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K.H. Wiltshire, J.E. Tanner, C.F.D. Gurgel and M.R. Deveney

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South Australian Research and Development Institute
SARDI Aquatic Sciences
2 Hamra Avenue
West Beach SA 5024
Telephone: (08) 8207 5400
Facsimile: (08) 8207 5415
http://www.pir.sa.gov.au/research

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Author(s):	K.H. Wiltshire, J.E. Tanner, C.F.D. Gurgel and M.R. Deveney
Reviewer(s):	A. Redondo-Rodriguez and G. Grammer (SARDI)
Approved by:	Assoc. Prof T. Ward Science Leader – Marine Ecosystems
Signed:	
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EXECUTIVE SUMMARY

Integrated multi-trophic aquaculture (IMTA) involves strategic co-culture of organisms so that wastes from one species are used to grow another, providing environmental and economic benefits. Seaweeds can be used in IMTA systems to remove and utilise dissolved inorganic nutrients from fish aquaculture, allowing environmentally sustainable expansion of this industry. Farming seaweeds is also of interest due to increasing demand for seaweed products, of which Australia is a net importer. Seaweed farming is, however, not an established industry in Australia, and to date no offshore seaweed aquaculture occurs here. A number of seaweed species native to Australia were recently investigated for their aquaculture potential, particularly with respect to application in IMTA, as part of a Fisheries Research and Development Corporation (FRDC) project conducted at SARDI Aquatic Sciences. The FRDC project found that the red seaweed *Solieria robusta,* a carrageenan producer, grew consistently well in laboratory trials designed to replicate conditions around fish farms and showed promising, although variable, growth in field trials. Field trials for that project were small-scale due to the limited availability of biomass. Further development of this species for aquaculture will require hatchery techniques to be refined and up-scaled to produce sufficient seeding stock for large-scale cultivation trials, and ultimately to provide seeding material for farms. *Solieria robusta* showed good nitrogen removal ability in the FRDC project laboratory trials, but more detailed knowledge of nutrient uptake dynamics is needed for incorporation in biogeochemical models, to determine the ratio of seaweed to fish that will result in greatest environmental benefit, and to help inform the best locations for seaweed aquaculture in relation to fish farms.

The current study investigated explant production and hatchery culture (grow-out) for seedling cultivation of *Solieria robusta.* We also investigated uptake rates of nitrogen (N) as both ammonium and nitrate. To investigate explant production, cuttings taken from either plant tips or stems were cultivated in one of two common enrichment media: Provasoli or von Stosch solution, at full, half and quarter strength. The effects of N source (ammonia or nitrate) and concentration (1 – 450 μM L^{-1}) on seedling grow-out was examined to determine the optimal N source and concentration for seedling growth. Nitrogen uptake rates were determined for ammonium and nitrate N over concentrations of 1 – 300 μM L^{-1}.

Tip explants grown in full strength Provasoli enrichment solution were the best performing in the explant production trial, and epiphyte contamination occurred less frequently in tip than stem

explants. Seedlings in the grow-out trial showed a trend towards lower growth at the higher N levels tested, indicating that the optimum N for growth of *Solieria robusta* may be at the lower end or beneath the levels tested, which were predominantly higher than would be experienced in cultivation around South Australian fish farms, although within the range used for tank culture of other seaweeds. Growth rates varied between specimens, indicating that strain selection will be important for optimizing growth performance. Specimens grown under higher N levels accumulated higher tissue N than specimens grown at lower N. No difference in growth photosynthetic performance or tissue N was observed between N sources, indicating that both can be utilised by *Solieria robusta* and either would be a suitable substrate for hatchery cultivation. Nitrogen source also did not affect uptake rates, which were linear over the range tested with no evidence of saturation dynamics. *Solieria robusta* showed high affinity for both ammonia and nitrate N, with good ability to remove N even at low concentrations.

Keywords: Seaweed, Rhodophyta, Integrated multi-trophic aquaculture, Nitrogen removal.

1. INTRODUCTION

1.1. Background

Several fish species are farmed in sea cages in Australia, and production is increasing to meet growing demand for seafood both nationally and internationally (Department of Agriculture and Water Resources 2016; Mobsby and Koduah 2017). There is a strong emphasis on environmentally sustainable management of Australian aquaculture, but community concern about environmental impacts of the industry remains (Rimmer and Ponia 2007; Department of Agriculture and Water Resources 2016). Improving the environmental performance of Australian aquaculture is a priority to achieve environmentally sustainable expansion and improve public perception, with advances to production technology or implementation of integrated multi-trophic aquaculture (IMTA) being two identified pathways (Department of Agriculture and Water Resources 2017).

IMTA is a system involving co-culture of organisms at complementary trophic levels, such that wastes from one (e.g. finfish) are recycled and utilised by others, such as filter-feeders (e.g. bivalves), which remove particulate wastes, and autotrophs (e.g. seaweeds), which remove dissolved inorganic nutrients (Soto 2009). The seaweeds, bivalves or other extractive species used in IMTA are also crops of commercial value, leading to reduced economic risk through diversification of product farmed (Ridler *et al.* 2007; Barrington *et al.* 2009; Soto 2009). Extractive species in IMTA systems grow faster than in monoculture, leading to greater overall profitability (Petrell and Alie 1996; Troell *et al.* 2003; Whitmarsh *et al.* 2006). IMTA has been shown to promote greater social acceptance of aquaculture activity (Ridler *et al.* 2007), and IMTA seafood can also be marketed at a premium price (Whitmarsh and Wattage 2006).

The feasibility of incorporating seaweeds into IMTA systems in South Australia (SA) was investigated by Wiltshire *et al.* (2015). Two main fish species are farmed in SA, with farms located in Spencer Gulf: southern bluefin tuna (tuna), *Thunnus maccoyii*, and yellowtail kingfish (kingfish), *Seriola lalandi.* These are predatory fish with high food conversion ratios relative to other aquaculture species such as salmonids, particularly tuna, which are fed baitfish rather than pellets. Each tonne of production releases 200 kg (kingfish) to 500 kg (tuna) of nitrogen, with 50–70% in dissolved form (Fernandes *et al.* 2007; Fernandes and Tanner 2008). Dissolved nitrogen is the nutrient limiting the environmental carrying capacity of fish aquaculture in southern Spencer

Gulf (Collings *et al.* 2007; Tanner *et al.* 2007; Middleton *et al.* 2013) and IMTA with seaweeds is applicable to mitigate dissolved nitrogen inputs to the environment (Neori 2008).

The species used in IMTA systems should be native to ensure they are suitable for the local environment and to avoid introduced cultivated species becoming pests (Barrington *et al.* 2009; Soto 2009). Few Australian seaweed species have been commercially cultivated, however, and off-shore cultivation is yet to be developed (Lee 2010). Application of IMTA in Australia is therefore likely to require development of novel species for aquaculture. Australia, and in particular southern Australia, has high seaweed diversity with a large proportion of endemic species (Phillips 2001). The potential value of this seaweed diversity, which may provide unique bioactive properties and high quality extracts, is recognised, further supporting the development of a local seaweed industry using novel species (Lee 2010; Lorbeer *et al.* 2013). Wiltshire *et al.* (2015) therefore investigated eight seaweed species native to South Australia's fish farming region to determine potentially suitable species and farming systems for the development of IMTA in Australia.

The species investigated comprised four from each of two distinct types: red (Rhodophyta: Florideophyceae) and brown (Ochrophyta: Phaeophyceae) seaweeds. Across a range of field and laboratory trials the red seaweed *Solieria robusta* (Greville) Kylin (Solieriaceae) and the brown seaweed (Ocrophyta) *Ecklonia radiata* (C.Agardh) J.Agardh (Lessoniaceae) were identified as the best potential candidates for aquaculture of their respective types (Wiltshire *et al*. 2015). *Solieria robusta*, a carrageenan producer, grew consistently well in laboratory trials designed to replicate conditions around fish farms and showed promising, although variable, growth in small-scale field trials (Wiltshire *et al*. 2015). Larger-scale trials will be needed to refine farming techniques, e.g. by determining the best culture systems and planting density to optimise growth and nutrient removal (Troell *et al.* 2009; Titlyanov and Titlyanova 2010), and to identify the most suitable areas for farming and position relative to fish farms or cages (Wiltshire *et al*. 2015). *Solieria robusta* will grow from cuttings (Wiltshire *et al*. 2015), but production of sufficient biomass for larger-scale trials, and ultimately to provide seeding material for farms, will require hatchery techniques to be refined and up-scaled. This study aimed to investigate methods for production and grow-out of explants in the laboratory to inform the best conditions for seedling growth in this species. Wiltshire *et al.* (2015) found that *S. robusta* grew faster with added nitrogen (N) as ammonia, but did not investigate growth with nitrate N, since ammonia is the primary form of N in fish waste (Fernandes *et al.* 2007; Fernandes and Tanner 2008), and only tested levels that are likely to be found in field culture in Spencer Gulf. They therefore did not determine the optimal

level of N addition or N source. For hatchery cultivation, a higher N level than may occur in the field could be optimal (e.g. 50 to ≥ 200 µM for several species, *c.f.* predicted Spencer Gulf level of 12 µM), and nitrate may be a better substrate because ammonia becomes toxic at higher concentrations (Rui *et al.* 1990; Liu *et al.* 2004; Carmona *et al.* 2006; Pribadi 2012; Ribeiro *et al.* 2013; Grote 2016).

