Arthrospira (Spirulina) in tannery wastewaters. Part 2: Evaluation of tannery wastewater as production media for the mass culture of *Arthrospira* biomass

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ABSTRACT

Mass blooms of *Arthrospira* (*Spirulina*) have been reported in waste stabilisation ponds treating tannery wastewaters and have been linked to a reduction in odour emissions in these systems. However, these blooms are unstable and unreliable, forming and disappearing in an apparently unpredictable manner, and they have remained poorly understood. Controlled production of *Arthrospira* biomass in this medium could not only be used to enable a more predictable control of odour in these systems (as detailed in Part 1 of this report), but could also provide a biomass product with external value. Techno-economic studies of microalgal biomass production have identified the cost of growth media formulation as a critical driver in the profitability of the algal biotechnology enterprise. Apart from the potential feed value of *Arthrospira* biomass as a product, the renewed interest in, and possibly marginal economics of, biofuels production from the microalgae has refocused attention on the possible advantages of wastewater use as low-cost production media.

Part 2 of this study reports the investigation of factors regulating *Arthrospira* growth in the tannery wastewater medium and thus requiring active control in order to optimise biomass production. It was shown that *Arthrospira* growth in this high-protein, low-carbonate medium is under ammonia control, rather than nutrient limitation, as may previously have been thought. It was also shown that an effective mass culture strategy in this medium would require a maximum effluent loading rate that operates as a function of the optimised ammonia removal rate. Growth optima were demonstrated for ammonia and bicarbonate levels of 20 mg·ℓ^{-1} and 12–17 g·ℓ^{-1}, respectively, and inhibition of growth was demonstrated at ammonia levels above 60 mg·ℓ^{-1}. Both autotrophic and mixotrophic growth of *Arthrospira* was observed and organic uptake may contribute to a stimulation of biomass production compared to growth in defined inorganic media. Heavy metal accumulation may present a toxicity hazard where biomass is targeted for use in animal feed rations. A heavy metals removal step was investigated involving the passage of the tannery effluent through an anaerobic sulphide-generating compartment in a primary pond, prior to its use in *Arthrospira* production. An acceptable *Arthrospira* feed-grade biomass was produced in this way. These results indicate potential cost-benefit advantages in the use of tannery effluent-based growth media for *Arthrospira* biomass production, and waste nutrient recovery may mitigate negative energy yield problems where the biomass is further processed in biofuels manufacture.

Keywords: Spirulina*; Arthrospira;* microalgal biomass; biofuels; tannery; wastewater; waste stabilisation ponds

INTRODUCTION

Massive blooms of microalgal growth and, in particular, of near mono-species blooms of *Arthrospira*, have been observed to occur in tannery waste stabilisation ponds (WSP) from time to time. However, these blooms are unstable and unreliable, forming and disappearing in an apparently unpredictable manner (Rose et al., 1996). Although zero-discharge evaporative WSP have been applied in the treatment of tannery wastewaters since the 1950s, particularly in water-stressed regions, little has been reported on factors controlling the growth and performance of microalgae in these systems (Shuttleworth, 1978; Rowswell et al., 1984), and these blooms have remained poorly understood.

The role of *Arthrospira* in the control of odour emissions from WSP has been described in Part 1 of this report (Dunn and Rose, 2013) and the effective use of this system to engineer more predictable management of the problem has been shown to depend, in part, on being able to optimise *Arthrospira* growth in the tannery wastewater medium. Additional opportunity exists for *Arthrospira* biomass production in these systems, with the demonstration that, under certain circumstances, an acceptable animal feed grade product may be produced that meets toxicological assessment criteria (Maart, 1993). Sporadically harvested biomass from a tannery WSP was also used in the development of a novel artificial feed ration in algae-based diets for the cultivation of the South African abalone *Haliotus midae* (Britz, 1996).

