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Metal biosorption in lignocellulosic biofuel biorefinery effluent: an initial step towards sustainability of water resources

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Abstract Biosorption of metals by microorganisms is a promising technology to remove accumulated non-process elements in highly recycled biorefinery process water. Removal of these elements would enable greater water reuse and reduce the environmental impact of effluent discharge. A model lignocellulosic ethanol biorefinery wastewater was created based on pulp mill effluent. This generated a wastewater with an environmentally realistic high loading of dissolved natural organic matter (900 mg/l), a potentially important factor influencing metal biosorption. Analysis of feedstock and pulp mill effluent indicated that Mn and Zn are likely to be problematic in highly recycled lignocellulosic ethanol biorefinery process water. Therefore, the growth of several bacteria and fungi from existing collections, and some isolated from pulp mill effluent were tested in the model wastewater spiked with Mn and Zn (0.2 mM). Wastewater isolates grew the best in the wastewater. Metal uptake varied by species and was much greater for Zn than Mn. A bacterium, *Novosphingobium nitrogenifigens* Y88^T, removed the most metal per unit biomass, 35 and 17 mg Mn/g. No other organism tested decreased the Mn concentration. A yeast, *Candida tropicalis,* produced the most biomass and removed the most total metal (38 % of Zn), while uptake per unit biomass was 24 mg Zn/g. These results indicate that microorganisms can remove significant amounts of metals in wastewater with high concentrations of dissolved natural organic matter. Metal sorption by autochthonous microorganisms in an anaerobic bioreactor may be able to extend water reuse and therefore lower the water consumption of future biorefineries.

Keywords Metal · Biosorption · Organic matter · Lignocellulosic ethanol biorefinery · Bioremediation

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Introduction

Biofuel production is an increasingly important industry, helping to meet escalating energy demands while contributing to economic growth and energy independence. Depending on the source, biofuels generally pose less of an environmental hazard than fossil fuels in terms of green house gas emissions, a factor that also favours their adoption [20]. However, the production of biofuels requires large quantities of high-quality freshwater, a resource of increasingly limited supply [51]. The sustainability and economic feasibility of biofuels can be improved by minimizing water use in the refineries. One mechanism for this is to increase reuse of water within the plant. Water recycling is a common practice in refining processes; however, evidence from the pulp and paper industry shows that the build up of non-process elements, such as metals and salts, can limit the extent to which water can be recycled. These constituents cause problems such as scaling and corrosion, and may also impact product quality [21, 24]. In a biorefinery, a significant amount of water is often lost to evaporation, accelerating the concentration of the non-process elements. If the metals and salts could be removed, water reuse could be greatly extended, and the environmental sustainability of biofuels would be enhanced.

Lignocellulosic biofuels are an increasing favourable option. By utilising woody by-products, such as sugar cane bagasse or corn stover, it eliminates the need for land-use change and also avoids direct competition with food resources [20, 45]. One potential lignocellulosic feedstock is wood. In a country such as New Zealand, this is the most common lignocellulosic biomass available, in the form of the pine, *Pinus radiata*.

Sorption by microorganisms is a promising technology to remove heavy metals from water. It also has the advantage of being a more cost-effective treatment than physicochemical means [14, 49]. Metals bind to the carboxyl, hydroxyl, carbonyl, thio, amine and phosphate functional groups on biological membranes and on extracellular polymeric substances (EPS) exuded by microorganisms [14, 49]. Research on sorption of metals by microorganisms usually focuses on removing metal from simple aqueous solutions [14, 37], although some researchers have studied uptake in more complex systems such as actual wastewaters that contain natural organic matter (NOM) [9, 23, 37, 42]. NOM is a broad term referring to the organic constituents of the biomass, and in the case of wood includes lignins and polysaccharides. NOM is likely to be found in very high levels in process waters in lignocellulosic refineries, and may have complicating effects on metals removal, owing to its strong metal-binding capacities [11]. Recent findings have, however, shown that ternary complexes can form among the NOM, microorganisms and metals [5], which could enhance metal biosorption.

The question of how microorganisms are capable of sorbing metals from water rich in NOM is also of fundamental interest in toxicology. It is generally recognized that NOM will bind metals, reducing bioavailability and thus toxicity to the organisms [11]. Hence, the biotic ligand model was developed [12] to incorporate the effects of NOM, pH and other cations into predictions of toxicity to aquatic wildlife. The processes governing the bioavailability of metals in a wastewater are likely similar to those in the natural environment and can provide insight into the bioavailability of the metals after they are released into the environment. Metals that can be taken up by a microorganism in a wastewater bioreactor may also be taken up by aquatic wildlife if the effluent is discharged untreated into waterways.

The first objective of this study was to generate a model lignocellulosic ethanol biorefinery wastewater and identify metals likely to accumulate to levels of concern. The second objective was to compare the ability of a variety of organisms to grow in, and to remove metals from, a model lignocellulosic biorefinery wastewater. Such an organism will have potential for further investigation as a tool for metal extraction from lignocellulosic biorefinery wastewater. These objectives will allow two key questions to be addressed: (1) Are there organisms capable of growing in lignocellulosic biorefinery effluent? (2) Can microorganisms remove a significant amount of metals from an effluent with high NOM content (i.e. is this metal bioavailable to the microorganisms)? These are the first steps toward future work that will characterize metal uptake mechanisms and develop predictive models for investigating the influence of metal concentration, metal mixtures, NOM concentration and NOM composition on remediation success of lignocellulosic biorefinery effluent.