In their investigation of N responses, Wiltshire *et al.* (2015) showed that *S. robusta* could effectively remove ammonia from the experimental media used, but the data collected did not allow calculation of uptake rates or kinetics. These more detailed data are needed to incorporate nutrient removal by seaweeds into biogeochemical models, and assist in determining the ratio of seaweed to fish biomass needed for nutrient mitigation. The current study therefore also determined uptake rates of *S. robusta* for both ammonia and nitrate N.

1.2. Objectives

- Compare the performance of *S. robusta* explants sourced from tip and stem segments and grown in commonly used enrichment media
- Determine the optimum N source and concentration for *S. robusta* seedling grow-out
- Determine *S. robusta* N uptake rates for nitrate and ammonia

2. METHODS

2.1. Seaweed material

Specimens of *S. robusta* were collected in September 2018 at ~3 m depth from Outer Harbor (34º 48′ 14″ S, 138º 28′ 24″ E) and transferred to 20 L aquaria in a controlled environment room at SARDI Aquatic Sciences (Figure 1). Specimens were maintained in filtered (0.5 µm) flow through natural seawater (at 4 L h^{-1}) from Gulf St Vincent at ambient salinity and 20°C with no supplemental nutrient added until use in experiments. Lighting was provided by cool white LED lamps filtered through medium green shade cloth to provide a photon flux density of 45 µE $m^{-2}s^{-1}$ photosynthetically active radiation (PAR) with a 12:12 light:dark cycle.

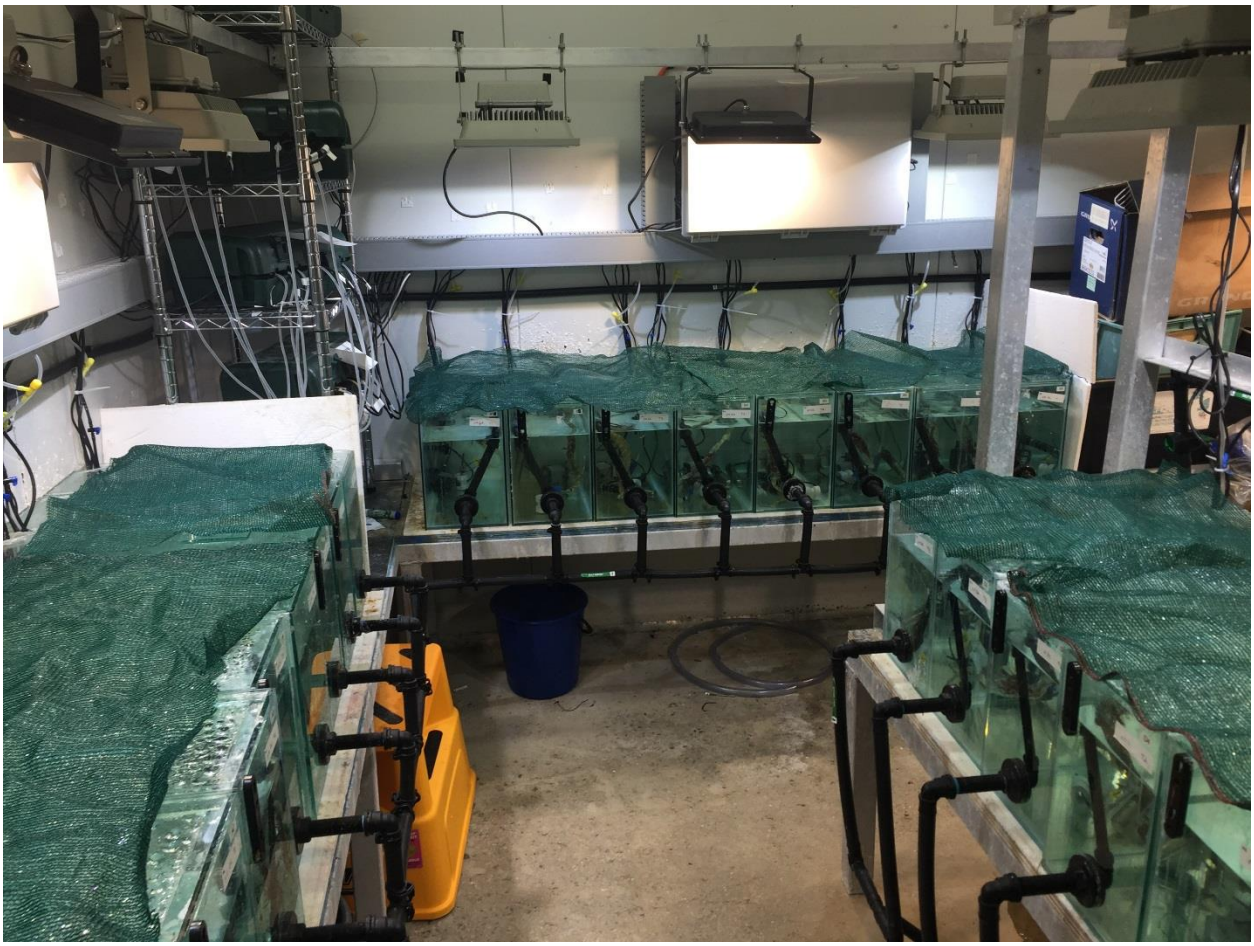

Figure 1. Controlled environment room at SARDI Aquatic Sciences.

2.2. Explant production and cultivation

Methods for explant production were based on those applied by Yong *et al.* (2011; 2014) to commercially cultivated *Kappaphycus* and *Eucheuma* spp, which are also Solieriaceae. Clean fronds of *S. robusta* were selected from the specimens, excised with a sterile scalpel blade and cleaned with a soft toothbrush in filtered seawater to remove micro-epiphytes. Explants of ~20 mm length were taken from two sources: frond tips or stems. Tip explants included branch tips, which in *S. robusta* contain apical cells (Womersley 1994), while stem explants were taken at a minimum distance of 50 mm from tips. Each flask contained one tip and one stem explant from the same specimen, with different specimens used as source material for each flask, and specimens randomly assigned to treatment flasks. Flasks contained 200 mL filtered natural seawater plus enrichment solution. The enrichment solutions used were modified Provasoli Enrichment solution (PES) (Berges *et al.* 2001) and Von Stosch medium (VSM) (Harrison and Berges 2005), at full, half and quarter strength each, with four replicate flasks used per treatment. These are common enrichment solutions used for the propagation of red seaweeds (Yong *et al.* 2014). Flasks were maintained at 20 °C in a culture cabinet (Climatron 520-DL) under lighting of 100 µE $m^{-2}s^{-1}$ PAR with a 12:12 light:dark cycle and with gentle aeration (Figure 2). Seawater in each flask was replaced three times weekly with fresh enrichment media added over a four week experimental period. The N source in each of the enrichment solutions used is nitrate. Samples for nitrate-N determination were taken from samples of freshly mixed seawater and enrichment solution of each treatment and stored frozen until analysis. Explant growth was determined form fresh weights recorded at the start and end of the experiment after gently patting dry on paper towel using the specific growth rate (SGR as % d^{-1}) formula: $SGR = 100 * \ln(FW_t - FW_0)/t$, where FW_t = final fresh weight, FW_0 = initial fresh weight, and t is time in days. Some explants did not survive over the four week period and were not included in the SGR analysis. The occurrence of epiphytes on explants was recorded at the end of the experiment.

Figure 2. Explant cultivation flasks in culture cabinet.

2.3. Seedling nitrogen responses

The seedling grow out experiment was conducted in the same controlled environment room as used to maintain the stock collection and under the same temperature and lighting regime, with filtered natural seawater at ambient salinity. Specimens of ~4 g fresh weight were transferred to mesh bags made from nylon mussel netting (Venus products) and assigned randomly to one of 24 aquaria (Figure 3). After two weeks of acclimation with no nutrient addition and continuous flow through seawater supply, nutrients were added by peristaltic dosing pumps daily at 9:00 AM for four weeks, during which water was supplied to aquaria for six hours nightly (6:00 PM to midnight) to give a total tank turnover of ~1.2 times per day. Dosing pumps added an aliquot of 1, 2, 5, 10, 20 or 30 mL from stock solutions of 0.2 M N as either ammonia (from $(NH_4)_2SO_4$) or nitrate (from $NaNO_3$), with two aquaria per treatment, with these treatments randomly assigned to aquaria. Stock solutions contained phosphate (P, as KH_2PO_4) in a 10:1 N:P ratio to avoid P limitation. Final N levels varied between replicate tanks due to variability in dosing pump accuracy and water turnover rates that meant some tanks did not achieve a full water exchange daily. Average N levels in each aquarium were determined from water samples collected weekly throughout the experiment. Effective quantum yield of PSII photochemistry (Genty *et al.* 1989)

was calculated for each specimen based on fluorescence values taken using a wireless waterproof Pulse Amplitude Modulated (PAM) fluorometer (Classic Fluorometer, Aquation Pty Ltd, Australia), following Maxwell and Johnson (2000) on the day before the experiment was concluded. SGR of seedlings was determined as for explants (see section 2.2) using fresh weights recorded at the start of the experiment, i.e. immediately prior to the commencement of nutrient addition, and after four weeks cultivation with added nutrient. After recording fresh weights at the end of the experiment, 2–3 g of material was taken from the specimens from each aquarium for tissue N analysis.

