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A Mixture of Manganese, Silica and Phosphorus Supplementation Alters the Plankton Density, Species Diversity, Gut Microbiota and Improved the Health Status of Cultured Marron (*Cherax cainii*, Austin and Ryan, 2002)

Smita Sadanand Tulsankar[1] · Md Javed Foysal[1,2] · Anthony J. Cole[1] · Marthe Monique Gagnon[1] · Ravi Fotedar[1]

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Abstract
Trace element supplementation to the freshwater environment can influence the plankton density and species diversity, contributing to the nutrition of aquaculture species, especially during the juvenile stage. An experiment was conducted under laboratory conditions to evaluate the effects of supplementing different mixtures of manganese, silica and phosphorus on the plankton density and species diversity and their impact on cultured juvenile marron (*Cherax cainii*, Austin and Ryan, 2002). Manganese, silica and phosphorus in concentrations of 0.0024, 0.41, 0.05 mg*L^{-1}; 0.0041, 0.82, 0.12 mg*L^{-1}; and 0.0058, 1.26, 0.25 mg*L^{-1} respectively termed as low, medium and high were supplemented to tank water containing a phytoplankton density of $3.77 \pm 0.16 \times 10^6$ cells*L^{-1} and 292.9 ± 17.6 individuals*L^{-1} of zooplankton, and plankton growth was observed every 24 h for 6 days. Afterwards, a 3-month trial was conducted studying the effects of these trace element concentrations and resulting plankton densities on marron growth, survival, moulting, gut microbiota and health indices. Silica supplementation at high concentration increased the diatom abundance, silica and phosphorus supplementation at higher concentration that resulted in a significant increase in plankton density and species diversity, leading to improved marron health indices than the control and the tanks receiving a low concentration. Marron-specific growth rate, weight gain and dissolved copper concentration in haemolymph were significantly higher in tanks with higher supplementation and higher plankton density. Marron survival, moult interval and total haemocyte count were not affected by the supplementation. Marron gut microbiota at higher trace element concentration supplementation showed a significant increase in abundance of phosphate solubilizing bacteria.

Keywords Aquaculture · Trace elements · Phytoplankton · Zooplankton · Freshwater crayfish · Gut microbiota

Introduction

Among the various aquaculture species farmed in Western Australia (WA), marron (*Cherax cainii*, Austin and Ryan, 2002) is a commercially important freshwater crayfish due to its high value, distinct taste and disease-free status [1]. Marron can consume any available food including plant and animal matter within a ponds system [2]. The effects of trace element supplementation on fish production have been studied previously [3–6]. To increase the marron production through plankton productivity, marron farmers use different organic including fermented barley straws and inorganic fertilizers, a common practice in aquaculture [7, 8]. Several studies have shown that manganese (Mn), silica (Si) and phosphorus (P) are vital for plankton productivity in freshwater ecosystems [9–15]. Their deficiency can diminish the phytoplankton growth [10], and their supplementation can increase plankton biomass initiating a modulation of community structure [10, 12].

Phosphate fertilizers are widely used [8] to increase the plankton productivity and in turn to improve the growth performance of cultured aquatic animals [6, 16, 17]. Under

✉ Smita Sadanand Tulsankar
smitasad@postgrad.curtin.edu.au

[1] Curtin Aquatic Research Laboratories, School of Molecular and Life Sciences, Curtin University, Bentley, Western Australia 6102, Australia

[2] Department of Genetic Engineering and Biotechnology, Shahjalal University of Science and Technology, Sylhet 3114, Bangladesh

controlled laboratory conditions and in the absence of planktons, juvenile signal crayfish (*Pacifastacus leniusculus*) culture remains largely unsuccessful, mainly due to the low survival and growth rates during the early life stages [18, 19], whereas a higher survival and growth rates of juveniles signal crayfish were obtained with the supply of live zooplankton [19].