Materials and methods

Model wastewater manufacture and metal selection

Although no full-scale lignocellulosic ethanol biorefinery currently exists, a report developed from pilot plant data indicates probable conditions of the wastewater [32]. The wastewater treatment system in this report includes anaerobic digestion followed by aerobic digestion. The aerobic treatment step was targeted as the locus for metals removal, with the rationale that the anaerobic step would be primarily aimed at carbon removal, so the aerobic step could be optimized for metals removal. A model wastewater was created by modifying a pulp mill effluent to resemble a lignocellulosic ethanol biorefinery effluent [32]. The pulp mill effluent was from a New Zealand thermomechanical mill that produces pulp from *P. radiata*. The metal content of the pulp mill effluent was measured by nitric acid digestion and inductively coupled mass spectrometry (ICP-MS) analysis (Table 1, RJ Hill Labs, Hamilton) according to APHA method 3125B and USEPA 200.8 [2, 10], whereas total metals in raw *P. radiata* wood chips were analysed by APHA method 3125B with boiling HNO_3 digestion according to APHA method 3030E [2]. The metal concentrations in these samples were used to select metals of interest for this study. On the basis of these data (see "Results and discussion" section) Mn and Zn were chosen as the metals of greatest relevance. Nutrient analyses were also performed on the effluent, to assist with building a characteristic model wastewater. Total ammoniacal-N was measured via phenol/hypochlorite colorimetry (NH_4-N = NH_4^+-N + NH_3-N). Total Kjeldahl nitrogen (TKN) was measured by sulphuric acid digestion with copper sulphate catalyst, then phenol/hypochlorite colorimetry. Nitrate-N + nitrite-N were measured as total oxidized nitrogen using automated cadmium reduction and flow injection analysis. Total nitrogen was calculated as TKN + nitrate-N + nitrite-N. Total phosphorus was measured as dissolved reactive phosphorus using molybdenum blue colorimetry. Total carbon (TC) was measured

by catalytic oxidation to CO_2 and IR detection, and total inorganic carbon (TIC) was determined by acidification to CO_2, purging and IR detection. Total organic carbon (TOC) was calculated as TC-TIC.

Immediately after collection this effluent was filtered with Whatman grade 3 filter paper (6 μm), then with Whatman GF/C glass microfibre (1.2 μm) and frozen in 2-l aliquots. The filtration of the raw effluent did not dramatically alter the concentrations of any metals, except Al (data not shown). To prepare the model wastewater, an aliquot was thawed, autoclaved and centrifuged at 5,000×*g* for 10 min. To each litre of the pulp mill effluent base, 400 μl acetic acid and 127 μl ethanol were added. The chosen amounts were based on concentrations of these constituents predicted before and after anaerobic treatment in a lignocellulosic ethanol pilot plant [32], to account for either of these locations as a likely position for an aerobic metal removal step within a biorefinery. To maintain a biological oxygen demand/nitrogen/phosphorous ratio of 100/3.5/0.6 and the original pH (5.4) of the effluent, NH_4Cl (77.5 mg/l), K_2HPO_4 (13.0 mg/l) and $NaHCO_3$ (498 mg/l) were added. Finally, the solution was 0.45-μm syringe filtered, to provide a homogenous model wastewater and confine this study to the dissolved phase only.

Organism collection and isolation

Organisms were sought in various culture collections around New Zealand. Some organisms were selected on the basis of known metal tolerance and suitability for mesophilic aerobic conditions. Other organisms, such as *Pseudomonas putida* mt-2, were included because they are easy to culture in a variety of conditions. Three species of algae, bacteria and mould and one species of yeast were initially collected and subjected to a preliminary test. Algae, bacteria and yeast that did not grow sufficiently to visibly 'cloud' the model wastewater after 48 h in an illuminated (70 μmol photon/m^2/s) shaking incubator set to 36 °C

Table 1 *Pinus radiata* ($n = 1$) and pulp mill effluent ($n = 3$) constituents, and calculated model wastewater composition

	P. radiata wood chips (mg/kg)	*P. radiata* pulp mill effluent (mg/l)	Model wastewater (mg/l)
Metals/salts			
Aluminium	27	0.11	0.11
Arsenic	<0.10	0.023–<0.021	0.023–<0.021
Barium	2.7	0.13	0.13
Boron	1.7	0.14	0.14
Cadmium	0.019	<0.0011	<0.0011
Calcium	470	54	54
Chromium	0.37	<0.011	<0.011
Copper	0.91	0.012–<0.0011	0.012–<0.0011
Iron	19	<0.42	<0.42
Lanthanum	0.010	<0.0021	<0.0021
Lead	0.031	0.0051	0.0051
Magnesium	140	9.4	9.4
Manganese	29	0.38	0.38
Potassium	870	45	51
Sodium	<50	60	196
Zinc	9.2	0.34	0.34
Nutrients and other			
Total nitrogen		2.9	23
Ammoniacal-N		0.048	20
Nitrate-N + nitrite-N		0.047	0.047
Kjeldahl N (TKN)		2.8	23
Phosphorus		3.2	5.5
Dissolved organic carbon (DOC)		890	1,010
Acetic acid		64	464
Ethanol		18	118
$NaHCO_3$ (buffering)			498
pH		5.4	5.4

Model wastewater manufacture and metal selection are described in the "Materials and methods" section

were not tested further. Moulds were initially tested under similar conditions for 2 weeks at 25 °C. As many of these organisms showed poor growth in the model wastewater, organisms were also isolated from two New Zealand pulp mill wastewater sources specifically for this study.

The first source of isolates was effluent from a mill that produces both bleached and unbleached kraft pulp. Organisms were isolated on Phytagel (Sigma) solid media, pH 5, at 35 °C and amended with high amounts of Mn and Zn (1 g/kg each) to attempt to select for highly metal-tolerant species. The second source of isolates was a 5-l sequencing batch reactor (SBR) maintained with the dissolved oxygen no less than 43 % of saturation, 35 °C and feeding at a rate of 3 l/day. The feed for the SBR was a mixture of bleached chemi-thermo-mechanical pulping and thermo-mechanical pulping effluent that had been partially predigested in a moving bed biofilm reactor, supplemented with 20 mg/l ethanol and 125 mg/l acetic acid. Mn and Zn were added in the same concentrations as those spiked in the model wastewater (0.20 mM, 11 mg/l Mn and 13 mg/l Zn). Weekly samples were taken from the SBR, and organisms were isolated by serial dilution in the model wastewater with Mn and Zn (0.2 mM), at 36 °C.