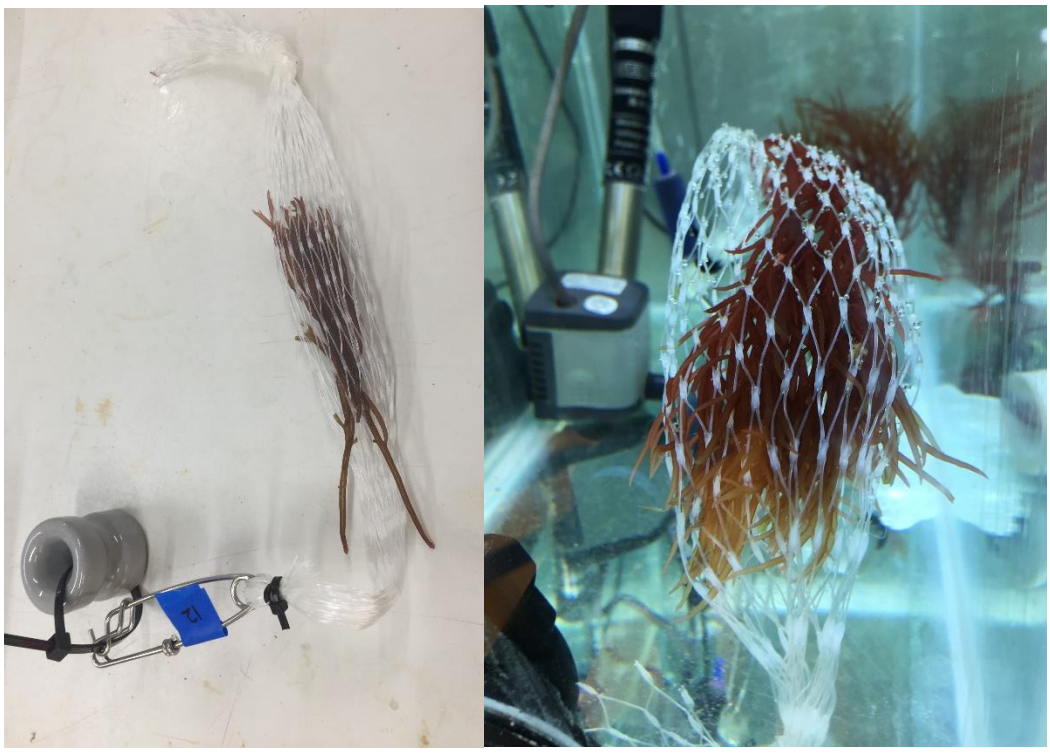

Figure 3. *Solieria robusta* specimens placed into mesh bags for seedling cultivation experiment.

2.4. Nitrogen uptake rates

Seaweed material for use in the uptake rate experiment was transferred from the stock collections to 2 L flasks containing filtered natural seawater, which were maintained in the same culture cabinet with the conditions used for the explant trial (Section 2.2) for two weeks acclimation with seawater replaced twice weekly. Uptake rates were determined using 200 mL flasks containing an average of 0.65 g fresh weight of *S. robusta* taken from the acclimated material. Flasks

contained 200 mL low-nutrient artificial sea salts (Sigma) with N of 10, 25, 50, 100, 200 or 300 µM as either ammonia (from $(NH_4)_2SO_4$) or nitrate (from $NaNO_3$), with three replicate flasks per treatment. Phosphate (P, as KH_2PO_4) was added in a 10:1 N:P ratio to avoid P limitation. Water samples of 50 mL for N analysis were taken from each flask after addition of nutrient and mixing and prior to specimen addition, and then after one hour. During the hour uptake period, flasks were maintained in the same culture cabinet with the conditions used for the explant trial (section 2.2). The tissue N status of the seaweed used in the uptake trial was determined from samples taken from seaweed specimens from which fronds were taken for use in the trial. The fresh weight of material in each flask was determined after gently patting dry on paper towel, and converted to dry weight using the average water content determined from the tissue N samples (see section 2.5). Uptake rates (V) were then determined as: $V = (M_0 – M_t) / (t \times DW)$, where M_0 and M_t are the moles of N at time 0 and t, calculated from concentration x volume at each time, t is the time interval and DW the seaweed dry weight.

2.5. Chemical analyses

Samples for tissue N content were frozen, freeze-dried overnight, and then ground to a fine powder using a Fritsch stainless steel ball mill. A 100 mg aliquot was analysed on a LECO Truspec CNS Elemental Analyser (LECO, St Joseph, MI, USA). Water content was determined from the difference in weight of specimens before and after freeze-drying.

Water nutrient samples were kept frozen until analysis and analysed on a Thermo Scientific™ Aquakem™ analyser for ammonia levels above 3 µM and nitrate levels above 15 µM, with lower level samples analysed by flow injection analysis (FIA) on a Lachat QuickChem 8000 Automated Ion analyser. Ammonia ($NH_3 + NH_4^+$) was determined using the idophenol blue method (Lachat 2003b) in both cases. Nitrate was determined using the sulphanilaminde method using hydrazine reduction for the Aquakem™ or a cadmium reduction column for FIA (Lachat 2003a).

2.6. Statistics

All statistical analyses were conducted using *R* v3.4.3 (R Core Team 2017). Survival and epiphyte occurrence in the explant trial were analysed with logistic generalised linear mixed models (GLMMs), and SGR by a linear mixed model, using the package *lme4* (Bates *et al.* 2014), in each case with enrichment solution, N concentration (as a measure of solution strength), and explant type as factors, and flask as a random effect. Fixed effects were assessed by comparing nested models using likelihood ratio tests (LRT) and $\alpha=0.05$. Where interaction terms were found to be

significant by LRTs, significance of terms comprising the interactions was not further assessed. SGR in the seedling grow-out trial showed evidence of a non-linear response to N concentration, with one outlying point showing large influence on the results based on Cook's distance. Results were analysed both with and without this influential point for comparison using both linear models and generalised additive models (GAM) in each case to assess non-linearity. GAMs were fitted using the *R* package *mgcv* (Wood 2006). N source and concentration were included as factors, and Akaike's information criterion with small sample size correction (AICc) was used to compare models, with the best model indicated by lowest AICc; however, more complex models were only considered better than simpler models where the reduction in AICc was >2 (Arnold 2010; Burnham *et al.* 2011). Uptake rates were fitted to Michaelis-Menten curve for each N source using the *R* package *drc* (Ritz et al. 2016), which applies iterative non-linear least squares estimation. The Michaelis-Menten model is given by $V = V_{max} \times S/(K_s + S)$, where S is the substrate concentration, K_s is the half-saturation constant and V_{max} is the maximum uptake rate. Michaelis-Menten fits were compared to linear models using AICc to determine whether the responses showed evidence of saturation. Where the responses did not show evidence of saturation kinetics, the linear model was used with initial N concentration and N source as factors and *F*-tests used to assess effects with $\alpha=0.05$. The slope of the response in this case equals affinity for the substrate, given by V_{max}/K_s when a Michaelis-Menten curve is fitted, which is a better guide to the ability of a seaweed to remove nutrient at low concentration than V_{max}, however, the individual saturation kinetics parameters cannot be estimated from the linear model (Harrison and Hurd 2001; Smit 2002).

3. RESULTS AND DISCUSSION

3.1. Explant production

SGR of *S. robusta* explants varied between tip and stem explants contingent on both enrichment solution (ES) type and strength (Figure 4,Table **1**). Overall greatest SGR (mean ± s.d. 2.2 ± 1.5 % d^{-1}) was achieved by tip explants with full strength PES. SGR increased with ES strength in tip fragments with PES and stem fragments with both ES types, although with differing slopes, while SGR declined with increasing ES for tip explants in VSM (Figure 4). Epiphytes were observed on only two tip explants, one each in half and full strength VSM, while 9 of 16 stem explants had visible epiphytes, including all stem explants in quarter strength VSM (Figure 5). The logistic GLMM showed that frequency of epiphyte occurrence varied with ES type and with explant source contingent on ES strength (Table **1**). Epiphyte occurrence was more common with VSM for both explant types, while epiphytes were observed more frequently in lower ES strength for stem explants and at higher ES strength for tip explants. Stem explants in full strength ES of either type were noted to have particularly heavy epiphyte growth, which would have contributed to their final mass and hence calculated SGR, further supporting the better overall performance of tip explants in PES. The majority of explants (32 of 40) survived, with losses spread across treatments (Table **2**). The logistic GLMM of survival supported the observation that losses were not explained by explant type, ES type or strength (Table **1**).