The input cost of production medium formulation was identified, early on, as a critical factor determining economic viability of the algal biotechnology enterprise (Oswald, 1995). This stricture contributes in part, at least, to the relatively low number of microalgal species production systems that have emerged as commercially viable mass culture operations, notwithstanding a period of intensive research over several decades. Media cost remains an important driver in the currently renewed focus on the feasibility and profitability potential of algal biomass in biofuels production (Chisti, 2007; Harun et al., 2010). The particular sensitivity of this factor in production profitability analysis has refocused interest in the use of wastes as possible sources of nutrients and, where possible, the linkage of waste treatment and algal production to achieve reductions in both operational and capital costs (Christensen and Sims, 2011). Use of waste nutrients may also mitigate the problem of negative energy yields in biofuels production.

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This report describes factors which regulate *Arthrospira* growth in tannery effluent and also attempts to explain the growth distribution patterns observed and the dynamics of microalgal blooms in the WSP system. Where successful, this might inform process development applications and the engineering of full-scale biomass production operations and/or odour control functions using the tannery wastewater substrate as a growth medium. Understanding factors regulating *Arthrospira* growth in these systems could also benefit the management and overall performance of the tannery WSP as an effective waste treatment operation.

EXPERIMENTAL

Characteristics of the 15 pond zero-discharge evaporative tannery WSP cascade used as the basis of this study have been described in Part 1 (Dunn and Rose, 2013). Growth studies were performed in 500 mℓ glass flasks using either Zarrouk's inorganic medium (Vonshak, 1997) or tannery effluent diluted in distilled water.

Analytical procedures followed APHA Standard Methods (APHA, 2005). Salinity was measured using an Atago salinity refractometer. Biomass production was measured gravimetrically and chlorophyll *a* was measured following the method of Lichtenthaler (1987). Measurements of photosynthetic productivity used the [^{14}C]-sodium bicarbonate CO_2 fixation method modified by Oren (1992) and sample measurement was undertaken in a Beckman LS3150T scintillation counter.

Radio-labelled organic nutrition studies were conducted by addition of [^{14}C]glycine (3.81 GBq·mmol^{-1}) and D[^{14}C]glucose (10.9 GBq·mmol^{-1}) (Amersham), to suspensions of washed *Arthrospira* cells in Zarrouk's inorganic medium (Vonshak, 1997) in 250 mℓ glass flasks, and containing a concentration range of either unlabelled glycine or glucose (Merck). One set of control flasks contained no added glycine or glucose. Flasks were incubated at 25°C in a constant environment room. One set of cultures for each treatment was exposed to continuous illumination at 160 µmol·m^{-2}·s^{-1}, while the other set was incubated in the dark. At time intervals of 5 min, 3 h and 6 h, 1 mℓ of culture medium was removed, filter washed (x3) with sterile defined medium and the cells transferred to aqueous scintillant (Packard). Culture supernatant was likewise transferred in 1 mℓ aliquots to aqueous scintillant. An insert tube containing ethanolamine (Merck) was placed in the flasks to trap generated CO_2, the contents of which were also transferred to aqueous scintillant. All samples were counted in a Beckman LS3150T scintillation counter and values adjusted to disintegrations per minute (dpm). Results reflect a mean of triplicate flasks and triplicate radio-labelled measurements from each flask. Total colony forming units (cfu) on nutrient agar (Difco) were determined for each flask at the commencement of each experiment to eliminate the possibility of bacterial mineralisation of the carbon source and, hence, the release of labelled CO_2 unrelated to *Arthrospira* metabolism.

Nutritional constituent analyses were undertaken by the Animal and Poultry Science Laboratory, University of KwaZulu-Natal. The heavy metal feed standard used here followed that set out in the German Ministry of Agriculture Law Collection No 117 (German Ministry of Agriculture, 1987) and subsequently consolidated in the European Union. Regulation (EC) No 882/2004 of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feeds.