The four isolates from wastewater were identified by extracting the DNA with ZR soil microbe DNA Mini-Prep™ kit (Zymo, Irvine, CA, USA). For bacterial species, PCR was used to amplify the 16S rDNA with the primer set 27F/1492R according to Lane [26]. DNA clean-up was carried out with a GFX™ PCR DNA kit (Amersham, GE Healthcare, Rydalmere, NSW, Australia). Sequencing was performed at Massey Genome Service (http://genome.massey.ac.nz/). For fungi, the internal transcribed spacer (ITS) regions of the rDNA were amplified using the primer set ITS1F/ITS4 [6]. PCR and sequencing were carried out as described by McCarthy et al. [31]. The raw sequences were edited with Sequence Scanner v1.0 (Applied Biosystems, Mulgrave VIC, Australia) and the sequences (405–835 bp) were identified by comparison to existing sequences using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The microbe was identified as the match with the highest sequence homology (≥99 %) and sequences were submitted to the GenBank database. Accession numbers for the isolates and the final list of organisms tested for survival and metal uptake in the model wastewater are described in Table 2.

Organism screening assay

A 24-h batch culture screening assay was used to evaluate the ability of each organism to grow in the model wastewater. The effect of metals was tested by comparison to treatments without metals added. Tests were conducted at 36 °C, as many bioreactors in pulp and paper mills operate under mesophilic conditions [18] and those in lignocellulosic biorefineries are also likely to [32]. Seven treatment groups (each with 4 replicates) consisted of: Difco nutrient broth (Becton–Dickinson and Co.) un-inoculated (negative control); nutrient broth (positive control); the model wastewater described above un-inoculated (model wastewater negative control); the model wastewater; the model wastewater plus 0.2 mM Mn (11 mg/l); the model wastewater plus 0.2 mM Zn (13 mg/l); and a final treatment of only the effluent base of the model wastewater without added nutrients to check for possible negative effects of the additions on growth.

One day prior to the assay, an aliquot of the model wastewater and the liquid inoculum was prepared using aseptic technique. The inoculum was started by transferring a colony from a nutrient agar plate to 50 ml of nutrient broth with 10 µl acetic acid and 3 µl ethanol added (pH ca. 5.5), to roughly simulate conditions of the model wastewater. Exceptions were the commercial yeast and the fungus, which were dissolved or pulverised, respectively, in sterile Milli-Q water just before inoculation. Two of the organisms, *P. putida* and *S. scionensis*, did not grow well in the desired inoculum media. The buffering agents (NH_4Cl, K_2HPO_4, $NaHCO_3$) for the model wastewater were added to the inoculum media in similar proportions to grow *P. putida*. *S. scionensis* did not grow in either of those conditions or nutrient broth at 36 °C. Therefore, nutrient broth at 33 °C was used to grow the *S. scionensis* inoculum.

Each assay was initiated with the following steps carried out in a laminar flow or biohazard hood. Model wastewater or nutrient broth (50 ml) was measured into a 250-ml Erlenmeyer flask. Flasks were previously cleaned by soaking in 10 % HNO_3 overnight then rinsing at least six times with distilled H_2O. Then, the metal stock (0.5 ml) was added to appropriate flasks. The stocks were made from chloride salts of the metals (Sigma-Aldrich, Fluka, St. Louis, Mo., USA) and acidified to pH less than 2 with HCl. Separate flasks were used for analytical confirmation of the initial metal concentrations. These flasks contained un-inoculated model wastewater and were preserved with HNO_3 immediately after the start of the test. The inoculum was diluted to achieve an optical density (OD) measurement of 0.3 at 600 nm and 0.5 ml was added to each flask. Flasks were covered with Breathseal plate sealer (Greiner Bio-one, Frickenhausen, Germany) and then placed in a shaking (100 rpm) incubator maintained at 36 ± 1 °C.

After 24 h, flasks were removed from the incubator. The contents of one flask per treatment were streaked on nutrient agar plates to check the purity of the organism and confirm the sterility of the negative controls. Then a 4-ml aliquot was pipetted from each flask into cuvettes for OD measurement at 600 nm. Another 4-ml aliquot was taken

Table 2 Organisms subjected to screening assay in the model wastewater

	Organism	Source	Reference/accession no.[a]
Bacteria	*Novosphingobium nitrogenifigens* Y88^T	Pulp & paper mill effluent bioreactor	[1]
	Sphingobium scionensis WP01^T	Hydrocarbon-contaminated sawmill soil	[28]
	Pseudomonas putida mt-2	Common soil microbe	[53]
	Klebsiella pneumoniae	Pulp & paper mill effluent	JQ640571
Yeasts	*Saccharomyces cerevisiae*	Commercial source	
	Sporopachydermia lactativora	Pulp & paper mill effluent	JQ640569
	Candida tropicalis	Pulp mill effluent bioreactor	JQ640572
Moulds	*Ochroconis gallopava*	Pulp & paper mill effluent	JQ640570

[a] If the organism was obtained from an existing collection, a citation on the isolation or characterization is provided. For organisms isolated for this study the GenBank accession number is provided

for flow cytometry (FC) analysis. The remaining solution (about 38 ml) was weighed and the pH was measured. This solution was then centrifuged at 2,000×*g* for 10 min to the pellet biomass and the supernatant was poured off. The supernatants of the inoculated treatments of the model wastewater were acidified to pH less than 2.0 with HNO_3. If a species grew well in the presence of metals (OD > 0.3) the acidified supernatant was sent for metal analysis by ICP-MS. The biomass pellet was twice washed with 3 ml distilled H_2O and centrifuged. The pellet was then lyophilised and weighed to obtain dry biomass weight. Metal uptake was determined by calculating the absolute metal mass in solution from the weight of the solution in order to avoid error from changes in concentrations caused by evaporation. Uptake was calculated by subtracting the metal mass in the supernatant from the metal mass in flasks set up specifically for the measurement of the initial concentrations. Separate controls were run with metals and without microorganisms to check for precipitation. A very small precipitate was observed after centrifugation. Acid digest and analysis of the precipitate indicated that it contained 1–4 % of the total metal added.