Although Mn, Si and P are essential trace elements for the growth of plankton and crustaceans, limited research has investigated their effect on the plankton density and species diversity as well as on the growth, survival, health and gut microbiota of freshwater crayfish [20], a study by Sierp [20] where authors observed the effect of adult marron (*C. tennuimanus*) on plankton and nutrient dynamics and the response of phytoplankton to phosphorus fertilization in ponds having high hardness. The main focus of related studies has been to analyze different concentrations of Mn and Si and their toxic effects on the decapods [21–25]. Trace elements have the ability to reshape the gut microbiota of guppy fish (*Poecilia reticulata*) for improved digestion, immunity and adaptation [26]. No research has been reported investigating the effects of different concentrations of Mn, Si and P supplementation on water quality, crayfish growth, survival, health indices and gut microbiota mediated through plankton density or species diversity. We hypothesized that trace element supplementation will positively influence the plankton density and community structure and the plankton densities will improve marron growth, survival, health indices and gut microbiota. To test this hypothesis, we conducted an indoor laboratory experiment for 96 days under controlled conditions.

Materials and Methods

Experimental Design

The experiment was designed based on the outcomes of our previous field trial in commercial earthen marron ponds [14], wherein 12 pre-selected trace elements were measured, of which manganese (Mn), silica (Si) and phosphorus (P) were found to be strongly correlated with plankton density and species diversity over the seasons. The mean dissolved concentrations of Mn, Si and P of 28 ponds over four seasons were used to determine low 0.0024, 0.41 and 0.05 mg*L^{-1}, medium 0.0041, 0.82 and 0.12 mg*L^{-1} and high 0.0058, 1.26 and 0.25 mg*L^{-1} concentrations, respectively. These concentrations were used to evaluate the influence of the trace elements on plankton density and diversity.

Plankton Collection and Preparation for Stock Culture

Planktons were collected from the commercial marron farm in Manjimup (34° 18′ 75″ S, 116° 06′ 61″ E) WA. Pond water was filtered through a phytoplankton net to obtain 20 L of phytoplankton sample, and zooplankton net was used to obtain 20 L of zooplankton sample separately. Collected planktons were cultured as a stock culture for the experiment in outdoor conditions at the Curtin Aquatic Research Laboratory (CARL), using 300-L water capacity plastic tanks, filled with 200 L of freshwater, with a supply of vigorous aeration and direct sunlight. A continuous culture system was used to culture the plankton and Aquasol® by Yates Pty Ltd was added to the tanks to boost and maintain the phytoplankton density at 11.45 × 10^6 cells*L^{-1}. Daily observations of tank conditions and plankton were made and the planktons were counted three times a week. The phytoplankton were fed to zooplankton to maintain the density.

Trace Element Supplementation and Plankton Culture

Under controlled laboratory conditions, sixteen 300-L water capacity tanks were filled with 150 L of water and were stocked with phytoplankton and zooplankton at a rate of 3.77 ± 0.16 × 10^6 cells*L^{-1} and 292.9 ± 17.6 individuals*L^{-1}, respectively. On the same day, Mn in the form of manganese (II) chloride tetrahydrate ($MnCl_2.4H_2O$), silica-sodium metasilicate nonahydrate ($Na_2SiO_3.9H_2O$) and phosphorus in the form of potassium hydrogen phosphate (K_2HPO_4) in three different concentrations as described in Table 1 were prepared by dissolving in distilled water in 100-mL beakers and were supplemented to the twelve treatment tanks. Mn, Si and P were supplemented

Table 1 Three different mix concentrations of Mn, Si and P were prepared using manganese (II) chloride tetrahydrate ($MnCl_2.4H_2O$), sodium metasilicate nonahydrate ($Na_2SiO_3.9H_2O$) and potassium hydrogen phosphate (K_2HPO_4) respectively to achieve the required three different concentrations (mg*L^{-1}) of Mn, Si and P