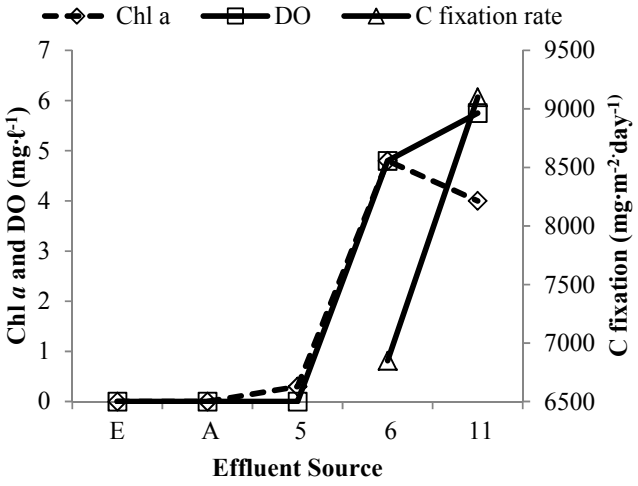

Figure 1

Distribution of microalgal biomass across the waste stabilisation pond cascade as indicated by chlorophyll a and dissolved oxygen levels, and carbon fixation rates, and comparing untreated effluent (E) and ponded effluent (Ponds A, 5, 6, 11).

RESULTS AND DISCUSSION

Microalgal growth in the WSP (Fig. 1) was restricted to the latter ponds in the cascade (Ponds C–11) and was composed largely of near mono-species blooms of *Arthrospira* sp., provisionally identified as *A. platensis*. *Dunaliella* spp. occurred in small numbers with *D. viridis, D. parva* and later *D. salina* appearing in correlation with rising salinity values across the system (Table 1). *Rhodobacterium* spp. provided the dominant component of the bacterial population and, in the absence of microalgal growth, gave a strong purple colour to those ponds. Biomass production in the system occurred against the background of a dynamic wastewater treatment environment which was characterised by sharply rising salinity, alkalinity and pH gradients, and with the removal of around 70% soluble chemical oxygen demand (sCOD) in both the anaerobic and facultative compartments (see Table 1, Part 1).

Identification of factors regulating the appearance of *Arthrospira* blooms in the WSP are important in understanding

TABLE 1
Analysis of effluent feed and ponded effluent at various points across the zero-discharge aste stabilisation ponding system

(mg·ℓ$^{-1}$)	Effluent	Pond A	Pond 5	Pond 6	Pond 11
Total alkalinity as $CaCO_3$	525	_	1 246	1 615	2 585
Dissolved Oxygen	< 0.005	< 0.005	< 0.005	3.98	4.71
Chemical Oxygen Demand	3 173	1 722	522	450	1 677
Sulphide as Na_2S	1 192	500	28	<1	6
Sulphate as SO_4	364	<1	715	1 365	943
Ammonia as NH_3	925	764	119	14	30
Phosphate as PO_4	15	7	7	7	12
pH	7.40	8.30	8.60	9.20	9.50
Total dissolved inorganic solids (g·ℓ$^{-1}$)	8	12	18	19	58

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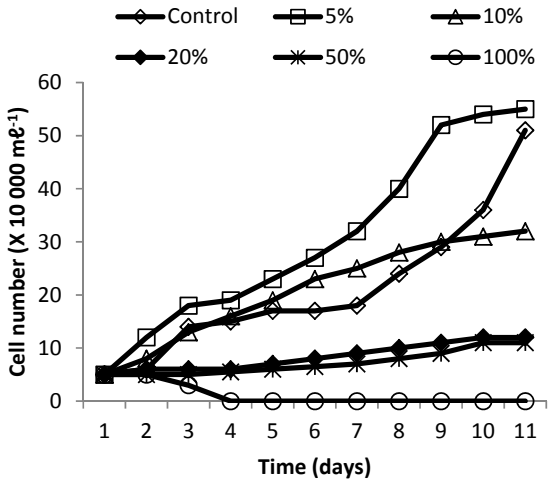

Figure 2

Growth of Arthrospira in a dilution range of untreated tannery effluent and in Zarrouk's defined mineral medium (control), measured by cell count.

