels for the process waters ranged from the lowest (0.19 mg·L^{-1} N) in Goose/Grant Lakes to the highest (33.50 mg·L^{-1} N) in TMP effluent (Table 1). In comparison, B3NV growth medium had a TN level of 123.0 mg·L^{-1} whereas the municipal wastewater effluent, for example from Mill cove wastewater treatment (MCWW) plant in Bedford, Nova Scotia, Canada typically contains nutrient concentrations of ~20 and 2.0 mg·L^{-1} for ammonium and phosphate, respectively (Park *et al.* 2015). The TP for the process waters ranged from 0.03 mg·L^{-1} P in both Goose/Grant and Landrie Lakes to 4.20 mg·L^{-1} P in secondary clarifier effluent (2°). There appears to be greater nutrient levels (TN and TP) in TMP wastewater effluent to support microalgal growth compared to other process waters. In addition, the primary, secondary, TMP and PM2 waste effluents contained higher levels of total sodium (500, 460, 1,100 and 200 mg·L^{-1} respectively) compared to Goose/Grant and Landrie Lake waters (3.3 and 7.3 mg·L^{-1} respectively) (Table 2). The pH for the process waters ranged from 5.94 to 8.03, and increased when the process waters were transported back to the laboratory, except for TMP. The pH values were similar among all process waters (pH 6.98–7.95) in microalgal cultures upon completion of growth trials. Furthermore, the parameters such as TOC, salinity (NaCl; ppm), (mS·cm^{-1}) and TDS were much higher in primary clarifier effluent (1°), secondary clarifier effluent (2°), and TMP effluent than PM2 effluent and Goose/Grant and Landrie Lake waters.

For this screening, the collected wastewater effluents and process waters were passed through a series of filters (as described in the 'Materials and Methods' section). Photos taken before and after filtration are shown in Figure 2. The unfiltered primary, TMP and PM2 wastewaters were quite turbid, due to the presence of suspended wood fibres and other woody particulate matter. In addition, primary, secondary and TMP wastewaters were discolored while PM2 was not. Filtration to a final stringency of 0.45 μm clarified PM2 wastewater and reduced the turbidity of primary and TMP waters, but did not completely eliminate it (Table 1 and Figure 2). The discoloration appeared to be intensified by filtration, particularly for secondary wastewater (Figure 2). Dissolved and/or colloidal organic matter most likely accounted for this discolouration.

The growth curves and total dry biomass weights determined for the experimental conditions employed and are shown in Figures 3 and 5, respectively.

Growth and biomass production

C. vulgaris and *Dictyosphaerium* sp. did not grow on any of the unfertilized process waters (Figure 3(a) and 3(d), respectively). However, fertilization with the B3NV growth medium stimulated growth in all of the process waters with the exception of TMP, where no growth of either strain was observed (Figure 3(b) and 3(e)). Of the fertilized process waters, both strains grew best in the lake waters and the positive control media, followed by PM2, secondary and finally primary (Figure 3(b) and 3(e)). No algal growth and/or bacterial contamination was observed in the negative controls (Figure 3(c) and 3(f)). Uninoculated primary, secondary and TMP process waters were noticeably discoloured while the other process waters were clear. Conspicuous growth of algae was observed in fertilized primary and secondary process waters but the pigmentation appeared paler compared to the other cultures, most likely due to the background discoloration and lower overall growth (Figure 3(b)). There was no apparent algae growth or pigmentation in the fertilized TMP water, consistent with the cell count data (Figure 3(b)).