Chemicals/trace elements (mg*L^{-1})	Low supplementation (LTS)	Medium supplementation (MTS)	High supplementation (HTS)
$MnCl_2.4H_2O$	0.0087	0.0148	0.0209
$Na_2SiO_3.9H_2O$	0.8771	1.7541	2.6954
K_2HPO_4	0.2812	0.6748	1.4058
Mn	0.0024	0.0041	0.0058
Si	0.4100	0.8200	1.2600
P	0.0500	0.1200	0.2500

only once at the start of the experiment. The experimental design included four treatments with four replicates: (1) only plankton (control; CTL); (2) plankton + low trace element supplementation (LTS); (3) plankton + medium trace element supplementation (MTS) and (4) plankton + high trace element supplementation (HTS). Continuous aeration and light were provided to grow the plankton. After trace element supplementation, the planktons were counted at 24, 48, 72, 96, 120 and 144 h to evaluate the plankton density and species diversity (Supplementary data #1). Trace element supplementation was conducted in the absence of marron in order to avoid any inadvertent phosphorous addition through feed.

The Table 1 shows the total weight of $MnCl_2.4H_2O$, $Na_2SiO_3.9H_2O$ and K_2HPO_4 used to achieve the required concentration of Mn, Si and P ($mg*L^{-1}$).

Introducing Marron to Tanks Under Laboratory Conditions

Marron were collected from the commercial marron farm in Manjimup. A total of 190 juvenile marron (weighing average initial weight 6.13 ± 0.23 g, average orbital-carapace length (OCL) 2.95 ± 0.07 cm and total length of 6.26 ± 0.11 cm) were collected and transported to the CARL. Marron were stocked in 300-L water capacity tanks to acclimate to the laboratory conditions at the CARL for 15 days. After 6 days (144 h) of trace element supplementation and on plankton counting, all tanks were stocked with nine marron per tank in individual holding cages made up of plastic containers and top covered with mesh, to avoid cannibalism and escape of marron. The cages had a volume of $2000cm^3$ (170 mm × 115 mm × 135 mm) with four gaps of 4–5 mm on each sides to allow the water exchange directly from the tank water into the cage. Marron were fed at 2% of their body weight, once a day in the evening. The uneaten feed and faeces were removed, 1 h after the feeding.

Water Quality Analysis

All water parameters were kept in an optimum range for the growth of marron [27]. The water parameters including temperature, dissolved oxygen (DO) and pH were checked daily. An Oxyguard® digital DO meter (Handy Polaris 2, Norway) was used for DO and temperature measurements, and an Ecoscan pH 5 meter (Eutech instruments, Singapore) was used to record pH. A DR/890 portable colorimeter with Permachem reagents (Hach, USA) was used to analyze the total ammonia nitrogen (TAN), nitrite (NO_2-N), nitrate (NO_3-N) and reactive phosphate (PO_4) once a week. The experiment was static, i.e. no water exchange was made. Tank water level was maintained at 150 L throughout the experiment by adding water to compensate for losses due to the evaporation.

Plankton and Trace Element Analysis

Throughout the experiment, plankton density was analyzed three times a week and was maintained at the same density as recorded after 144 h by either addition of plankton or removing by filtering plankton out, using respective plankton nets. For phytoplankton analysis, 2 L of tank water was filtered to obtain 100 mL of sample. For zooplankton analysis, 5 L of tank water was filtered to obtain 100 mL of sample. The filtered water was re-stocked into the same tank. The plankton species were identified to the lowest possible taxonomic level using keys from a manual by Ingram [28] and a book by Canter-Lund [29]. The plankton density ($cells*L^{-1}$) was calculated by using the equations from Ingram [28] and Tulsankar [30]. The dissolved trace element concentration in tank water at initial, after supplementation, at the end of the experiment and in marron haemolymph were analyzed at Murdoch University, Perth, WA. The water samples were collected in 100-mL plastic containers directly from the tanks and were filtered through 0.45 μm Millipore filters to eliminate suspended particles. The haemolymph samples were collected using 1-mL syringe containing 0.2 mL of sodium citrate anticoagulant (100 mM glucose, 30 mM trisodium citrate, 26 mM citric acid, 15.5 mM NaCl and 10 mM EDTA) inserted in between the third and fourth pair of pereopod and were kept on ice during the sampling and transportation. Inductively, coupled plasma optical emission spectrometry (Agilent, ICP-OES, spike recovery limit 80–120%) with the standard methods described in APHA [31] was used to analyze the dissolved concentrations with the detection limits of Mn (<0.0002 $mg*L^{-1}$); P (<0.02 $mg*L^{-1}$) and Si (<0.02 $mg*L^{-1}$) in water and Ca (<5 $mg*L^{-1}$); Cu (<0.1 $mg*L^{-1}$); Fe (<0.5 $mg*L^{-1}$); Mn (<0.05 $mg*L^{-1}$) and P (<5 $mg*L^{-1}$) in haemolymph. The results of dissolved trace elements in haemolymph were calculated based on the dilution factor [32].