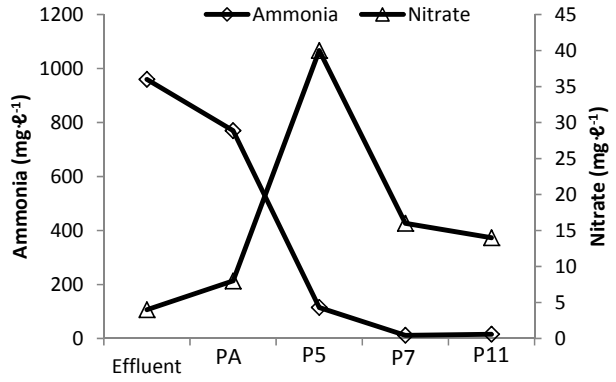

Figure 3

Reduction in ammonia levels across the tannery pond with a rise in nitrate indicating active nitrification and comparing effluent with Ponds A, 5, 7 and 11.

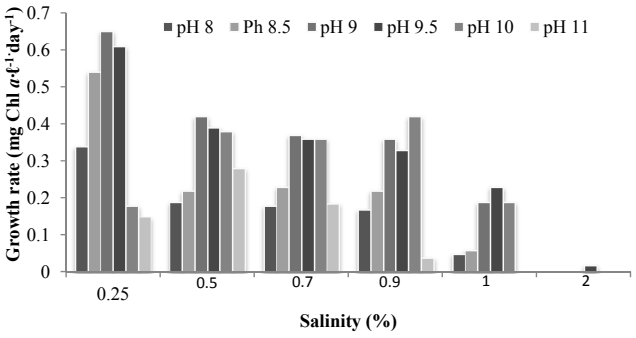

Figure 4

Growth performance of Arthrospira in a 5% dilution of untreated tannery effluent comparing the effects of increasing pH and salinity concentration.

the possible use of tannery effluent as growth media for industrial-scale biomass production. Growth regulation in the effluent medium was shown to be concentration-related in a series of shaker flask studies (Fig. 2). While undiluted effluent did not support growth, as may be anticipated from pond observations, dilutions of effluent from 50% showed increasing production potential. Interestingly, growth at the 5% and 10%

TABLE 2
Composition of untreated tannery wastewater, waste stabilisation pond effluents and Zarrouk's defined inorganic growth medium

(mg·ℓ$^{-1}$)	Defined Medium	Effluent	Pond 5	Pond 7	Pond 11
Calcium	13.3	226	-	-	-
Carbon	2 800	957	1 246	1 615	2 585
Chloride	527	4048	7 157	11 651	24 184
Iron	0.4	10.7	6.2	0.65	6.14
Magnesium	7.0	260	171	191	190
Nitrogen	500	717	125	63	54
Phosphorus	62	19	28	23	24
Potassium	125	127	118	276	259
Sodium	3 800	3 090	2 890	6 790	8 690
Sulphur	151	195	167	209	316

effluent dilutions showed higher yields than in the Zarrouk's defined medium control alone, thus indicating the possibility of an organic mixotrophic, as well as an inorganic autotrophic, nutritional component to *Arthrospira* growth in this medium (Chen and Yang, 1997; Vonshak et al., 2000).