Filterable dry weights of cultures were also determined on day 11 (Figure 4). For both strains, the greatest biomass accumulation was observed in the fertilized lake waters and the positive control followed by PM2, consistent with the cell count data (Figure 4(a) and 4(b); all >1 g dry wt L^{-1}). In cultures made from these process waters, a small amount of filterable biomass was detected in the unfertilized treatment (~0.1–0.2 g dry wt L^{-1}) indicating some algal growth under these conditions. With the exception of the *C. vulgaris* cultures prepared from PM2 water, the measured dry weight was always greater in the unfertilized treatments compared to the uninoculated negative controls. The dry weight data obtained from the primary, secondary and TMP process waters were more difficult to interpret (Figure 4(a) and 4(b)). For *Dictyosphaerium* sp., the apparent accumulation of algal biomass was observably higher in fertilized primary and secondary water (0.9–1.0 g L^{-1}) but lower in fertilized TMP water (~0.5 g L^{-1}, Figure 4(b)). However, a total of ~0.2 g L^{-1} of filterable dry weight was detected in both the unfertilized and uninoculated treatments, indicating a sizable fraction of the dry weight measured in the fertilized treatment (perhaps 20–30% in the primary water and about 50% in the TMP water) was suspended wood fibres and other particulates. For *C. vulgaris* a similar pattern emerged (Figure 4(a)). As with *Dictyosphaerium* sp., the apparent accumulation of algal biomass appeared authentic, as the background level of filterable material in the uninoculated treatment was relatively low. However, in the *C. vulgaris* cultures prepared from primary and TMP water, the relatively high level of apparent algal biomass accumulation of 0.5–0.8 g·L^{-1} was confounded by relatively high dry weights of 0.4–0.6 g·L^{-1} in the uninoculated treatments, indicating the presence of significant background suspended particulate matter.

The general conclusion from the first experiment was that neither strain would grow in any of the waste pulp and paper process waters unless first amended with B3NV growth medium. Even in the presence of nutrients, *C. vulgaris* did not grow in TMP water while *Dictyosphaerium* sp. grew very poorly. Despite the addition of the same final concentration of nutrients, growth in primary, secondary and PM2 wastewaters was always lower than in the laboratory media and the lake waters, suggesting the presence of at least one or two growth inhibitors (Figure 3(b) and 3(e)).

The results of the microtitre trials are shown in Figure 5 for *C. vulgaris* and Figure 6 for *Dictyosphaerium* sp. When comparing the cell density of *C. vulgaris* and *Dictyosphaerium* sp. in the flask cultures in the unfertilized process water to the fluorescence measurement in microplates, there was no apparent algae growth in all process waters in both methods (Figures 3(a), 3(d), 5(a)–5(f) and 6(a)–6(f)). When the process waters were fertilized with B3NV, algal growth and pigmentation were apparent in both flask and microplate cultures for both species except for the process water TMP (Figures 3(b), 3(e), 5 and 6). Similarly, no algal growth was observed in the negative controls for either