Flow cytometry analysis

To facilitate cell counting using flow cytometry (FC), cells were stained with SYTO® green fluorescent nucleic acid stains according to the manufacturer's instructions (Molecular Probes™, USA). SYTO 9 was used to stain bacteria, whereas SYTO BC was used to stain yeast. Cell counts are all semiquantitative as the staining of all cells and the accuracy of counts were not verified for each species. Flow cytometry cannot be performed easily with the filamentous fungus, so this analysis was not done for the fungal species. Samples (1.0 ml) were stained with a 50 µM SYTO® dye solution in phosphate buffered saline (PBS, pH 7.2).

A FACSVantage SE flow cytometer (BD Biosciences, USA), which was equipped with DiVa electronics and software, a 488-nm laser powered at 300 mW and a helium neon laser providing 633-nm excitation were used to perform sample analysis. The instrument sheath fluid was PBS delivered through a 70-µm nozzle at 131 kPa. Samples were generally diluted to ca. 1×10^6 cells/ml in PBS prior to fluorescent dye staining. TruCount™ fluorescent beads (BD, USA) were included with samples (ca. 5,000 beads per sample tube) to facilitate quantitation. For all sample analyses, the event threshold was set on the 90° scatter (side scatter, SSC) channel at a value of 200. SYTO dye fluorescence was measured on the FC FL1 channel (530/30 nm). Single-parameter histograms and dual-parameter dot plots of the various channel events were monitored and used for setting gates. SYTO-stained events could then be discriminated on SSC versus FL1 fluorescence dotplots by highlighting these in different colours using the DiVa software. The red-fluorescent FL4 channel (660/20 nm), together with the FL1 channel, was used for isolation of TruCount™ bead fluorescence from labelled-cell fluorescence. The total number of measured events per FC analysis was predetermined by the operator's ability to visually resolve populations, enabling manual construction of gates around populations of interest. The total number of measured events was generally 10,000, at a rate of 1,000 events/s. The number of cells for each identified population was calculated using the following formula: cells/ml = (cell count × bead count × sample dilution factor)/(actual beads/ml).

Metal analysis

ICP-MS was performed at the Department of Chemistry, University of Canterbury, Christchurch, NZ. The samples were diluted 20× with 1 % HNO_3 and were analysed for Mn and Zn by ICP-MS (Agilent 7500 cx). Quality assurance and control procedures included analysis of two

spiked samples for each 20 samples. The average per cent recovery with standard deviation was 112 ± 9 % for both Zn and Mn.

Statistics

Statistical analyses were performed using R [38]. Analysis of variance (ANOVA) followed by Tukey's HSD test was used to find significant differences between individual treatment groups ($p < 0.05$).

Results and discussion

Model wastewater manufacture

Biosorption has significant potential as a technology for removing non-process metals from future biorefinery process water, a mechanism that will extend reuse of process water in the plant. Accordingly, a model effluent from a future lignocellulosic ethanol biorefinery was created to test the possibility of using microorganisms to sorb accumulated metals. Pulp mill effluent was used as the base of the model wastewater because it was the best surrogate organic matter available for a biorefinery that would process *P. radiata*. The effluent provided a relatively high NOM concentration (DOC of 890 mg/l) to represent a highly recycled process water. Table 1 shows the composition of this pulp mill effluent base and the final model wastewater. The N and P were relatively low (2.9 mg/l total N and 3.2 mg/l PO_4^{3-}) in the mill effluent used as the base of the model wastewater, and in order to provide sufficient nutrients for microbial growth additional N (as NH_4Cl) and P (as K_2HPO_4) were added. The pilot plant report [32] did not contain predictions of N and P concentrations in the effluent, but did suggest that these nutrients would need to be added in large amounts to the anaerobic digester, and it would be likely that some would remain in the effluent from this digester. Effluent samples from the pilot plant had a pH of 4.9–5.4 similar to that in the model wastewater (pH 5.4).

Metal selection

Metals accumulating in pulp mill process waters are believed to come primarily from the wood [21]. Therefore *P. radiata* wood chips and pulp mill effluent from a plant that processes *P. radiata* were used to indicate the metals most likely to accumulate in process water from a lignocellulosic ethanol biorefinery that utilizes this feedstock. Mn and Zn were the heavy metals in highest concentrations in the pulp mill effluent, and the Mn concentration was the highest of the metals in the *P. radiata* wood chips (followed by Al > Fe > Zn; Table 1). Mn and Zn were also among the heavy metals in highest concentrations in historical data from another pulp mill that primarily processes *P. radiata* [52].

Much of the focus on removal of non-process metals from closed or partly closed cycle pulp and paper mills has focused on transition metals, mainly Mn and Fe because they interfere with hydrogen peroxide bleaching [16, 27]. Kanto Öqvist et al. [22] compared metal concentrations in the process waters from a mill with part closure to those from a mill with full closure and found the average Mn concentrations were 1.45 and 8 mg/l, respectively. They also found that many wastewater constituents including metals, organic acids and nutrients were about tenfold higher in the fully closed mill compared to the partially closed mill. This factor of 10 can be used to estimate the metal concentrations in highly recycled process water.

Mn is problematic in pulp mills not only because it interferes with whitening reagents as described above, but also because it builds up on plumbing as scale [21, 35]. Additionally, if Mn levels in effluent leaving the mill are high enough they can harm aquatic wildlife downstream. Mn is toxic to the model aquatic crustacean *Ceriodaphnia dubia*, having a medial lethal (LC_{50}) concentration of 9.1 mg/l after 48 h. Zn, like Mn, is essential for life, but it can also damage freshwater ecosystems at much lower concentrations. A Zn concentration of 0.039 mg/l is toxic to the alga *Selenastrum capricornutum*, causing a 50 % inhibitory effect on cell division (EC_{50}) after 48 h [13].

On the basis of their relative levels in lignocellulosic effluents, and associated negative impacts on mill performance and the aquatic environment, Mn and Zn were chosen as the metals of greatest relevance to the potential bioremediation of a lignocellulosic biofuel biorefinery. These two metals were added to the model wastewater in equimolar amounts (as described in the "Materials and methods" section, 11 mg/l Mn and 13 mg/l Zn) based on their concentrations in the mill effluent (Table 1) and using the rough concentration factor of 10 from Kanto Öqvist et al. [22] to estimate the accumulation of metals in highly recycled process water.