Inorganic nutrition

The inorganic constituents of the undiluted effluent and of the WSP water are compared to defined Zarrouk's medium in Table 2. This shows that while carbon (as $CaCO_3$) may have been limiting in the untreated effluent, concentrations rose through the ponding system and correlated with increasing photosynthetic activity. While nitrogen was clearly not limiting in the undiluted effluent, its reduction in the latter ponds appeared to be related to nitrification and to ammonia stripping as pH rose across the system (Fig. 3). Phosphorus appeared to be limiting and may require supplementation, but reversible calcium binding may account for a rise in soluble phosphate seen in the latter ponds (Table 2). Sodium levels, from salt used in hide preservation, are high in these wastewaters, and Richmond (1986) has noted that *Arthrospira* growth may not be inhibited as long as the potassium:sodium ratio remains below 5, which obtained here. Neither calcium nor magnesium exceeded toxic levels reported by Mitchell and Richmond (1988).

The above assumptions were tested in a grid matrix shaker flask study series in which the 5% effluent dilution was adjusted over a range for salinity, pH, ammonia and bicarbonate status (Figs. 4–6). The salinity/pH matrix (Fig. 4) showed that while growth is optimal at lower salinities, and pH values between 9 and 9.5 that obtain in the untreated effluent, growth is still possible at salinities around 1% and at pH 11, which occurred in the latter ponds.

While a 5-fold stimulation of *Arthrospira* growth by bicarbonate addition (Fig. 5) confirms carbon limitation in the effluent medium, the addition of phosphate made little difference until growth was stimulated by the higher levels of bicarbonate added. Thereafter, phosphate addition up to 86 mg·ℓ$^{-1}$ gave best growth response at a maximum bicarbonate addition of 12 g·ℓ$^{-1}$. Levels of phosphate addition higher than that appear to be inhibitory.

Figure 6 indicates that ammonia is used directly as a nitrogen source by *Arthrospira*, particularly when growth is stimulated by bicarbonate addition (Soletto et al., 2005). While the best response was observed with the addition of 20 mg·ℓ$^{-1}$

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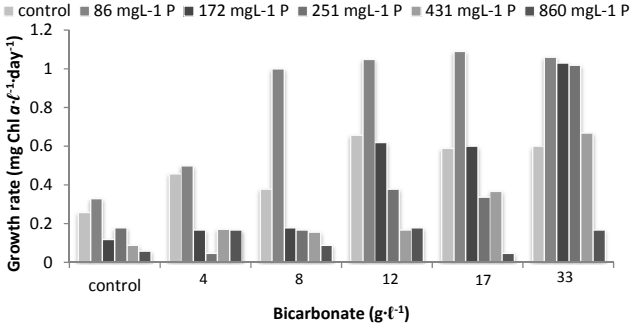

Figure 5
Growth performance of Arthrospira (mg·ℓ⁻¹ Chl a) in a 5% dilution of untreated tannery effluent comparing increasing concentrations of bicarbonate and phosphate and with 5% untreated tannery effluent as the control.

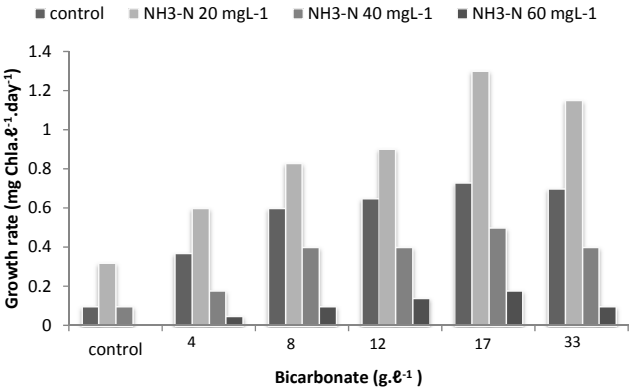

Figure 6
Growth performance of Arthrospira (mg/l Chl a) in a 5% dilution of untreated tannery effluent comparing increasing concentrations of bicarbonate and ammonia and with 5% untreated tannery effluent as the control. No growth occurred in the 80 mg·ℓ⁻¹ and 100 mg·ℓ⁻¹ ammonia concentration series.

ammonia at the 12 g·ℓ⁻¹ bicarbonate addition, ammonia toxicity increased and was found to completely inhibit growth above 60 mg·ℓ⁻¹. This value is somewhat lower than the 100 mg·ℓ⁻¹ maximum previously reported by Abeliovitch (1983). The shift of ammonia to the ionised form, that may be anticipated as alkalinity rises across the system, is observed in the flask study to some extent but, together with ammonia stripping in the open ponds, may also account for the reduction in growth inhibition seen in the latter ponds.