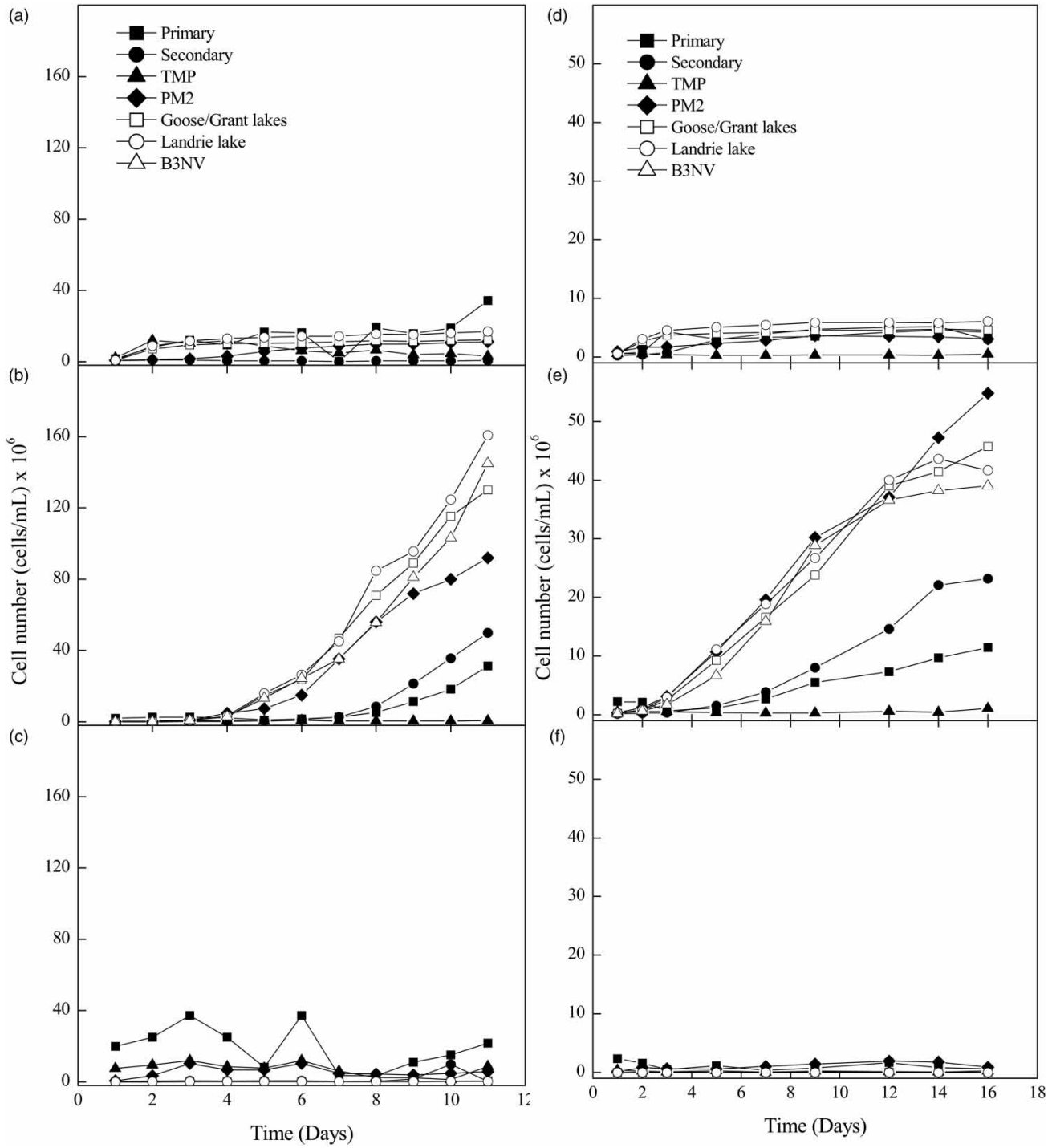

Figure 3 | Growth of *Chlorella vulgaris* (a, b and c) and *Dictyosphaerium* sp. (d, e and f) in duplicate shake flasks in six process waters ($n = 2$, SEM). Three treatments were carried out for each of the waters: (a & d) process water with algae added; (b & e) process water plus addition of nutrients with algae; and (c & f) process water plus addition of nutrients with no algae added.

species (Figures 3(c) and 3(f), 5(a)–5(f) and 6(a)–6(f)). The overall trend observed in the microtitre plates was similar to the trend in the shake flasks: with the exception of TMP, growth of both strains in the other process waters was robust provided that nutrients were added first. Growth was either weak or absent in unfertilized process