Organism screening

Two critical factors are required for a model metal-remediating organism for a lignocellulosic ethanol effluent metal-removing bioreactor: the organism needs to be able to live and grow in the wastewater (with or without supplementary nutrients) and needs to be metal tolerant. Many organisms obtained from existing culture collections did not grow after 48 h in the wastewater, so they were not subject to further screening. This included the algae *Scenedesmus* sp. and *Chlorella* sp., species isolated from

municipal wastewater and found to be tolerant of Zn [34], and *Microcystis wesenbergii* which is responsible for algal blooms in freshwater systems [50]. A number of moulds also failed to grow in the model wastewater. *Trichoderma viride*, *Trametes versicolor* and *Antrodia xantha* [17, 25] have the ability to grow on chromate copper arsenate-treated wood and were thus hypothesized as potential bioremediators of metal-enriched wastewaters.

Clearly organisms adapted to a wastewater environment were needed. Therefore, to obtain a metal-tolerant organism that would grow in the model wastewater, organisms were isolated from metal-enriched wastewaters. This included organisms specifically isolated for the purposes of this study (*C. tropicalis*, *S. lactativora*, *K. pneumoniae* and *O. gallopava*), and some that had been previously isolated from similar effluents (*N. nitrogenifigens* [1] and *S. scionensis* [28]). To test the relative efficacy of our choice of organisms we also examined the utility of more common model bacteria (*P. putida*) and yeast (*S. cerevisiae*). These have the advantage in that they are already well characterized and easy to culture.

The two "non-effluent" organisms, the brewer's yeast (*S. cerevisiae*) and the bacteria *P. putida*, grew poorly in the model wastewater (Fig. 1a). Only the organisms isolated from similar wastewaters exhibited significant growth (OD $>$ 0.2) in the model wastewater: *N. nitrogenifigens*, *K. pneumoniae, C. tropicalis, S. lactativora* and *O. gallopava*. All species grew well (OD $>$ 1.0) in the nutrient broth positive control (not shown), except for *N. nitrogenifigens* and *S. scionensis*. *S. scionensis* is another wastewater isolate [28] that could grow in the wastewater but only at lower temperatures (<33 °C; not shown). For brevity, biomass measurements are shown only for the species that grew well (Fig. 1b) since the biomass data exhibited similar trends to growth. The only species severely affected by the metals was *K. pneumoniae*, for which growth was reduced by 85 % upon the addition of Zn, whereas Mn only decreased growth by about 25 %. *O. gallopava* also showed a 25 % reduction in biomass with either of the metals. Growth was always greater in the model wastewater compared to growth in the effluent without the nutrient addition, showing the necessity of this adjustment.

Clearly four of the wastewater isolates—*N. nitrogenifigens*, *C. tropicalis*, *S. lactativora* and *O. gallopava*—have characteristics that enable them to survive and grow in the tested metal-enriched wastewater. *N. nitrogenifigens* was isolated from a pulp mill effluent bioreactor by other researchers [1]. *C. tropicalis* has been studied as the saccharification and fermentation organism for generating bioethanol from rice straw [36], and could therefore function as both the fermenting organism and as part of the wastewater treatment. This organism is also potentially pathogenic so care would need to be taken if it is used on large scale [3]. Less is known about the yeast *S. lactativora*. This yeast does not have the ability to ferment sugars, although it will utilise glucose, xylose and ethanol as carbon sources [41]. The fungus, *O. gallopava*, has been found in areas of high temperature and low pH, including hot springs [54], thermal effluents of nuclear power reactors [40], wood pulp samples [46] and is also known to be pathogenic [54]. It may have unique traits for inhabiting these extreme environments, which may enable it to live in a wide range of conditions.

Flow cytometry analysis

FC analysis offers a different way to measure growth, by providing actual counts of the number of cells. Cell counting using FC showed similar major trends as the biomass and OD data, but there were important differences, primarily in the results from the Zn treatments. Figure 2 shows the FC results of the three best growing species. The FC results indicated that the Zn treatment resulted in lower numbers of yeast cells relative to the Mn and the 'no metals added' treatments (Fig. 2). This was true for both yeast species. The OD measurements were also lower in the Zn treatments, suggesting less growth. However, the biomass measurements (Fig. 1b) did not show these differences. This could be due to higher production of EPS in Zn treatments. EPS secretion in response to a metal stressor has previously been shown in yeast [33], and in fish, mucus secretion has been noted in response to Zn exposure [15]. EPS would increase the biomass without increasing the cell number. Also, EPS would likely increase clumping, which would also appear as fewer cells in FC. The role of EPS in metal uptake is not clear as it has been found to either enhance metal sorption [48] or to hinder sorption, presumably by forming clumps of cells and decreasing surface area available for binding [47].

An alternative explanation for the lower number of cells with constant biomass in Zn treatments could be that the cells were larger. In the yeast *S. cerevisiae*, the cells quickly take up Zn and store large quantities in the vacuole [29]. This may increase cell size. Additionally, the FC forward scatter parameter (FSC) is often directly proportional to cell size, whereas SSC indicates internal granularity or complexity [43]. FSC intensity from the main population of *S. lactativora* increased from 20,000 to about 50,000, as shown in representative plots (Fig. 3). A similar doubling of intensity was seen in the plots for *C. tropicalis*. This suggests that the cells are becoming larger and more complex from the added Zn, supporting the suggestions from the FC counts and biomass data that there were fewer, larger cells in the Zn-exposed yeast species. For *N. nitrogenifigens* though, the FC analysis and OD measurements