Organic nutrition

A possible role for organic nutrition in this system was investigated in radio-labelled nutrient uptake shaker flask studies (Fig. 7). Washed inocula of the WSP *Arthrospira* isolate were incubated in Zarrouk's medium, in both the presence and absence of light, together with [^{14}C]-glucose and [^{14}C]-glycine. This was done to simulate possible nutritional uptake by *Arthrospira* from carbohydrate and amino acid sources derived from the hide substrate. Substantial binding of the radio-labelled glucose and glycine in the light was reduced with the addition of increasing concentrations of the unlabelled substrate and suggested a competitive quenching effect. While label uptake was lower in the dark, similar trends were observed, and the presence of the label in ethanolamine insert tubes in the flasks indicated that these substrates had been

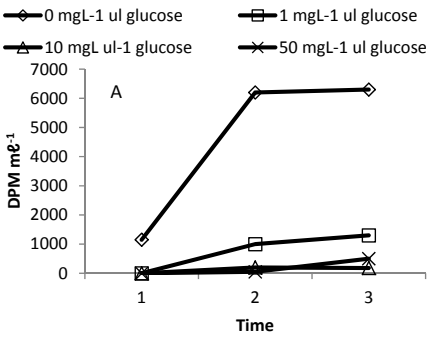

Figure 7A
Uptake of D-[^{14}C]-glucose (degradations/minute) by Arthrospira incubated in the light in defined medium, without the addition of unlabelled glucose and also with the addition of increasing concentrations of unlabelled glucose at Time 1 = 5 min, Time 2 = 3 h and Time 3 = 6 h.

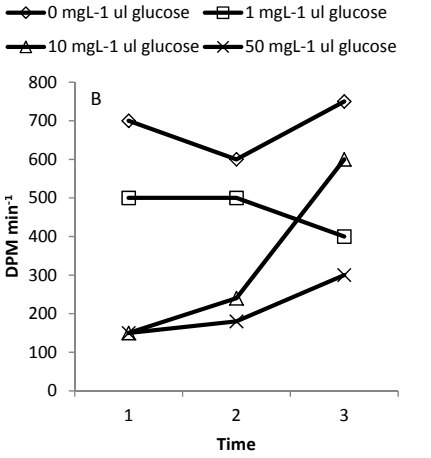

Figure 7B
Uptake of D-[^{14}C]-glucose (degradations/minute) by Arthrospira incubated in the dark in defined medium, without the addition of unlabelled glucose and also with the addition of increasing concentrations of unlabelled glucose at Time 1 = 5 min, Time 2 = 3 h and Time 3 = 6 h.

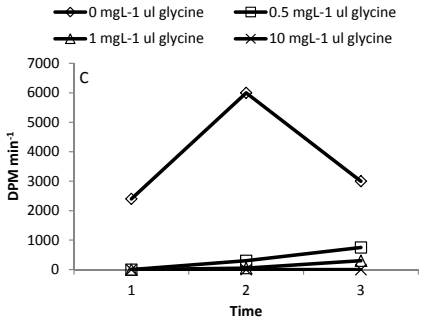

Figure 7C
Uptake of [^{14}C]-glycine (degradations/minute) by Arthrospira incubated in the light in defined medium, without the addition of unlabelled glycine and also with the addition of increasing concentrations of unlabelled glycine at Time 1 = 5 min, Time 2 = 3 h and Time 3 = 6 h.