3.2. Seedling cultivation

Nitrogen concentrations in the seedling cultivation trial ranged from 0.7–330 µM in aquaria with ammonia addition, and 0.9–460 µM in aquaria with nitrate addition. Growth of *S. robusta* was variable, with overall mean SGR ± s.d. of 1.4 ± 0.7 % d^{-1}. There was a generally declining trend in SGR of *S. robusta* with N concentration for both nitrate and ammonia addition, but the aquarium with highest nitrate N (460 µM) demonstrated the greatest SGR (2.5 % d^{-1}), leading to a non-linear pattern in the response of SGR to N (Figure 6). GAM models with a smooth effect for N concentration provided a better fit to the data than linear models as assessed by AICc (Table **3**). The effect of N source was tested by comparing a GAM of the overall SGR response for both N sources with one that also included a term for the difference between N sources (Wood 2006). The model without the term for N source was best as assessed by AICc (Table **3**), indicating that the SGR response did not depend on N source, while there was smooth effect of N concentration (approximate significance = 0.02, effective df = 2.38). The aquarium with high SGR at high N

concentration was a very influential point based on Cook's distance. Excluding this outlying point, the SGR response to N still exhibited some non-linearity (Figure 7), but the difference in AICc between linear models and GAMs including the same factors was < 2, indicating that the simpler linear model was preferable (Table **3**). In this case the linear model showed a marginally significant effect of N concentration ($F_{1,19}$ = 4.45, p = 0.048) on SGR, which did not depend on N source ($F_{1,19}$ = 0.07, p = 0.791) or the interaction ($F_{1,19}$ = 0.02, p = 0.884). Although statistically significant, the effect size was small, with a coefficient of -0.003 showing there was a minimal decline in SGR with increasing N over the concentrations tested. Effective quantum yield measured by PAM fluorometry was variable across aquaria and did not show any clear trends. This observation was supported by linear models which showed no significant effect of N source ($F_{1,20}$ = 0.36, p = 0.557), concentration ($F_{1,20}$ = 0.14, p = 0.711), or interaction ($F_{1,20}$ = 1.44, p = 0.244) on effective quantum yield. Tissue N ranged from 1.3–4.7 % DW^{-1}, increasing with N concentration (coefficient: 0.005, $F_{1,20}$ = 11.2, p = 0.003; Figure 8), with this result not dependent on N source ($F_{1,20}$ = 0.65, p = 0.429) or the interaction ($F_{1,20}$ = 2.95, p = 0.102).

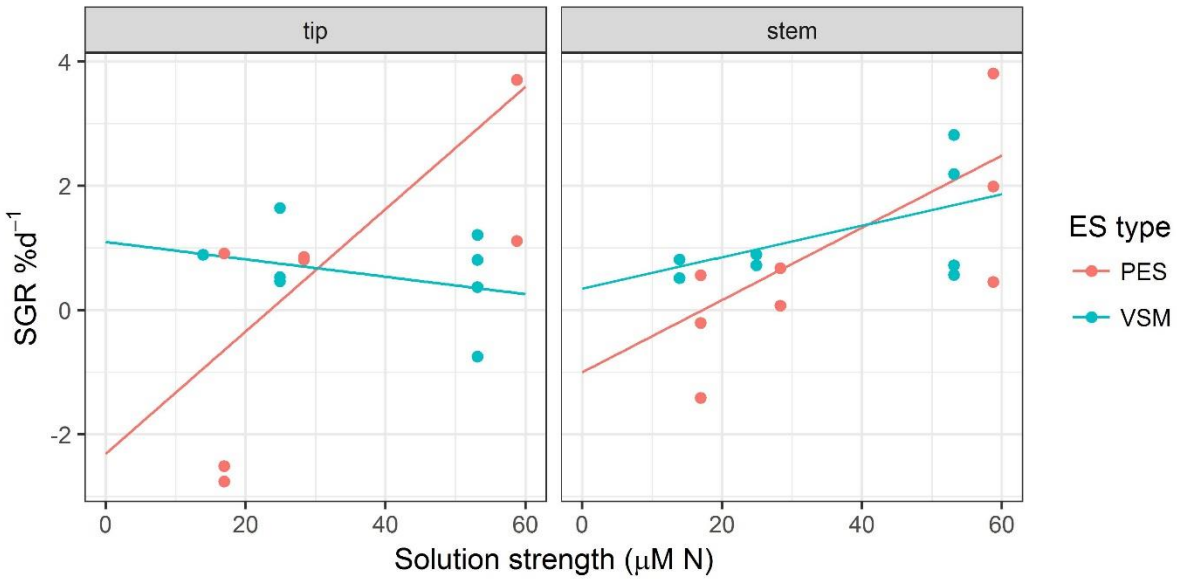

Figure 4. Specific growth rate (SGR, % d^{-1}) of tip and stem explants of *Solieria robusta* grown in Provasoli enrichment solution (PES) or Von Stosch media (VSM) of different concentration: full, half, and quarter strength, expressed as µM of nitrogen (N) and fitted linear model.

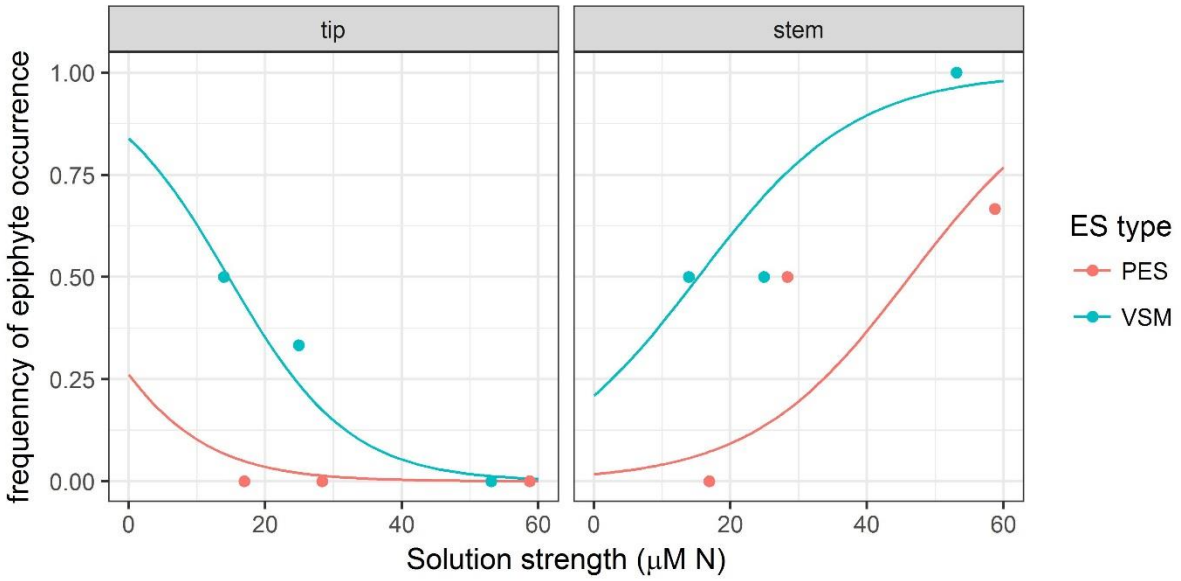

Figure 5. Occurrence of epiphytes on tip and stem explants of *Solieria robusta* grown in Provasoli enrichment solution (PES) or Von Stosch media (VSM) of different concentration: full, half, and quarter strength, expressed as µM of nitrogen (N) and fitted logistic model.

Table 1. Likelihood ratio test (LRT) results from explant production trial. The LRT statistic is the difference in explained deviance between models, with probability (p) determined from a χ^2 distribution with degrees of freedom (df) equal to the difference in the number of model parameters. Significant effects are marked with (*).