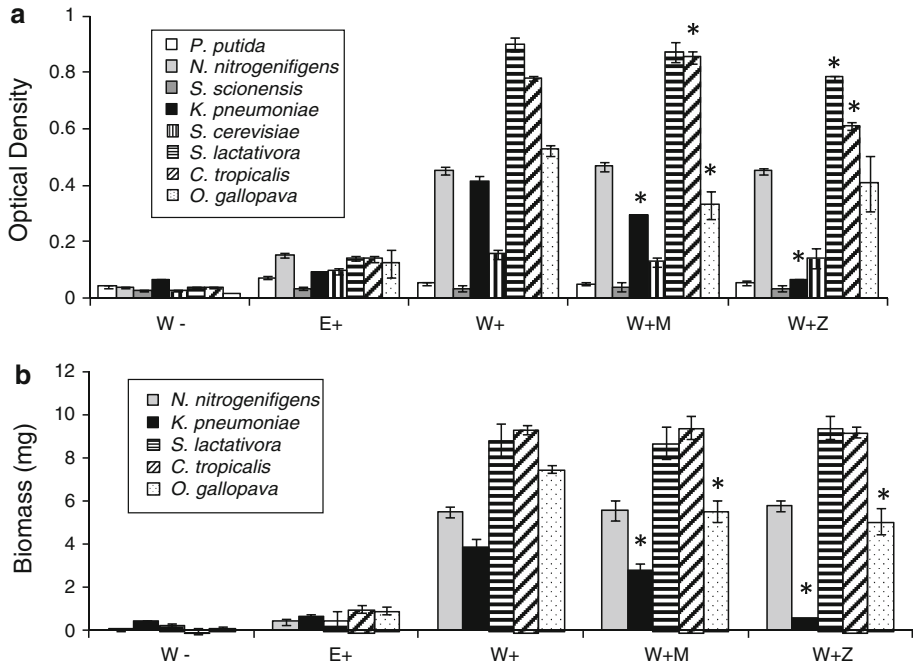

Fig. 1 Growth of various organisms indicated by optical density (**a**) and biomass of selected species (**b**) after 24 h in model wastewater (*W*) with no metal, 0.2 mM Mn (*M*) or 0.2 mM Zn (*Z*) added, or in the pulp mill effluent base of model wastewater (*E*). Solutions were inoculated (+) with the indicated species or not inoculated (−). *Asterisks* indicate differences ($p < 0.05$) for Tukey's comparisons of the treatments with metal against the inoculated wastewater without added metal. Each *bar* represents the mean of 4 replicates ± standard deviation

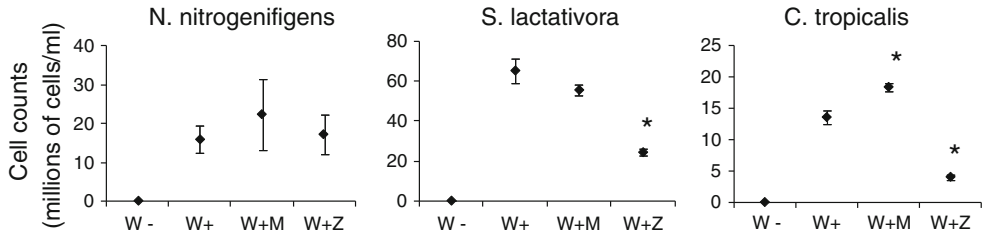

Fig. 2 Changes in flow cytometry cell counts of selected species from metal exposure. Cells were grown in the model wastewater (*W*) with no metal, 0.2 mM Mn (*M*) or 0.2 mM Zn (*Z*) added. Solutions were inoculated (+) with the indicated species or not inoculated (−). *Asterisks* indicate differences ($p < 0.05$) for Tukey's comparisons of the treatments with metal against the inoculated wastewater without added metal. Each *bar* represents the mean of 4 replicates ± standard deviation

did not show these changes. None of these changes were obvious after exposure to Mn (not shown).

Metal sorption

Metal uptake varied significantly by species (Fig. 4). *C. tropicalis* took up the greatest amount of either metal from solution, 38 % of the Zn (5.0 mg/l, Fig. 4a), followed closely by the bacteria, *N. nitrogenifigens* which removed 32 % of the Zn (4.2 mg/l). The other two species removed less Zn, 14–16 % (1.8–2.1 mg/l). The two yeasts studied had markedly different uptake patterns despite similar changes observed via FC following Zn exposure. *C. tropicalis* removed about threefold more Zn than *S. lactativora*. The spores of the genus *Sporopachydermia* have a relatively thick cell wall with a unique ultrastructure compared to other yeasts, a property that gives this yeast its name [41]. Tests were done using vegetative cells of this species not spores, but the cells may retain some unique properties that have a role in the lower Zn sorption observed in this organism. *C. tropicalis*, a common yeast which is part of the normal human flora, has previously been found to be resistant to heavy metals [4, 19, 39]. *N. nitrogenifigens* was isolated from effluent that was unlikely to have metals in high concentrations, although it was isolated on solid media with 5 mM NiCl [1].

The yeast *C. tropicalis* took up the most total metal from solution (Fig. 4a), but the bacteria *N. nitrogenifigens*, which produced only two-thirds of the biomass of the *C. tropicalis* (Fig. 1b), took up almost the same amount of Zn. This made it the most efficient biosorber in our experimental system on a per unit biomass basis (Fig. 4b). *N. nitrogenifigens* removed 35 mg Zn/g biomass and 17 mg Mn/g biomass, whereas *C. tropicalis*, the next best biosorber, removed 24 mg Zn/g biomass. This could be due to the higher surface area of the smaller cells, or because of differences in the cell wall composition.