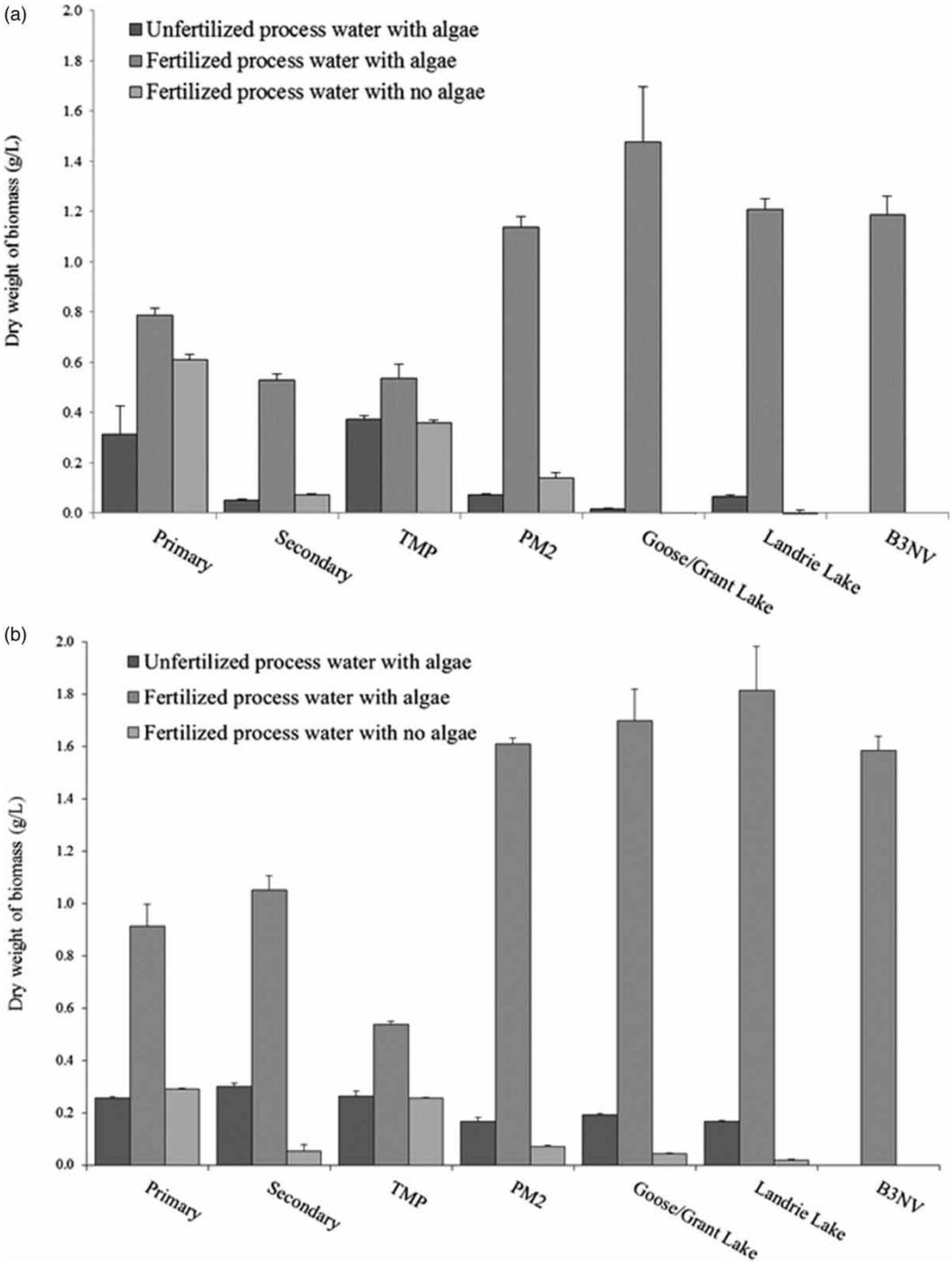

Figure 4 | Biomass analysis of *Chlorella vulgaris* (a) and *Dictyosphaerium* sp. (b) after 11 days of growth in shake flasks in six different process waters ($n = 2$, SEM).

waters (Figures 5 and 6). This experimental work shows that algal growth can be screened on wastewater effluents and monitored over a short period of time using the high-throughput microplate method. Wastewaters can then be downselected for a more detailed flask-level experiment or microplate method to study the physiology of algae using different potential inhibitors of microalgal growth such as phenols/tannins.

Even though wastewater in general has great potential as a growth medium for algal biomass or feedstocks