Response	Factor	df	LRT	p
SGR	ES type x Concentration x explant source*	1	5.04	0.025
Epiphytes	ES type x Concentration x explant source	1	<0.001	1
	ES type x Concentration	1	0.174	0.676
	ES type x explant source	1	0.616	0.433
	Concentration x explant source*	1	5.63	0.018
	ES type*	1	5.12	0.024
Survival	ES type x Concentration x explant source	1	0.674	0.411
	ES type x Concentration	1	0.465	0.495
	ES type x explant source	1	0.355	0.552
	Concentration x explant source	1	0.435	0.509
	ES type	1	0.667	0.414
	Concentration	1	0.647	0.421
	Explant source	1	<0.001	1

Table 2. Number of explants surviving in each treatment out of a total of 4 in each case

	Number surviving	
ES type and Concentration	**Tip explant**	**Stem explant**
PES full	3	2
PES half	2	2
PES quarter	3	3
VSM full	4	4
VSM half	2	3
VSM quarter	2	2

3.3. Uptake rates

Uptake rates (V) of *S. robusta* did not show evidence of saturation over the range of concentrations tested (Figure 9), with linear models providing a better fit to the data than Michaelis-Menten curves as assessed by AICc (Table 4). Saturation kinetics parameters for *S. robusta* therefore could not be calculated, but substrate affinity can be determined from the regression slope of V on N concentration. The linear model showed that affinity was 0.89, and did not vary between N sources, with no effect of N source ($F_{1,32} = 0.68$, $p = 0.415$) or the interaction ($F_{1,32} = 0.01$, $p = 0.922$), while the effect of N concentration on uptake rate was significant ($F_{1,32} = 129.7$, $p < 0.001$). Analysis of tissue N showed that specimens used in the uptake trial had average N content (mean ± s.d.) of 2.4 ± 0.1%.

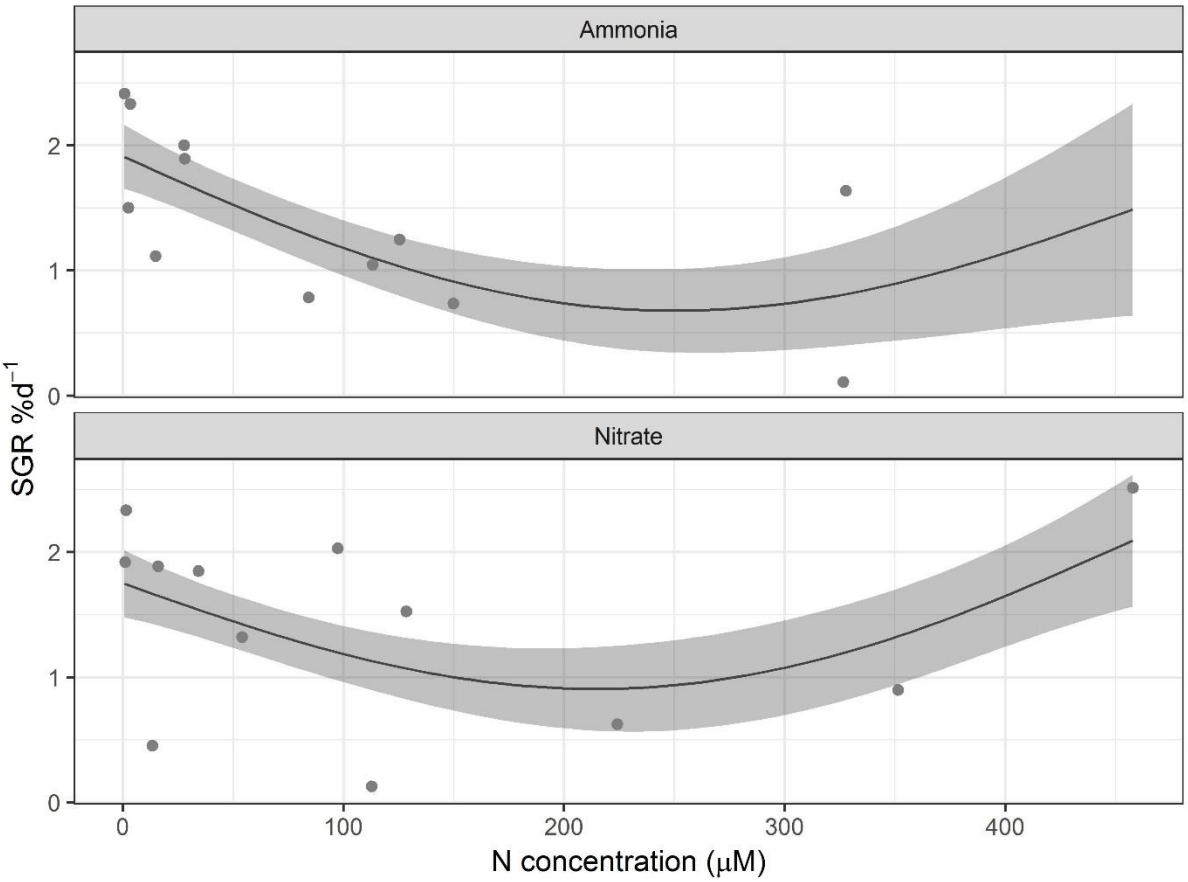

Figure 6. Specific growth rate (SGR, % d^{-1}) of *Solieria robusta* seedlings grown with different concentrations (μM) of nitrogen (N) as ammonia or nitrate showing GAM fitted to all data, with shaded area showing 95% confidence intervals of the fit.

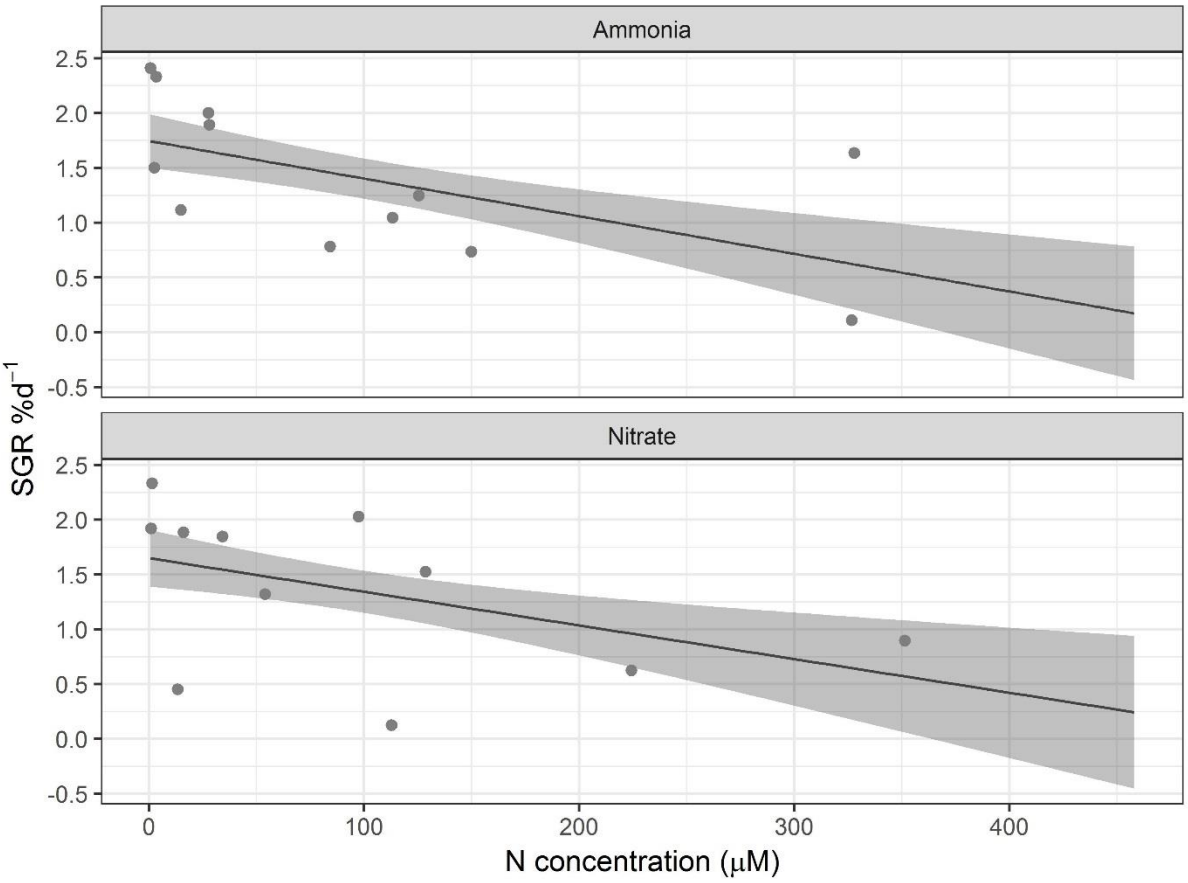

Figure 7. Specific growth rate (SGR, % d^{-1}) of *Solieria robusta* seedlings grown with different concentrations (μM) of nitrogen (N) as ammonia or nitrate showing linear model fitted to data after removing influential outlying point, with shaded area showing 95% confidence intervals of the fit.

Table 3. AICc results comparing models for specific growth rate of *Solieria robusta* seedlings with different nitrogen (N) sources (nitrate and ammonia) and concentrations using all data and a data set with highly influential outlier removed. ∆AICc is the difference in AICc from the model with lowest AICc for each dataset†.