Marron Growth Analysis

Marron growth data were recorded fortnightly, and mortality was recorded daily. Marron-specific growth rate (SGR; g %/day), weight gain percentage (WG; g %) and survival rate (SR; %) were calculated by using the following equations

$$\text{Specific growth rate (SGR, g\%/day)} = 100 \times (\text{Ln}\,(W_t) - \text{Ln}\,(W_i))/\text{T},$$

$$\text{Weight gain (WG, g\%)} = 100 \times (W_t - W_i)/W_i.$$

$$\text{Survival rate (SR, \%)} = 100 \times (n_t/n_0)$$

where W_t is final weight (g), W_i is initial weight (g), n_t is the number of marron alive at (T) days and n_0 is the number of marron stocked initially.

Marron moulting data such as dates and times were recorded daily. Moult interval (T_m; days) was measured on the basis of days required to moult, between two successive moults using the following equation:

Moult interval (T_m; days)

$$T_m = T_{n+1} - T_n$$

where T_n = date of n moult, T_{n+1} = date of $n + 1$ moult.

Marron Health Indices

Marron health indices were analyzed at the end of the experiment, by testing haemolymph for total haemocyte count (THC) and differential haemocyte count (DHC), hepatopancreas wet and dry weight indices moisture content (HM %), wet weight (Hiw), dry weight (Hid), tail muscle moisture content (TM %), wet weight (TMiw) and dry weight (TMid) indices. Haemolymph samples were collected from one randomly selected marron per tank, and the haemolymph was drawn by using a 1-mL syringe inserted in between the third and fourth pair of pereopods. THC and DHC were analyzed according to [33]. One marron per tank was collected randomly, and hepatopancreas and tail muscle from each individual were collected and weighed. To obtain the dry weight, the samples were dried in crucibles at 105 °C in the oven for 24 h. The health indices were calculated as described by [34].

Marron Hindgut Microbiota Analysis

At the end of the experiment, a total of 24 marron, six per treatment were randomly selected. Gut content collection and separation of hindgut was performed inside a biosafety cabinet and the gut contents with mucosa were immediately lysed using Tissue Lyser II (Qiagen, Hilden, Germany). A subsequent pool of two marron gut contents from each respective tank was created by homogenization and transferred into 1.5-mL Eppendorf tube. Bacterial DNA was extracted using Blood and Tissue Kit (Qiagen, Hilden, Germany). DNA concentration was measured in Nanodrop Spectrophotometer (Thermo Fisher Scientific, USA) and diluted into 50 ng/μL for PCR. Fifty microliters of PCR master mix was prepared for each sample that contained 25 μL Hot Start 2X Master Mix (New England BioLabs Inc., Ipswich, MA, USA), 2 μL of bacterial DNA, 1 μL of each forward and reverse primers (V3-V4) and 21 μL of DEPC treated water. Forty cycles of PCR amplification were completed in a thermal cycler (BioRad S100, Bio-Rad Laboratories, Inc., Foster City, CA, USA). PCR products clean-up and amplicon barcoding was performed with a secondary PCR according to the Illumina standard protocol (Part # 15044223 Rev. B). Samples were then sequenced on Illumina MiSeq platforms (Illumina Inc., San Diego, CA, USA) using a v3 kit (600 cycles).