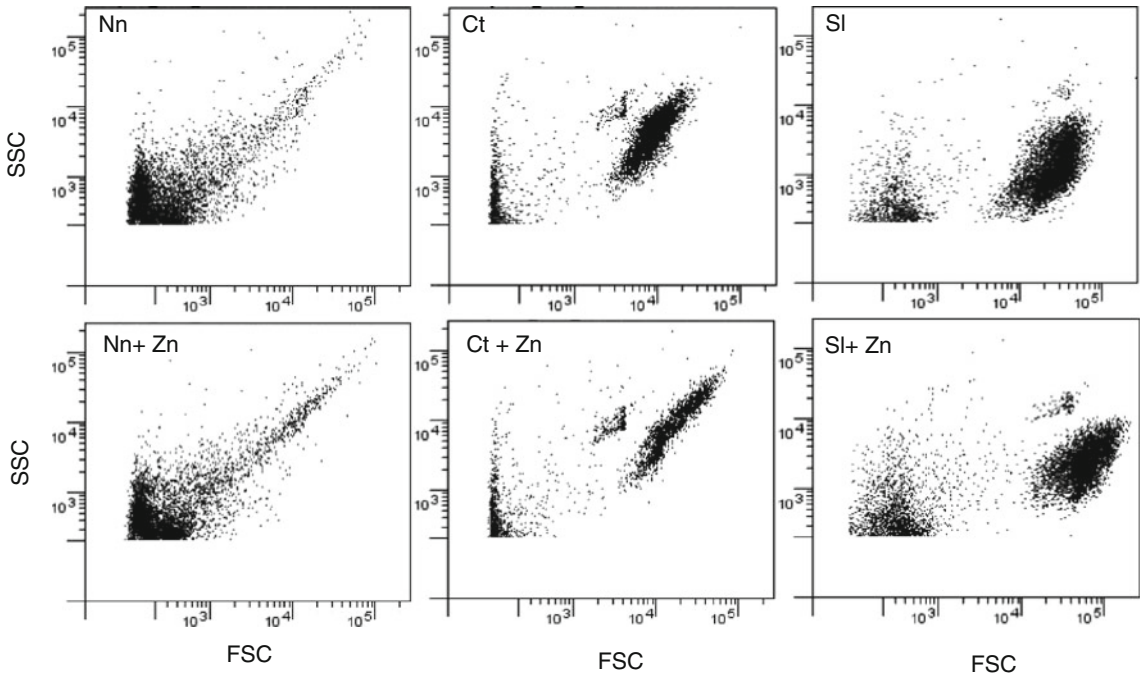

Fig. 3 Representative plots displaying an increased side scatter intensity (*SSC*) and forward scatter intensity (*FSC*) from exposure to Zn for *S. lactativora* (*Sl*) and *C. tropicalis* (*Ct*), but not for *N. nitrogenifigens* (*Nn*)

Mn uptake in general was much lower than that of Zn (Fig. 4a). Only *N. nitrogenifigens* accumulated a significant amount, accounting for 20 % of the total Mn. Lower sorption of Mn compared to other metals had been observed before and is consistent with the binding strength predicted by the Irving–Williams series [7, 30], which has been used to indicate binding affinity of metals to the functional groups on the cell surface. In another study, *Pseudomonas aeruginosa* also sorbed more Zn than Mn, with a maximum biosorption capacity of 77.5 mg Zn/g biomass and 38.2 mg Mn/g biomass, at pH 7.0 [44]. In this study, *N. nitrogenifigens* removed 35 mg Zn/g biomass and 17 mg Mn/g biomass.

Metal biosorption is thought to occur primarily by metal binding to functional groups on the cell membranes, such as the carboxyl group, which depends on pH [14]. As the pH increases more deprotonated sites become available for binding by metal ions with a positive charge, increasing the metal binding capacity. The pH of the wastewater was initially low and remained low in the wastewater negative control (un-inoculated), but the pH increased to approximately 8.4 for all organisms that grew well (OD > 0.3) (Fig. 5), favouring metal uptake. The final pH was mildly correlated to Zn uptake ($r^2 = 0.65$ based on % uptake, $r^2 = 0.52$ based on uptake per unit biomass). However there were not sufficient statistical differences among the pH values to make a definitive correlation.

In addition to adsorption of metals to the cell membrane, cells may absorb metal inside the cell, which may enhance the removal capacity from the medium. At the same time, high intracellular concentrations of metals can be toxic. Some organisms have the ability to actively detoxify heavy metal ions and either store them in the periplasm or precipitate them in the form of bicarbonates and hydroxides. In *P. putida* active efflux of Zn and binding in the periplasm have been observed [8]. Metal tolerance would seem to be an asset for a metal-remediating organism, but the mechanism by which an organism copes with toxic amounts of metals determines if it will be a good remediator. If an organism simply effluxes the undesirable metal back outside the cell and into the culture media it will not be useful for metal removal. On the other hand, an organism that stores the metals inside the cell would be ideal.

The high NOM in this model wastewater could have inhibited metal removal by the microorganisms. Organisms consumed the carbon in the acetic acid and ethanol in the model wastewater, while the more recalcitrant NOM from the pulp mill effluent was likely to remain throughout the exposure, and would continue to have an impact on metal speciation, by binding free metal ions. There was approximately ten times as much NOM in the initial effluent base of the wastewater as there was organism biomass at the end of the test. Still, *N. nitrogenifigens* and *C. tropicalis* removed 30–40 % of the Zn, indicating that a large proportion of the metal was bioavailable. This suggests that these organisms are very effective at sequestering metals, outcompeting NOM for a large portion of the available

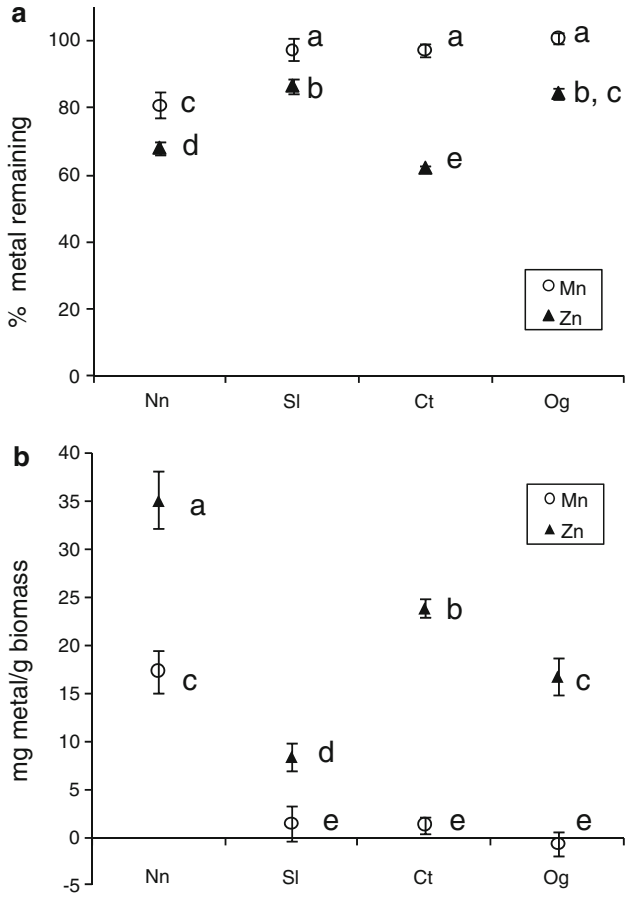

Fig. 4 Total metal uptake (**a**) and metal uptake per unit biomass (**b**) by *N. nitrogenifigens* (*Nn*), *S. lactativora* (*Sl*), *C. tropicalis* (*Ct*), *O. gallopava* (*Og*). Initial metal concentrations were 0.2 mM. Each point represents the mean of 4 replicates ± standard deviation. Data points sharing the *same letter* are not significantly different from each other on the basis of Tukey's multiple comparisons test ($p < 0.05$)