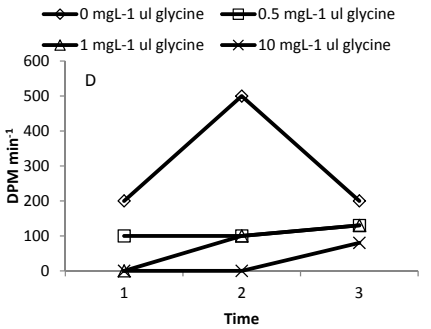

Figure 7D
Uptake of [^{14}C]-glycine (degradations/minute) by Arthrospira incubated in the dark in defined medium, without the addition of unlabelled glycine and also with the addition of increasing concentrations of unlabelled glycine at Time 1 = 5 min, Time 2 = 3 h and Time 3 = 6 h.

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actively metabolised to CO_2. These results suggest that mixotrophic organic nutrition plays some role in this system and may account for the enhanced growth rates observed in Fig. 2 in the 5% and 10% effluent dilutions (Lodi et al., 2005).

Biomass

Photosynthetic productivity during *Arthrospira* blooms in the WSP reached up to 9 000 $mg \cdot m^{-2} \cdot day^{-1}$ carbon fixation (or about 33 $t \cdot ha^{-1} \cdot yr^{-1}$), which compares with the 8 000–12 000 $mg \cdot m^{-2} \cdot day^{-1}$ reported for *Arthrospira* produced under controlled production conditions in sewage wastes (Fox, 1988) and an average yield of 26 $t \cdot ha^{-1} \cdot yr^{-1}$ for *Arthrospira* grown in sewage for poultry feed (Saxena, 1983).

Arthrospira biomass was harvested from the tannery WSP, sundried and the analysis of cell constituents is reported in Tables 3 and 4. While sun drying is associated with some protein and pigment loss in the dried biomass, the results are roughly comparable with heat-dried material (Vonshak, 1997), and indicate the broad nutritional potential of tannery effluent-grown *Arthrospira* biomass as a ration in animal feeds. Alternatively, its potential value for biofuels production can also be estimated from these results (Christensen and Sims, 2011).

Heavy metal contamination

Production of microalgal biomass in effluent-based growth media is vulnerable to toxicity effects, which is of particular importance where an animal feed-grade product may be targeted (Yi-Chao Lee and Chang, 2011). Heavy metal accumulation is a potential problem in wet-blue leather production and removal of metals from the *Arthrospira* growth medium was investigated by passing the effluent through an anaerobic compartment in the WSP prior to feeding to the HRP (Maart, 1993). Removal is reported in Table 5 with nickel being the only metal analysed showing levels slightly above the feed standard. Metal sulphide precipitation and adsorption are possible mechanisms accounting for the removal effect observed. Chromium in all samples analysed over a 3-year period was in the chromium III form. Chromium VI was not found in the system.

CONCLUSIONS

- Wastewaters generated in wet-blue tanning operations can support dense blooms of *Arthrospira*, making this a potentially attractive growth medium for biomass production used in odour control in WSP systems, and as a value-added product in its own right.
- Mixotrophic organic nutrition was observed to provide a growth stimulatory effect compared to defined mineral media and, if this is translated to mass culture production, commercially attractive yields may be attained in this system.

TABLE 3
Analysis of sundried *Arthrospira* biomass harvested from the tannery WSP

Cell component	Composition
Protein (%)	57
Carbohydrate (%)	14.9
Lipid (%)	7.6
Phycobiliprotein ($g \cdot kg^{-1}$)	4.51
Chl *a* ($g \cdot kg^{-1}$)	1.68
Carotenoids ($g \cdot kg^{-1}$)	2.9
Xanthophyll ($g \cdot kg^{-1}$)	1.68
Ash (%)	17

TABLE 4
Amino acid composition of sundried *Arthrospira* biomass harvested from the tannery WSP and compared with results reported by Richmond (1986) and Saxena et al. (1983) (g amino acid· 16 g N^{-1})