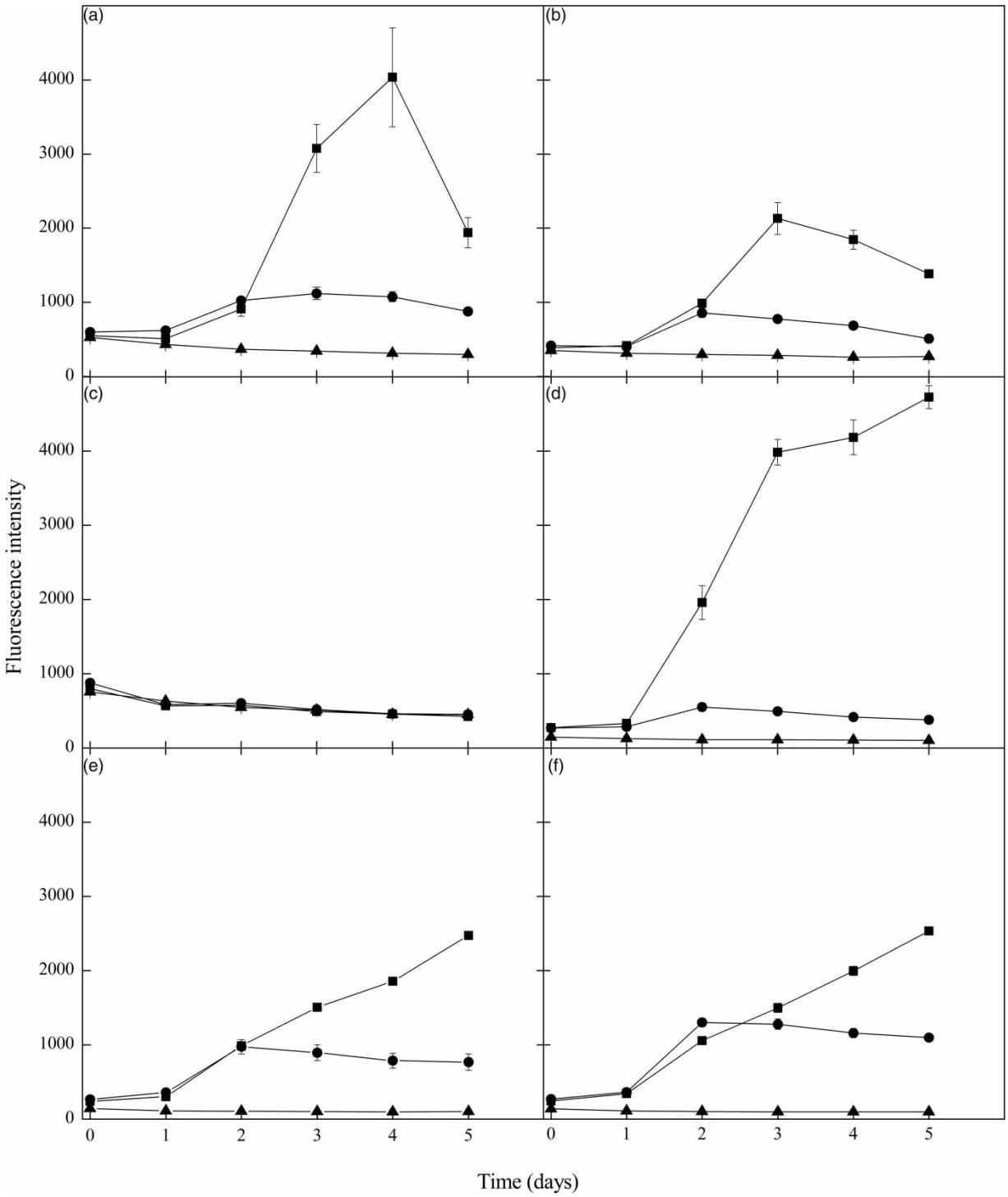

Figure 5 | Growth of *Chlorella vulgaris* in microtitre plates in six different process waters ($n = 4$, SEM). Three treatments were used: unfertilized process water with algae (circles); fertilized process water with algae (squares); and fertilized process water without algae (triangles). (a: primary, b: secondary, c: TMP, d: PM2, e: Goose/Grant Lakes, f: Landrie Lake).

production, a major problem is the broad variation in the wastewater quality and levels of toxic constituents. This preliminary assessment has revealed that not all types of pulp and paper process waters are suitable for algae cultivation but, in our view, further investigation is warranted. The results from this study clearly showed that the little to no growth detected in unfertilized primary, secondary and PM2 process waters was due to nutrient limitation since