Data set	Model type	Factors included	AICc	∆AICc
Full	**GAM**	N concentration (smooth) x source	56.37	6.47
		N concentration (smooth), source	53.21	3.31
		N concentration (smooth)*	49.90	
	Linear	N concentration x source	60.55	10.65
		N concentration, source	59.92	10.02
		N concentration	57.11	7.21
		N source	58.38	8.49
No outlier	**GAM**	N concentration (smooth) x source	53.35	6.59
		N concentration (smooth), source	49.88	3.11
		N concentration (smooth)	46.77	
	Linear	N concentration x source	53.64	6.88
		N concentration, source	50.36	3.59
		N concentration*	47.46	0.70
		N source	54.80	8.03

Table 4. AICc results comparing models for Michaelis-Menten curves and linear models fit to *Solieria robusta* uptake rate data with different nitrogen (N) sources (nitrate and ammonia) and concentrations. ∆AICc is the difference in AICc from the model with lowest AICc for each dataset†.

Model type	Factors included	AICc	∆AICc
Non linear	Michaelis-Menten curve per N source	360.6	1.9
Linear	N concentration x source	362.4	3.7
	N concentration, source	359.7	1.0
	N concentration*	358.7	
	N source	434.0	75.4

†The most parsimonious model in each case is marked with (*) based on the criteria that lower AICc is better, but a simpler model with ∆AICc <2 should be preferred over a more complex model (Arnold 2010; Burnham *et al.* 2011).

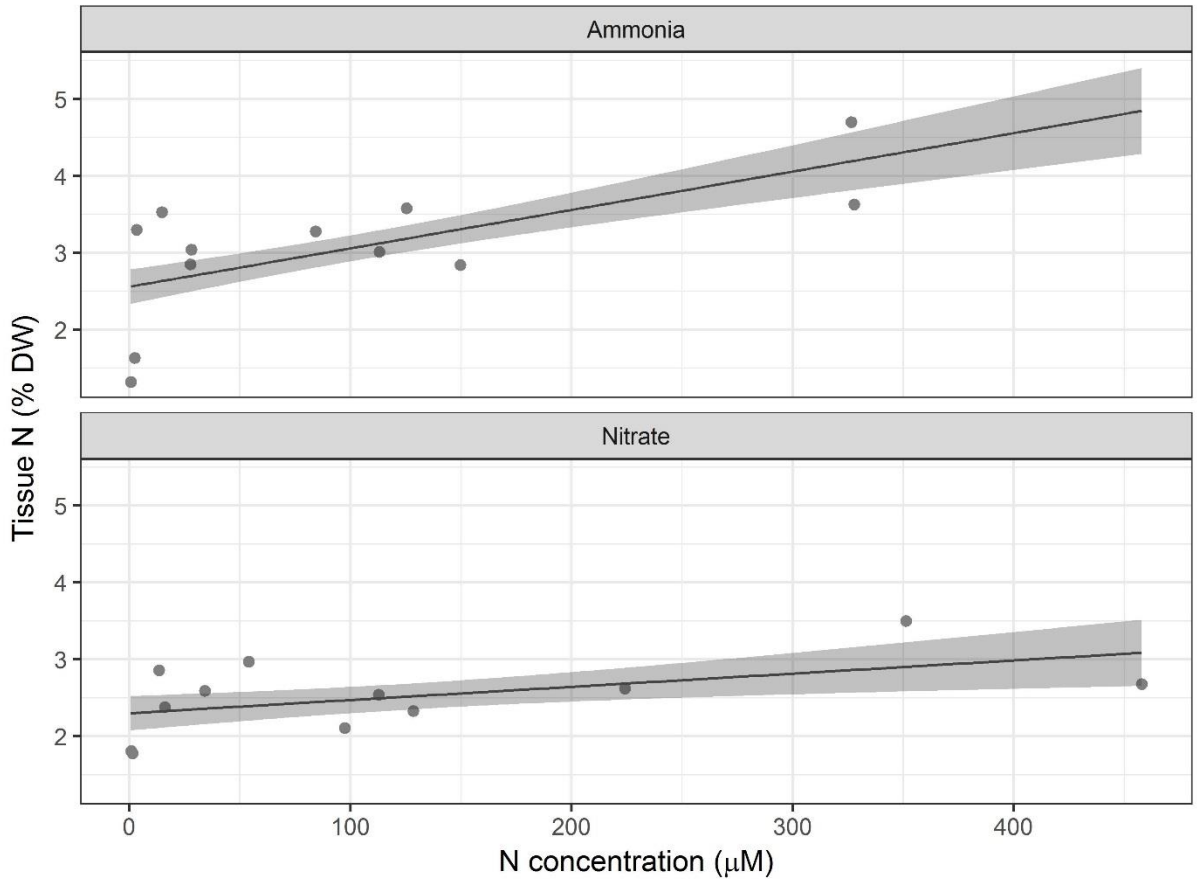

Figure 8. Tissue nitrogen (N) (%DW) of *Solieria robusta* seedlings grown with different concentrations (μM) of nitrogen (N) as ammonia or nitrate showing linear model fitted to data, with shaded area showing 95% confidence intervals of the fit.

3.4. Summary

Our explant production experiment showed that PES was a better enrichment solution than VSM for growth of tip explants of *S. robusta*. Commercially cultivated Solieriacae: *Kappaphycus* and *Eucheuma* spp. tip explants have also been shown to grow better in PES than VSM (Yong *et al.* 2011; Yong *et al.* 2014). Full-strength PES may be detrimental for some red seaweeds (de Paula *et al.* 2001; Harrison and Berges 2005), but this depends on the exact formulation of PES used (Berges *et al.* 2001; Harrison and Berges 2005), and on the frequency of addition, with pulse application being more beneficial than continuous supply (de Paula *et al.* 2001). We used PES with the modifications recommended by Berges *et al.* (2001), and pulse application (three times weekly) of both ES types. VSM contains some additional metal salts that are not included in PES;

these salts may be detrimental to some seaweeds, leading to poorer growth performance in this ES (Yong *et al.* 2011; Yong *et al.* 2014). The more frequent occurrence of epiphytes in VSM than PES may be due, at least in part, to VSM favouring growth of opportunistic algae over *S. robusta* explants, while more frequent and heavier epiphytes on stem than tip explants may be due to stems harbouring more microscopic contaminants than tips, or to tip but not stem explants being able to out compete opportunistic algae given suitable culture conditions. Tip explants included apical cells, which are active growth sites, likely contributing to their better performance than stem explants.

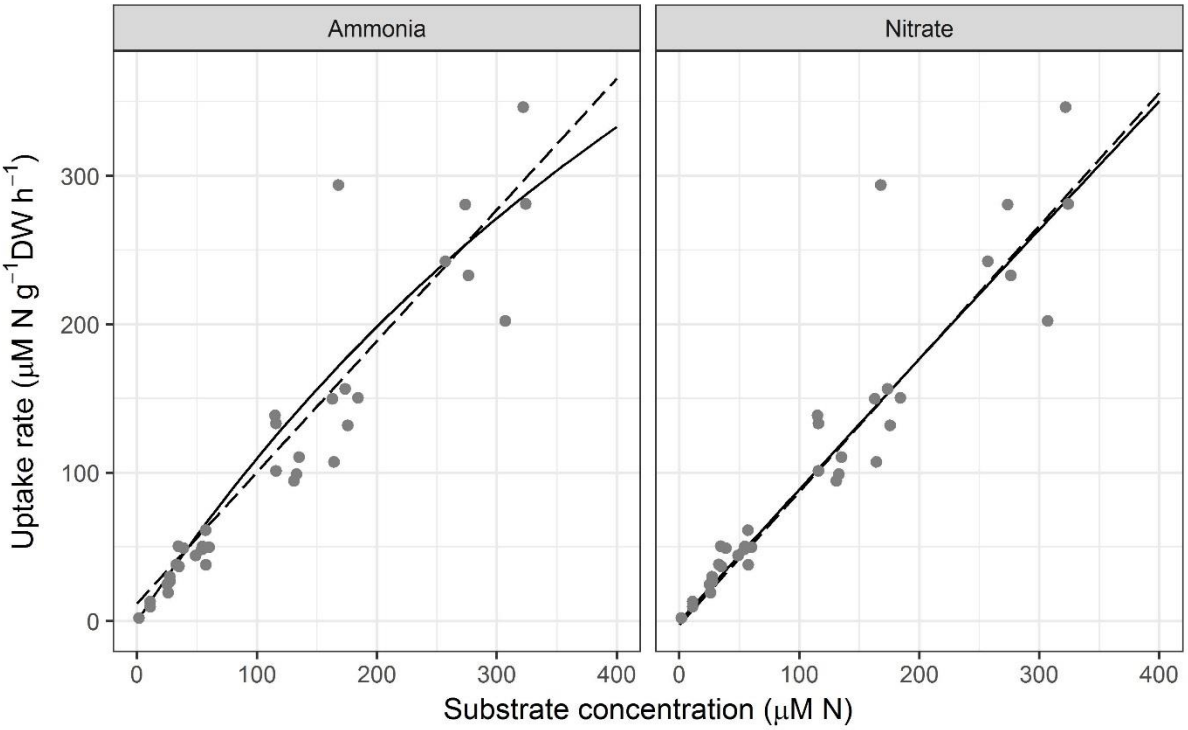

Figure 9. Uptake rate (μM N g^{-1} DW h^{-1}) of *Solieria robusta* for ammonia and nitrate as a function of substrate concentration as μM of nitrogen (N), with Michaelis-Menten (solid line) and linear (dashed line) fits to the data.