Bioinformatics and Statistical Analysis

Raw sequences were checked for initial quality in FastQC pipeline [35], trimmed for quality reads (parameters: -q 20 –l 200) in Sickle [36] and merged in MeFiT program [37]. MICCA pipelines used for filtering, open reference clustering and picking of OTUs at 97% similarity threshold [38]. SILVA 1.32 release used for phylogenetic assignment of operational taxonomic units (OTUs) at different taxa levels [39]. Multiple sequence alignment, FastTree (version 2.1.8) GTR+CAT phylogenetic tree was performed and constructed in PASTA algorithms [40, 41]. Rarefaction depth value was set to 32,996 bp and alpha-beta diversity was calculated using QIIME (v1.9.1) and R packages. Alpha diversity was calculated in terms of observed species, Shannon and Chao1 measurements. Beta ordination was calculated as Bray-Curtis dissimilarity of weighted UniFrac while permutational multivariate ANOVA (PERMNOVA) and non-metric multidimensional scaling (NMDS) analysis were performed to calculate and visualize the clustering of samples. Non-parametric statistical distance metric was calculated using ANOSIM with 1000 permutations. Relative and differential abundance of bacterial communities was calculated using phyloseq [42] and LEfSe [43], respectively.

Data Analysis

All the numerical data were analyzed using R software (v3.5.1) and are presented as mean ± standard error (SE). One-way analysis of variance (ANOVA) with Turkey's HSD post hoc tests was used to determine the significant differences between treatments. Paired *t*-test was used to determine the dissolved trace element concentrations in water before and after supplementation. All tests were considered statistically significant at $p < 0.05$.

Results

Water Quality and Plankton Density

Temperature, DO and pH were observed at the constant level throughout the experiment (Table 2). On trace element supplementation, plankton density was significantly ($p < 0.005$) highest in HTS tanks.

Plankton Community

The plankton stock culture included Chlorophyceae consisting *Cladymonas* spp., *Scenedesmus* spp., *Haematococcus* spp., *Eudorina* spp., *Selenastrum* spp., *Scenedesmus* spp. and *Volvox* spp.; Trebouxiophyceae: *Chlorella* spp.; Zygnematophyceae consisting *Closterium*

Table 2 Water parameters and plankton density in four treatment tanks throughout the experiment (mean ± S. E.; $n = 4$). The temperature and pH in tank water were similar to that found in marron ponds in autumn which was the season with the highest plankton density [14, 44]

Parameters	CTL	LTS	MTS	HTS
Temperature (°C)	21.5 ± 0.10	21.5 ± 0.10	21.5 ± 0.09	21.5 ± 0.11
DO (mg*L^{-1})	8.34 ± 0.04	8.42 ± 0.05	8.36 ± 0.05	8.37 ± 0.06
pH	7.59 ± 0.04	7.62 ± 0.04	7.60 ± 0.04	7.60 ± 0.04
Phytoplankton density (× 10^6 cells*L^{-1})	3.77 ± 0.16^a	4.05 ± 0.22^a	6.05 ± 0.42^b	7.38 ± 0.64^c
Zooplankton density (ind.*L^{-1})	293 ± 17.6^a	357 ± 16.2^b	413 ± 18.7^b	493 ± 25.7^c

^{a,b,c} The significant differences between all treatments $p < 0.05$; ind. is individual. *Abbreviations*: *CTL*, control tanks; *LTS*, low trace element supplementation tanks; *MTS*, medium trace element supplementation tanks; *HTS*, high trace element supplementation tanks

spp. and Bacillariophyceae consisted of *Navicula* spp., *Fragilaria* spp., *Pinnularia* spp., *Nitzschia* spp., *Gyrosigma* spp., *Cymbella* spp. and *Gomphonema* spp., *Keratella quadrata*, *Keratella cochlearis*, *Daphnia* spp., Copepoda adults and nauplii were also observed in juvenile marron tanks.

The ammonia, nitrite and nitrate fluctuated within acceptable range for marron (mean ± S. E.) as shown in Fig. 1A, B, C and D. Nitrate-nitrogen (NO_3-N) concentration exponentially increased over the study time, whereas reactive phosphate (PO_4) concentration was highest in HTS tanks ($n = 4$).