Amino acid	This study	Richmond	Saxena
Alanine	3.5	5.82	9.76
Aspartate	4.9	6.3	12.51
Arginine	3.26	5.98	4.06
Glutamate	8.83	8.94	10.44
Glycine	2.43	3.46	7.78
Histidine	0.69	1.08	1.87
Isoleucine	2.61	4.13	3.91
Leucine	4.25	5.8	8.3
Lysine	2.38	4	3.94
Methionine	0.95	2.17	1.52
Phenylalanine	2.06	3.95	2.76
Proline	2.01	2.97	5.35
Serine	2.24	4	7.21
Threonine	2.5	4.17	5.41
Tyrosine	1.94	4.6	2.1
Valine	3.12	6	6.86
Total protein	57.1	71	50–55

TABLE 5
Heavy metal concentrations in tannery effluent before and after treatment in the anaerobic compartment of a facultative pond unit located prior to the high rate pond, and also in *Arthrospira* biomass grown before and after the anaerobic treatment step. Standard deviation in brackets. (* German Ministry of Agriculture, 1987).

	Untreated effluent ($mg \cdot \ell^{-1}$)	Treated effluent ($mg \cdot \ell^{-1}$)	Biomass before treatment ($mg \cdot kg^{-1}$)	Biomass after treatment ($mg \cdot kg^{-1}$)	Feed standard ($mg \cdot kg^{-1}$)
Chromium	4.68 (0.1)	0.25 (0.02)	25.8 (0.5)	<1	10–15
Cadmium	0.08 (0.02)	0.02 (0.01)	5.96 (0.23)	<1	1.0
Cobalt	0.35 (0.03)	0.18 (0.01)	22.4 (0.6)	3.3 (0.2)	4–6
Iron	39.9 (2.1)	0.41 (0.03)	2 012 (12.2)	795 (13.4)	1 250–2 500
Lead	0.76 (0.03)	0.11 (0.01)	219 (2.3)	2.3 (0.2)	10
Nickel	0.41 (0.01)	0.21 (0.01)	49.2 (1.5)	17.5 (0.8)	10–15
Zinc	1.51 (0.1)	<0.1	218.5 (2.8)	22.5 (1.1)	250–500

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- *Arthrospira* growth in the tannery wastewater was shown to operate under direct ammonia control; this was concentration dependent and could be relieved by at least three routes, including direct uptake by *Arthrospira* as a nitrogen source, nitrification/denitrification and ammonia stripping. Optimum growth was achieved at ammonia and bicarbonate concentrations of around 20 $mg \cdot \ell^{-1}$ and 12–17 $g \cdot \ell^{-1}$ respectively and inhibition of growth at ammonia levels above 60 $mg \cdot \ell^{-1}$.
- Follow-up studies involved the construction of a 2 500 m^2 HRP unit alongside the tannery WSP. Loading rates to the HRP were based on the demonstration of ammonia toxicity levels in the current study and required the determination of a maximum effluent loading rate that operates as a function of the optimised ammonia removal rate. A biomass productivity of 12.87 $g \cdot m^{-2} \cdot day^{-1}$ (dry mass) with an average yield of around 46.8 $t \cdot ha^{-1} \cdot yr^{-1}$ was measured for the optimised HRP system. The details of this scale-up study are reported elsewhere (Rose and Dunn, 2013).
- Heavy metal contamination may be mitigated by pre-treatment of the tannery effluent growth medium in a sulphide-generating anaerobic compartment of the WSP.
- While the studies detailed in this investigation relate to a particular site, the broad trends in *Arthrospira* growth were observed in a number of comparable tannery ponding systems operating elsewhere. It seems that these observations could be reasonably generalised to the WSP treatment of wet-blue tannery wastewaters at those, and possibly other, sites.
- Follow-up process development studies and scale-up application of *Arthrospira* production in tannery wastewaters has been undertaken and is reported elsewhere (Rose and Dunn, 2013).

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