Nutrient supply is an important factor in seaweed growth, and, in field cultivation, seaweeds are often N limited (Harrison and Hurd 2001). Optimal seaweed growth rates in tank or hatchery culture typically occur at higher N concentrations than are found in natural seawater, but the best N source and concentration varies between species (Harrison and Hurd 2001; Liu *et al.* 2004; Carmona *et al.* 2006). Ammonia is used preferentially by some species, and may promote faster

growth, but is toxic at higher concentrations, with toxicity modified by pH and salinity, and tolerance varying between species (Rui *et al.* 1990; Harrison and Hurd 2001; Liu *et al.* 2004; Pribadi 2012). Ammonia toxicity is the reason that nitrate is the preferred N source in common enrichment solutions such as PES and VSM (Berges *et al.* 2001). In many cases there is little difference in seaweed performance when grown with ammonia or nitrate N, and better growth with nitrate enrichment is sometimes observed at lower N concentrations where ammonia toxicity should not occur (Hanisak 1990; Harrison and Hurd 2001). The effect of nitrate N on *S. robusta* growth had not been investigated, and while Wiltshire *et al.* (2015) examined growth of *S. robusta* at ammonia concentrations predicted to occur around SA fish farms, they did not test levels higher than 12 µM or determine optimal N for *S. robusta* growth. Wiltshire *et al.* (2015) found that *S. robusta* grew faster with high (12 µM) than low (0.8 µM) or nil N addition as ammonia, independent of light intensity over the range 50 - 365 µE m^{-2} s^{-1} PAR. Our lowest levels of N addition achieved similar concentrations (0.7–0.9 µM N) to the low level ammonia addition of Wiltshire *et al.* (2015), while other aquaria in our current experiment had concentrations similar to, although slightly above their high level (15–16 µM N). We did not, however, observe any increase in growth with added N over this range, rather growth generally declined with increasing N over the range of concentrations tested, although the specimens in some individual tanks grew considerably better than others at lower N concentrations. Our experiment was conducted within the optimal temperature range for *S. robusta* growth (16-22°C; Wiltshire *et al.* 2015), and with a light intensity similar to the lowest level tested by Wiltshire *et al.* (2015). While Wiltshire *et al.* (2015) did not find that light over the range tested affected growth, it is possible light may have become limiting at high N concentrations, which may have suppressed growth, but should not have led to a decline in growth. Interactive effects of temperature with light and nutrients on *S. robusta* growth have not been tested, and the ammonia addition experiment of Wiltshire *et al.* (2015) was performed at a slightly lower temperature (18 °C) than the current experiment, so temperature may also have contributed to our observed result. It should be noted that the decline in SGR with N was small, and N concentration explained only a low proportion of the variance in the data (adjusted $r^2 = 0.27$ for the best GAM using all data, and 0.24 for the best linear model using the data with excluded outlier). The variation between growth rates of specimens regardless of N concentration, especially the relatively high SGR achieved in the tank with high nitrate, suggests that other factors were influencing growth. As lighting, temperature, and salinity did not vary between aquaria, different performance may have been due to individual characteristics of the specimens used. In cultivation of the related *Kappaphycus* and *Eucheuma* species, strain selection has been

applied to develop high performance varieties (de Paula *et al.* 2001; Ask and Azanza 2002; Yong *et al.* 2014) and this is likely to be an important step in domesticating *S. robusta* for cultivation. Growth performance of Solieriaceae may also vary with reproductive phase, but determining the reproductive phase in these species is difficult unless reproductive structure are present (Ask and Azanza 2002; Zitta *et al.* 2012). The *S. robusta* specimens collected for our experiments were not fertile, and therefore their reproductive phase is unknown; the collections may have contained a mix of plants in different phases. To determine optimal N for *S. robusta*, an experiment focusing on the lower range of N concentrations tested here and performed under higher light intensity may be informative, as would further investigation of possible interactive effects of temperature. Initial screening to identify specimens with promising growth rates, especially if material with known reproductive phase can be obtained, would be useful to assist in identifying the best specimens and or reproductive phase to use for propagation.

The lack of saturation of nutrient uptake rates observed in our experiment can occur in red seaweeds that are N limited. A linear response to N concentration can arise, even at concentrations > 500 µM, when N-limited specimens perform surge uptake (Harrison and Hurd 2001; Smit 2002). Tissue N in the specimens used for our uptake trial was > 2% DW, and so above the level historically considered indicative of N limitation in seaweeds (Hanisak 1990) but the *S. robusta* seedlings grown with N addition accumulated higher (to > 4%) tissue N contents than this. The tissue N level indicative of nutrient limitation, referred to as the critical N level, is known to vary between seaweeds and is typically between 0.7 and 3.2% (Harrison and Hurd 2001). The critical N level for a seaweed species can be determined by measuring the growth rate and tissue N of specimens grown under a range of N concentrations, and then finding the tissue N at which growth rate plateaus (Harrison and Hurd 2001). The critical tissue N for *S. robusta* remains uncertain because we did not observe an increased growth response to added and tissue N in the seedling trial. While we also could not determine V_{max} from our uptake rate data, the maximum uptake rate of *S. robusta* observed in the uptake trial was > 200 µM N g^{-1} DW h^{-1}, which is above the 100 µM N g^{-1} DW h^{-1} considered useful for seaweeds applied to IMTA (Kang *et al.* 2013), and, given that N concentrations around fish farms in SA are likely to be ≤ 12 µM, the data provided by the uptake trial allows calculation of uptake rates within this range for incorporation into biogeochemical models. The uptake rate experiment was conducted under light intensity and temperature conditions that are within the range expected in field cultivation, although the temperature used was at the upper end of the seasonal range for current Spencer

Gulf fish aquaculture locations (Wiltshire *et al.* 2015). Uptake rates for a given nutrient concentration typically double for a 10 °C increase in temperature over the range relevant for seaweed growth, with this rule often used to predict uptake at other temperatures (Harrison and Hurd 2001). Repeating the uptake rate measurement at a range of relevant temperatures would, however, provide data to allow more accurate modelling.

3.5. Conclusions

The experiments reported here provide more evidence for the suitability of *S. robusta* to be used for nutrient remediation, given its ability to remove both ammonia and nitrate N over a wide range of concentrations, and provide data to allow N removal by this species to be incorporated into biogeochemical models. This will assist in determining the ratio of seaweed to fish biomass needed to offset N inputs to the environment. We also demonstrated successful explant production, with tip explants grown with PES performing best. The optimum N source and concentration for *S. robusta* growth is, however, still unclear. Variable growth rates observed here, and in the field experiments of Wiltshire *et al.* (2015), show that selecting the best performing specimens for propagation, as has been done for related commercial seaweeds, will be important in developing this species for aquaculture.

REFERENCES

Arnold TW (2010) Uninformative Parameters and Model Selection Using Akaike's Information Criterion. 74, 1175-1178.

Ask EI, Azanza RV (2002) Advances in cultivation technology of commercial eucheumatoid species: a review with suggestions for future research. *Aquaculture* 206, 257-277.

Barrington K, Chopin T, Robinson S (2009) Integrated multi-trophic aquaculture (IMTA) in marine temperate waters. In 'Integrated mariculture: a global review. FAO Fisheries and Aquaculture Technical Paper. No. 529.'. (Ed. D Soto) pp. 7-46: Rome, FAO)

Bates D, Maechler M, Bolker BM, Walker S (2014) lme4: Linear mixed-effect models using Eigen and s4. R package version 1.1-6.

Berges JA, Franklin DJ, Harrison PJ (2001) Evolution of an artificial seawater medium: Improvements in enriched seawater, artificial water over the last two decades. *Journal of Phycology* 37, 1138-1145.

Burnham KP, Anderson DR, Huyvaert KP (2011) AIC model selection and multimodel inference in behavioral ecology: some background, observations, and comparisons. *Behavioral Ecology and Sociobiology* 65, 23-35.

Carmona R, Kraemer GP, Yarish C (2006) Exploring Northeast American and Asian species of *Porphyra* for use in an integrated finfish–algal aquaculture system. *Aquaculture* 252, 54-65.

Collings GJ, Cheshire A, Tanner J (2007) Carrying capacity modelling. In 'Aquafin CRC - Southern bluefin tuna aquaculture subprogram: Tuna environment subproject - Development of regional environmental sustainability asessments for tuna sea-cage aquaculture. Technical report, Aquafin CRC project 4.3.3, FRDC project 2001/104. SARDI Aquatic Sciences Publication Number F2007/000803-1. SARDI Research Report Series No. 235.'. (Ed. J Tanner) pp. 286. (Fisheries Research and Development Corporation and South Australian Research and Development Institure (Aquatic Sciences): Adelaide)

de Paula EJ, Erbert C, Lima Pereira RT (2001) Growth rate of the carrageenophyte *Kappaphycus alvarezii* (Rhodophyta, Gigartinales) in vitro. *Phycological Research* 49, 155-161.