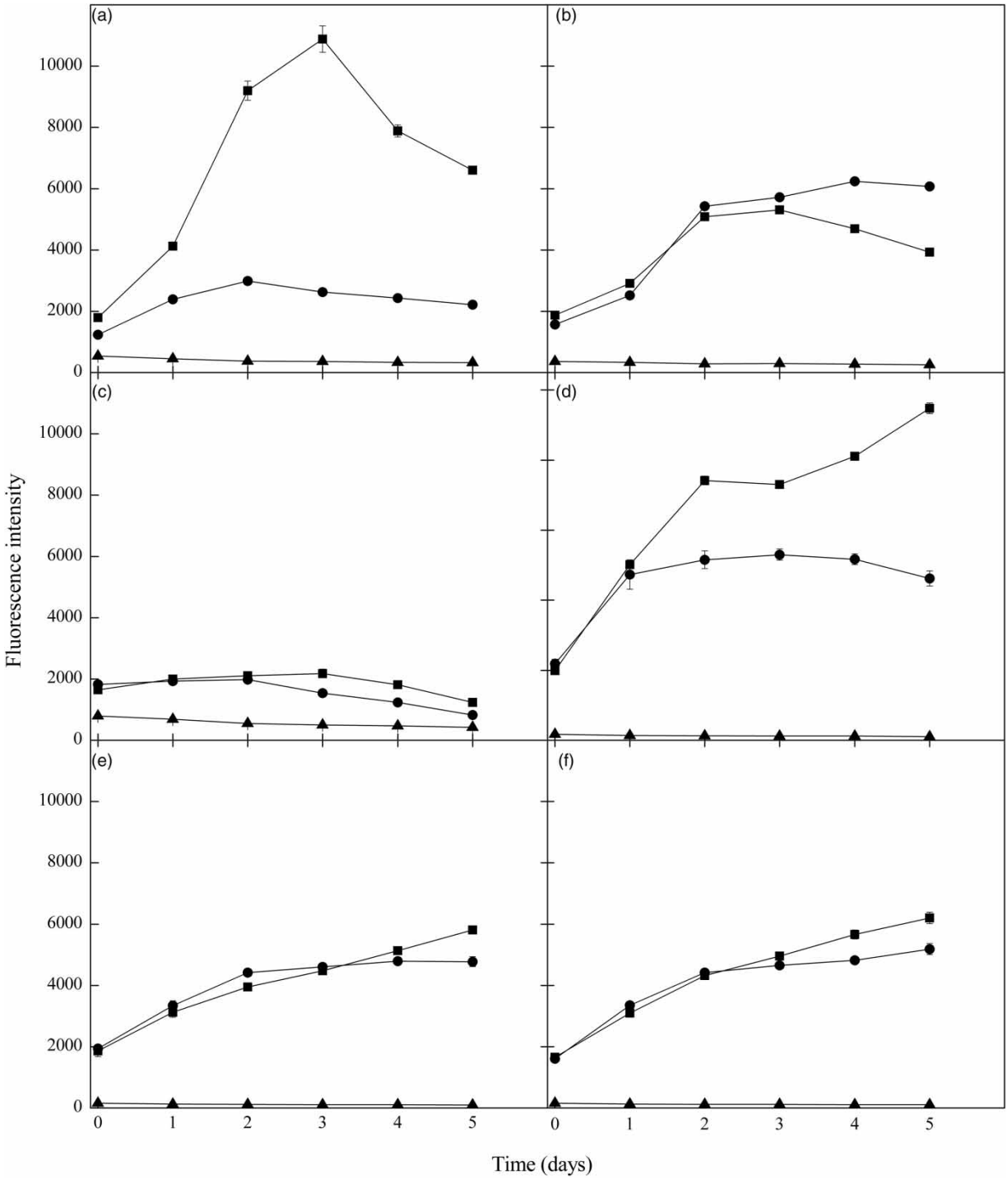

Figure 6 | Growth of *Dictyosphaerium* sp. in microtitre plates in six different process waters ($n = 4$, SEM). Three treatments were used: unfertilized process water with algae (circles); fertilized process water with algae (squares); and fertilized process water without algae (triangles). (a: primary, b: secondary, c: TMP, d: PM2, e: Goose/Grant Lakes, f: Landrie Lake).

addition of B3NV nutrients provoked some growth in all three, although not to the same extent as the amended lake waters and the control (Figure 3(a)–3(f)).