Trace Element Concentrations in Water

Before supplementing trace elements, the mean concentration of pre-selected trace elements in tank water was Mn 0.0006 ± 0.0001, P 0.03 ± 0.00 and Si at 2.05 ± 0.07 mg*L^{-1}. On supplementation and throughout the experiment, the dissolved concentration of some trace elements increased, for example Mn in MTS, where others either decreased or increased as depicted in Table 3. All of the trace elements that were supplemented showed an increase in concentration except Si under LTS. At the end of the experiment, the P concentration showed a progressive increase in concentration, whereas Si decreased, and Mn did not change.

Marron Growth, Survival and Moulting Days

Individual marron was weighed every fortnight, where total weight (g), OCL (cm) and total length (cm) were recorded for each marron. There were no significant differences in survival

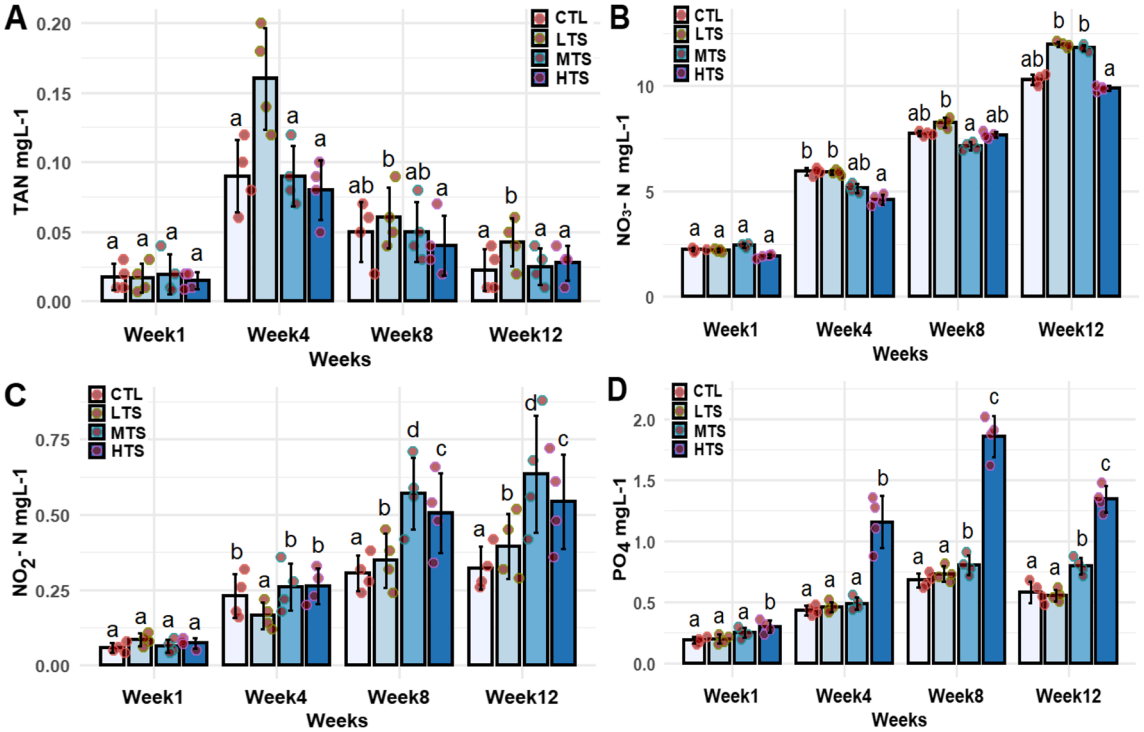

Fig. 1 Water quality parameters of four different treatments. (A) TAN, (B) NO_3-N, (C) NO2-N and (D) PO_4. Different letters a, b and c indicate significant differences between treatments ($p < 0.05$). Abbreviations: CTL, control tanks; LTS, low trace element supplementation tanks; MTS, medium trace element supplementation tanks; HTS, high trace element supplementation tanks

Table 3 Dissolved trace element concentrations (mg*L^{-1}) in experimental tank water at different times (mean ± S. E.; n = 4)