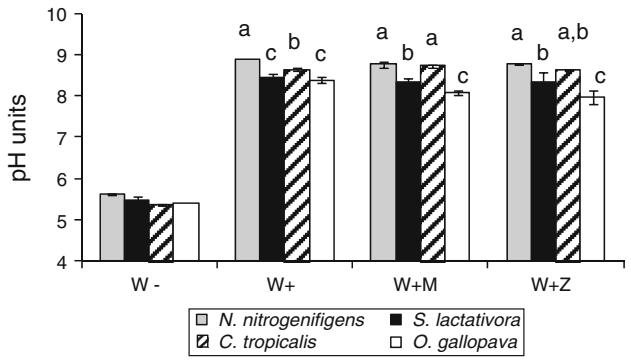

Fig. 5 The pH after 24 h in wastewater inoculated (+) with the indicated species or not inoculated (−). Organisms were grown in model wastewater (*W*) with no metal, 0.2 mM Mn (*M*) or 0.2 mM Zn (*Z*) added. Each *bar* represents the mean of 4 replicates ± standard deviation. Tukey's multiple comparisons tests were performed comparing data within the treatments groups only. Data points with the *same letters* are not significantly different from each other ($p < 0.05$)

metal. Bioavailability is a key concept to consider in terms of the remediating organism, but also is an important factor for organisms in any receiving environment. High bioavailability in such waters, particular freshwaters with limited competitive ions and complexing ligands, is likely to lead to high sensitivity of susceptible aquatic wildlife [12]. This is a problem that could be ameliorated with microbial biosorption bioreactors, which may sequester metals in a non-bioavailable form.

C. tropicalis and *N. nitrogenifigens* were naturally occurring wastewater isolates, and were also the most successful of the tested organisms in terms of being potential remediators of metal-enriched wastewaters. This suggests that any autochthonous species capable of growing in a bioreactor digesting metal-rich effluent should be similarly successful in sorbing metals. Any organisms that grow in the conditions with elevated metal concentrations would obviously need some mechanism to resist metal toxicity. Conversely, any allochthonous, hyperaccumulating organism will probably only grow in a specific set of conditions and may need to be maintained in a monoculture free of other organisms that could out compete the hyperaccumulator. This system would require a great deal of maintenance, whereas a reactor growing autochthonous microbes would be easier to maintain. Indeed, other researchers have found that naturally occurring microbes were useful for metal removal in the environments they were found in [23, 37, 39]. This study shows that uptake does vary by organism and metal, so bioreactor studies will be done in the future to ascertain that uptake is similar in the mixed culture of a reactor as it is for the individual model organisms *N. nitrogenifigens* and *C. tropicalis*. Nevertheless, investigation of individual model organism responses to metal-enriched effluents is useful in that it permits an understanding of the mechanisms of uptake, and bioremediation potential. Such experiments could be used to optimize metal removal in a bioreactor scenario.

In a biorefinery, water will be used in various stages in the refining process and will likely be collected into one treatment system with a solids removal step, followed by anaerobic digestion of the liquids. The resulting effluent stream could then be treated with a polishing step optimized to remove metals using microbial biomass, before the water is reused [18]. The results presented here suggest that at least a third of the dissolved Zn could be removed in such a polishing bioreactor, even in waters with high concentrations of metal-binding NOM. This treatment may be sufficient to keep Zn accumulation under control in a system that has relatively low metal inputs and continuously reuses the water. However, metal removal is dependent on several factors not explored here and depends on the metal. As seen here, removal of Mn is more

challenging than Zn. Finally, the biomass with the metals will need to be removed from the reactor and treated for safe disposal. Recovery of the metals from the biomass is possible [49], but whether or not this is necessary or practical depends on the value or toxicity of the metal(s). Zn for example is toxic to algae, but biomass with Zn could be a nutrient supplement for animals. If the metals cannot be separated from the biomass, the bioreactors will have provided the benefit of concentrating the metal into a much smaller volume for disposal.

Conclusions

Biorefinery process water recycling is likely to be limited by accumulation of dissolved salts and metals. Zn and Mn are the metals most likely to accumulate to levels of concern in a biorefinery processing *P. radiata*. *N. nitrogenifigens* and *C. tropicalis* were able to remove 30–40 % of the Zn in a model lignocellulosic biofuel biorefinery effluent, despite the high dissolved NOM concentration. Any autochthonous species (such as *N. nitrogenifigens* and *C. tropicalis*) are likely to be the easiest to maintain, and will be the most practical choice for a bioreactor, assuming that species in a mixed culture will sorb metals with similar success to the autochthonous species investigated in this study. Bioreactor studies will be done to validate that the removal can be maintained over extended periods in continuous culture. Such metal-sorbing bioreactors could be used in a biorefinery to maintain water quality and enable greater reuse of the process water, thus enhancing the sustainability of the biofuel. Future work will also be directed towards factors that affect metal uptake, including different types and amounts of NOM, so that a model could be used to optimize removal in a bioreactor.

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Conflict of interest This research was funded by the Foundation of Research, Science and Technology (FRST). The funding agency had no scientific input into the study, and the authors declare that they have no conflict of interest.

Ethical standard All experiments were conducted in accordance with New Zealand law, with particular attention to the Ministry of Agriculture and Forestry/Environmental Risk Management Authority policies regarding PC1 and PC2 containment facilities.

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