Although speculative, we suspect a few factors may have accounted for the lower growth observed. First, the sodium levels in the primary, secondary, TMP and PM2 waste

effluents were very high (500, 460, 1,100 and 200 mg·L^{-1} Na respectively) compared to Goose/Grant and Landrie Lake waters (3.3 and 7.3 mg·L^{-1} respectively) and this may have contributed to reduced microalgal growth in waste effluents despite the addition of nutrients. Salt stress is a major abiotic environmental factor that limits plant growth and productivity. Microalgae differ in their adaptability to salinity and other stress conditions. Church *et al.* (2017) reported that high levels of sodium or salinity stress decreased *C. vulgaris* growth rate in wastewater and slightly reduced nutrient removal rates. A recent study looked at the salinity tolerance and nutrient reducing ability of nine common freshwater microalgal species from the genera *Chlorella*, *Chlorococcum*, *Desmodesmus*, *Scenedesmus*, and *Monoraphidium* and found that these green microalgal species are halotolerant – able to proliferate in environments with high salt concentrations (Figler *et al.* 2019). In the same study, preliminary experiments showed that *Chlorella* spp. were able to grow significantly even in the presence of 5,000 mg·L^{-1} NaCl but there was significant growth inhibition at 10,000, 15,000, and 20,000 mg·L^{-1} salt concentrations (Figler *et al.* 2019). The source of sodium may be sodium chlorate, which is largely used by the pulp and paper industry to produce chlorine dioxide, to bleach wood pulp for the manufacture of higher quality and more environmentally friendly white paper products.

The primary and secondary process waters were discoloured, likely due to the presence of high concentrations of DOM. Although we did not address this directly in this study, it is possible that the DOM could have attenuated some of the incident light through direct absorption and thereby reduced the available PAR reaching the cells, lowering the photosynthetic rate and growth. Indeed, DOM absorbs low-wavelength light through approximately first order kinetics (Hugh MacIntyre, Dalhousie University, personal communication). We observed less growth in both strains in the discoloured waters compared to PM2, which was clear, supporting this hypothesis.

It is possible that the high apparent DOM in these effluents is due to a high level of tannins, which are highly reactive with proteins and can constitute up to 50% of the chemical oxygen demand (COD) in pulp and paper waste effluents (Field *et al.* 1988). The final discharge from pulping has a distinct colour due to tannins and dissolved lignin, which tends to absorb a significant amount of light and heat. In addition, less oxygen can be retained than unprocessed water. All of these have a negative effect on the aquatic flora and fauna (Ali & Sreekrishnan 2001; Thompson *et al.* 2001). Indeed, Ali & Sreekrishnan (2001) reported reduced algal productivity in natural waterways receiving high-tannin-bearing pulp and paper effluents due to reductions in solar radiation penetration. The toxicity effects of tannins on some marine and freshwater organisms have also been reported (De Nicola *et al.* 2007). For example, it has been shown that tannin is able to produce a change in the expected algal morphotype (i.e. fusiform) within a specific range of concentrations (75–185 mg·L^{-1}) and higher tannin concentrations completely inhibited growth of the alga *Phaeodactylum tricornutum* (Libralato *et al.* 2011).

The incrementally lower growth seen in both strains in fertilized primary process water, compared to secondary, may be due to the higher turbidity in primary water (Figure 2). As shown in Figure 2, primary water remained visibly turbid, despite a final filtration to 0.45 μm pore size, while secondary water, although deeply discoloured, was more transparent. Thus, the higher turbidity might have also caused light attenuation through a scattering effect, lowering the available PAR even further and resulting in reduced growth in primary water compared to secondary. The issue of coloured wastewaters has been reported in several studies. A study on olive-oil mill wastewater reported that the fat content and dark colour were the main inhibitors of the growth of *Scenedesmus obliquus* (Hodaifa *et al.* 2012). Another study also showed the dark colour and high turbidity of palm oil mill wastewater inhibited light penetration, causing light limitation for microalgal photosynthesis and growth (Nur *et al.* 2017).

The results observed in TMP water for both strains were different from the other process waters. There was little to no growth of either strain in unfertilized TMP water (Figure 3). This was surprising, since the observed nutrient levels in TMP, 34 mg·L^{-1} N (from ammonia) and 0.6 mg·L^{-1} P (from phosphate), were comparable to what is typically assayed in municipal wastewater effluent, which has been shown to support rapid growth and high yields of green microalgae (McGinn *et al.* 2012). Unlike the other process waters, there was little to no growth of either strain in TMP water even in the presence of B3NV nutrients. These observations suggest that the absence of growth in TMP water was not due to nutrient limitation but more likely due to the presence of at least one inhibitor of algal growth, a by-product of the TMP process. Wood pulping effluents have been reported to contain a wide variety of pollutants including phenols, furfurals, alcohols, fatty acids, peroxides, sterols, acids and resins (Toczyłowska-Mamińska 2017). Phenols at concentrations comparable to those reported from pulping effluents have been shown to