Treatments	CTL	LTS	MTS	HTS
After supplementation				
Mn	^{2}0.0006 ± 0.0001	^{2}0.0007 ± 0.0001	^{2}0.0013 ± 0.0001	^{2}0.0009 ± 0.0002
P	^{1}0.03 ± 0.00^{a}	^{1}0.03 ± 0.01^{a}	^{1}0.05 ± 0.00^{b}	^{1}0.19 ± 0.01^{c}
Si	^{2}2.05 ± 0.07^{a}	^{2}2.37 ± 0.15^{b}	^{2}2.97 ± 0.09^{c}	3.50 ± 0.23^{d}
At the end of the experiment				
Mn	^{1}0.0001 ± 0.0004	^{1}0.0002 ± 0.0001	^{1}0.0003 ± 0.0001	^{1}0.0003 ± 0.0001
P	^{2}0.42 ± 0.02^{a}	^{2}0.43 ± 0.02^{a}	^{2}0.46 ± 0.04^{a}	^{2}0.63 ± 0.05^{b}
Si	^{1}0.46 ± 0.03^{a}	^{1}0.49 ± 0.17^{a}	^{1}2.10 ± 0.35^{b}	3.53 ± 0.23^{c}

^{a,b,c,d} The significant differences between the treatments; ^{1,2} The significant differences over the time ($p < 0.05$). *Abbreviations*: *CTL*, control tanks; *LTS*, low trace element supplementation tanks; *MTS*, medium trace element supplementation tanks; *HTS*, high trace element supplementation tanks

rate among the treatments. Marron SGR and WG were highest for HTS tanks (Table 4).

Marron Health Indices and Trace Element Concentrations in Haemolymph

The percentage of granular cells was significantly lower in LTS tank marron. HTS tank marron had significantly improved haemolymph indices compared to CTL and LTS (Table 5). Total concentrations of Fe and Mn in marron haemolymph were below the detectable level, whereas the Cu concentration was significantly higher in HTS tank marron compared to other treatments. Hepatopancreas moisture content and dry weight indices showed that the marron in HTC tanks were healthy.

Sequence Quality and Alpha-Beta Diversity of the Gut Microbiota

After quality trimming, an average of 98,486 sequences and 358 OTUs per samples were obtained, ranging from 78,786 to 126,446 reads and 288 to 448 OTUs from 12 samples,

Table 4 SGR (g; %/day), weight gain (g; %), OCL increment (cm), total length increment (cm) and tail length increment (cm), survival (%), moult intervals (Tm; days) and health indices of juvenile marron cultured for 90 days (mean ± S. E.; n = 4)

Parameters	CTL	LTS	MTS	HTS
SGR				
0–30 days	^{4}0.29 ± 0.04	^{4}0.32 ± 0.06	^{3}0.40 ± 0.11	^{4}0.50 ± 0.08
30–60	^{1}0.02 ± 0.03	^{1,2}0.04 ± 0.02	^{1}0.03 ± 0.01	^{1,2}0.08 ± 0.04
60–90	^{1,2}0.05 ± 0.01	^{1}0.02 ± 0.01	^{1,2}0.10 ± 0.03	^{1}0.03 ± 0.01
0–60	^{3}0.15 ± 0.01^{a}	^{3}0.17 ± 0.02^{a}	^{2}0.21 ± 0.05^{ab}	^{3}0.29 ± 0.03^{b}
0–90	^{2,3}0.12 ± 0.01^{a}	^{2,3}0.13 ± 0.01^{a}	^{1,2}0.17 ± 0.03^{ab}	^{2,3}0.20 ± 0.03^{b}
Weight gain				
0–30 days	^{2}9.01 ± 1.39	^{2}9.94 ± 1.92	^{2}13.0 ± 3.63	^{2}16.4 ± 2.78
30–60	^{1}0.44 ± 0.99	^{1}1.12 ± 0.56	^{1}0.68 ± 0.41	^{1}2.60 ± 1.34
60–90	^{1}1.51 ± 0.46	^{1}0.58 ± 0.40	^{1}3.00 ± 1.02	^{1}0.72 ± 0.44
0–60	^{2}9.46 ± 0.85	^{2}11.1 ± 1.40	^{2}13.6 ± 3.51	^{2}19.4 ± 2.58
0–90	^{2}11.1 ± 1.10^{a}	^{2}11.8 ± 1.39^{a}	^{2}17.0 ± 2.69^{ab}	^{2}20.2 ± 2.89^{b}
Survival				
0–30 days	94.5 ± 5.50	^{2}97.2 ± 2.78	100 ± 0.00	97.2 ± 2.78
30–60	94.4 ± 5.55	^{2}100 ± 0.00	97.2 ± 2.78	91.3 ± 5.39
60–90	80.9 ± 3.94	^{1}85.4 ± 3.47	91.3 ± 5.39	87.3 ± 4.63
0–60	88.9 ± 6.41	^{2}97.2 ± 2.78	97.2 ± 2.78	88.9 ± 6.41
0–90	72.2 ± 7.17	^{1}83.3 ± 5.55	88.9 ± 6.41	77.8 ± 7.86
T_m (1st)	20.1 ± 1.11	20.5 ± 1.55	19.6 ± 1.46	19.6 ± 1.40
T_m (2nd)	17.5 ± 0.65	16.5 ± 0.29	16.0 ± 0.71	16.3 ± 0.63