Department of Agriculture and Water Resources (2016) Aquaculture industry in Australia Accessed: 5 November 2018. URL: http://www.agriculture.gov.au/fisheries/aquaculture/aquaculture-industry-in-australia

Department of Agriculture and Water Resources (2017) National aquaculture strategy. Department of Agriculture and Water Resources,, Canberra.

Fernandes M, Lauer P, Cheshire A, Angove M (2007) Preliminary model of nitrogen loads from southern bluefin tuna aquaculture. *Marine Pollution Bulletin* 54, 1321-1332.

Fernandes M, Tanner J (2008) Modelling of nitrogen loads from the farming of yellowtail kingfish *Seriola lalandi* (Valenciennes, 1833). *Aquaculture Research* 39, 1328-1338.

Genty B, Briantais J-M, Baker NR (1989) The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta (BBA) - General Subjects* 990, 87-92.

Grote B (2016) Bioremediation of aquaculture wastewater: evaluating the prospects of the red alga *Palmaria palmata* (Rhodophyta) for nitrogen uptake. *Journal of Applied Phycology* 28, 3075-3082.

Hanisak MD (1990) The use of *Gracilaria tikvahiae* (Gracilariales, Rhodophyta) as a model system to understand the nitrogen nutrition of cultured seaweeds. *Hydrobiologia* 204-205, 79-87.

Harrison PJ, Berges JA (2005) Marine culture media. In 'Algal culturing techniques'. (Ed. RA Andersen) pp. 21-33. (Elsevier Academic Press: London)

Harrison PJ, Hurd CL (2001) Nutrient physiology of seaweeds: Application of concepts to aquaculture. *Cahiers de Biologie Marine* 42, 71-82.

Kang Y, Hwang J, Chung I, Park S (2013) Development of a seaweed species-selection index for successful culture in a seaweed-based integrated aquaculture system. *Journal of Ocean University of China* 12, 125-133.

Lachat (2003a) Quickchem Method 31-107-04-1-D (Revision Date 16/09/2003). Lachat Instruments, Colorado, USA.

Lachat (2003b) Quickchem Method 31-107-06-1-B (Revision Date 19/09/2003). Lachat Instruments, Colorado, USA.

Lee B (2010) Cultivated Seaweed and Seaweed Industry Development in Australia. Rural Industries Research and Development Corporation, Publication No. 10/164 and Project No., PRJ-004681, Barton, ACT.

Liu D, Amy P, Sun JJJoOUoC (2004) Preliminary study on the responses of three marine algae, *Ulva pertusa* (Chlorophyta), *Gelidium amansii* (Rhodophyta) and *Sargassum enerve* (Phaeophyta), to nitrogen source and its availability. 3, 75-79.

Lorbeer AJ, Tham R, Zhang W (2013) Potential products from the highly diverse and endemic macroalgae of Southern Australia and pathways for their sustainable production. *Journal of Applied Phycology*, 1-16.

Maxwell K, Johnson GN (2000) Chlorophyll fluorescence—a practical guide. *Journal of Experimental Botany* 51, 659-668.

Middleton JF, Doubell M, James C, Luick J, Van Ruth P (2013) PIRSA Initiative II: carrying capacity of Spencer Gulf: hydrodynamic and biogeochemical measurement modelling and performance monitoring. Final Report for the Fisheries Research and Development Corporation. South Australian Research and Development Institute (Aquatic Sciences), SARDI Publication No. F2013/000311-1, SARDI Research Report Series No. 705, Adelaide.

Mobsby D, Koduah A (2017) Australian fisheries and aquaculture statistics 2016. Fisheries Research and Development Corporation project 2017-095. ABARES, Canberra.

Neori A (2008) Essential role of seaweed cultivation in integrated multi-trophic aquaculture farms for global expansion of mariculture: an analysis. *Journal of Applied Phycology* 20, 567-570.

Petrell RJ, Alie SY (1996) Integrated cultivation of salmonids and seaweeds in open systems. *Hydrobiologia* 326-327, 67-73.

Phillips JA (2001) Marine macroalgal biodiversity hotspots: why is there high species richness and endemism in southern Australian marine benthic flora? *Biodiversity and Conservation* 10, 1555-1577.

Pribadi TDK (2012) Macroalgal Ecophysiology in Response to High Ammonium Concentrations and Different Irradiances.

R Core Team (2017) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Ribeiro ALNL, Tesima KE, Souza JMC, Yokoya NS (2013) Effects of nitrogen and phosphorus availabilities on growth, pigment, and protein contents in *Hypnea cervicornis* J. Agardh (Gigartinales, Rhodophyta). *Journal of Applied Phycology* 25, 1151-1157.

Ridler N, Wowchuk M, Robinson B, Barrington K, Chopin T, Robinson S, Page F, Reid G, Szemerda M, Sewuster J, Boyne-Travis S (2007) Integrated multi-trophic aquaculture (IMTA): a potential strategic choice for farmers. *Aquaculture Economics and Management* 11, 99-110.

Rimmer M, Ponia B (2007) A review of cage aquaculture: Oceania. In 'Cage aquaculture - Regional reviews and global overview. FAO Fisheries Technical Paper No 498.'. (Eds M Halwart, D Soto and J Arthur) pp. 208-231. (FAO: Rome)

Ritz C, Baty F, Streibig JC, Gerhard D (2016) Dose-Response Analysis Using R. *PLOS ONE* 10, e0146021.

Rui L, Jiajun L, Chaoyuan W (1990) Effect of ammonium on growth and carrageenan content in *Kappaphycus alvarezii* (Gigartinales, Rhodophyta). *Hydrobiologia* 204, 499-503.

Smit AJ (2002) Nitrogen Uptake by *Gracilaria gracilis* (Rhodophyta): Adaptations to a Temporally Variable Nitrogen Environment. Botanica Marina. pp. 196.

Soto D (Ed.) (2009) Integrated mariculture: a global review. FAO Fisheries and Aquaculture Technical Paper. No. 529. (Rome, FAO)

Tanner J, Clark TD, Fernandes M, Fitzgibbon Q (2007) Innovative solutions for aquaculture: Spatial impacts and carrying capacity - further developing, refining and validating exisiting models of environmental effects of finfish farming. South Australian Research and Development Institure (Aquatic Sciences), SARDI Aquatic Sciences Publication Number F2007/000537, Adelaide.

Titlyanov E, Titlyanova T (2010) Seaweed cultivation: Methods and problems. *Russian Journal of Marine Biology* 36, 227-242.

Troell M, Halling C, Neori A, Chopin T, Buschmann AH, Kautsky N, Yarish C (2003) Integrated mariculture: asking the right questions. *Aquaculture* 226, 69-90.

Troell M, Joyce A, Chopin T, Neori A, Buschmann AH, Fang J-G (2009) Ecological engineering in aquaculture — Potential for integrated multi-trophic aquaculture (IMTA) in marine offshore systems. *Aquaculture* 297, 1-9.

Whitmarsh D, Wattage P (2006) Public attitudes towards the environmental impact of salmon aquaculture in Scotland. *European Environment* 16, 108-121.

Whitmarsh DJ, Cook EJ, Black KD (2006) Searching for sustainability in aquaculture: An investigation into the economic prospects for an integrated salmon–mussel production system. *Marine Policy* 30, 293-298.

Wiltshire KH, Tanner JE, Gurgel CFD, Deveney MR (2015) Feasibility study for integrated multitrophic aquaculture in southern Australia. Report to the Fisheries Research & Development Corporation. South Australian Research and Development Institute (Aquatic Sciences), Publication No. F2015/000786-1. Research Report Series No. 883, Adelaide.

Womersley HBS (1994) The marine benthic flora of southern Australia Rhodophyta Part IIIA Bangiophyceae and Florideophyceae (Acrochaetiales Acrochaetiales, Nemaliales, Gelidiales, Hildenbrandiales and Gigartinales sensu lato). (Australian Biological Resources Study: Canberra)

Wood SN (2006) Generalized additive models: An introduction with R.

Yong WTL, Ting SH, Chin WL, Rodrigues KF, Anton A (2011) In Vitro micropropagation of *Eucheuma* seaweeds. 2nd International Conference on Biotechnology and Food Science. (Ed. International Proceedings of Chemical Biological and Environmental Engineering). (IACSIT Press: Singapore)

Yong WTL, Ting SH, Yong YS, Thien VY, Wong SH, Chin WL, Rodrigues KF, Anton A (2014) Optimization of culture conditions for the direct regeneration of *Kappaphycus alvarezii* (Rhodophyta, Solieriaceae). *Journal of Applied Phycology* 26, 1597-1606.

Zitta C, Oliveira E, Bouzon Z, Hayashi L (2012) Ploidy determination of three *Kappaphycus alvarezii* strains (Rhodophyta, Gigartinales) by confocal fluorescence microscopy. *Journal of Applied Phycology* 24, 495-499.