inhibit the growth of two species of *Chorella* (Scragg 2006) and there are numerous other reports in the literature of the deleterious effects of phenolics and phenol derivatives on algal growth more generally (Pinto *et al.* 2002) The Port Hawkesbury mill uses hydrogen peroxide (H_2O_2) to bleach pulp produced from the TMP process. Hydrogen peroxide is used for brightening the mechanical pulp and as reinforcement in the caustic extraction stage in the Kraft process, and later as a distinct bleaching stage in the Kraft process. Within the wastewater effluent streams, the quantity of residual hydrogen peroxide is variable and depends upon peroxide bleaching efficiency and on pulping and bleaching conditions. Interestingly, hydrogen peroxide is a natural growth inhibitor for most algae if concentrations are high enough. Nearly all species of algae exposed to H_2O_2 in toxicity tests appear to be adversely affected. The degree of effect is both concentration and time dependent. Peroxide is a strong oxidizing agent and may have been present at high enough concentrations in the process waters to inhibit algal growth.

Numerous studies have reported the cultivation of microalgae using sewage water, dairy wastewater, swine effluent, anaerobic digestate and others. However, the use of pulp and paper effluent for the cultivation of microalgae is the least explored (Tarlan *et al.* 2002; Gentili 2014). The possibility of mixing wastewater from different industries for the production of algal biomass has been discussed by Gentili (2014) as a way of increasing the nutrient levels. Even though the wastewater from the pulp and paper industry is rich in carbon, it is limited in nitrogen and phosphorus. Although diluting or mixing pulp and paper waste effluents with other high-nutrient wastewaters has a great potential for growing algae for biomass and lipid production together with effective wastewater treatment, there is the danger of the addition of toxins and other unwanted chemicals.

CONCLUSIONS

The main goal of this research was to determine whether microalgae are able grow in pulp and paper mill waste effluents. The advantage of using microalgae in biotechnology is the reduction in biomass production costs through wastewater phycoremediation. The results from this study indicate that the wastewaters generated from the Port Hawkesbury pulp and paper mill did not produce microalgal biomass unless they were first amended with nutrients required for algal growth. Pulp and paper industry wastewaters are often low in nutrients such as nitrogen and phosphorus, so a potential solution for this is to mix in wastewaters with higher nutrient concentrations such as municipal or dairy wastewaters. Furthermore, pulp and paper wastewaters are commonly highly coloured due to the high lignin and DOM content and therefore require colour removal, which can be carried out using mixotrophic or mixed cultures of microalgae. Some pulp and paper wastewaters, such as from TMP, do not support algal growth even after amendment with nutrients and in despite of the relatively high concentrations of available nitrogen and phosphorus. This is likely due to the presence of chemical residues from the pulping process, such as sodium, peroxide, and tannin, which might inhibit algal growth. Even though wastewater has great potential as a growth medium for algal biomass or feedstocks production, a major problem is the broad variation in the wastewater quality and levels of toxic constituents. The application of microalgae to wastewater and effluent bioremediation research is evolving and is focused on coupling bioremediation with the production of important value-added products. The use of microalgae for phycoremediation shows promise for the removal of nutrients, contaminants, chemicals and heavy metals from wastewaters in the pulp and paper and other industries.

In addition, projects like the present study, which aim to screen multiple algal strains on multiple types of process waters would benefit, both in terms of time and resource investments, from using 96-well (or greater) microtitre plates instead of traditional shake flasks for greater throughput and replicability of experimental treatments.

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AUTHOR'S CONTRIBUTIONS

SB and RR conceived the experimental design of the study. SB and RR collected the wastewater effluents from the pulp and paper mill. RR performed the experiments and collected the data. All authors critically analyzed the data. SB wrote the initial draft of the manuscript and PM critically revised it. SB and PM read and approved the final manuscript before submission.

STATEMENT OF INFORMED CONSENT, HUMAN/ ANIMAL RIGHTS

No conflicts, informed consent, human or animal rights are applicable to this study.

DATA AVAILABILITY STATEMENT

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

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