^{a,b,c} The significant differences between treatments; ^{1,2,3,4} The significant differences over the culture days ($p < 0.05$). *Abbreviations*: *CTL*, control tanks; *LTS*, low trace element supplementation tanks; *MTS*, medium trace element supplementation tanks; *HTS*, high trace element supplementation tanks

Table 5 Total haemocyte count ($\times 10^6$ cells*mL^{-1}), differential haemocyte count (%), hepatopancreas and tail wet weight and dry weight indices, and trace element concentrations (mg*L^{-1}) in haemolymph of the juvenile marron at the end of the experiment (mean ± S.E.; $n = 4$)

Parameters	CTL	LTS	MTS	HTS
THC (10^6cells*mL^{-1})	1.54 ± 0.27	1.78 ± 0.30	1.69 ± 0.36	1.58 ± 0.16
Hyaline cells (%)	55.1 ± 0.55	58.5 ± 1.24	54.5 ± 0.41	55.3 ± 0.66
Granular cells (%)	35.4 ± 1.33^{b}	30.4 ± 0.94^{a}	35.5 ± 0.20^{b}	35.1 ± 0.55^{b}
Semi-granular cells (%)	9.50 ± 0.79	11.1 ± 0.38	10.0 ± 0.20	9.63 ± 0.52
CaH	483 ± 12.0	463 ± 12.5	443 ± 36.3	350 ± 14.6
CuH	2.00 ± 0.30^{a}	2.77 ± 0.40^{a}	3.67 ± 0.30^{a}	6.17 ± 1.00^{b}
PH	12.8 ± 0.60	11.8 ± 1.90	14.6 ± 1.40	13.4 ± 0.10
HM (%)	78.1 ± 3.60^{c}	78.0 ± 3.00^{c}	67.2 ± 2.58^{b}	57.5 ± 2.09^{a}
Hiw (%)	7.35 ± 0.40	6.26 ± 0.67	6.21 ± 0.40	7.39 ± 0.17
Hid (%)	1.65 ± 0.34^{a}	1.36 ± 0.23^{a}	2.06 ± 0.30^{ab}	3.14 ± 0.17^{b}
TM (%)	80.6 ± 0.94	81.2 ± 0.91	79.1 ± 1.36	80.6 ± 0.42
TMiw (%)	33.1 ± 2.58	36.0 ± 0.61	34.3 ± 1.03	35.2 ± 0.87
TMid (%)	6.36 ± 0.35	6.78 ± 0.38	7.17 ± 0.46	6.82 ± 0.22

^{a,b,c} The significant difference between the treatments. Where CaH, CuH, PH represent the calcium (Ca), copper (Cu) and phosphorus (P) in haemolymph. HM (hepatopancreas moisture), Hiw (hepatopancreas wet weight), Hid (hepatopancreas dry weight), TM (tail muscle moisture), TMiw (tail muscle wet weight) and TMid (tail muscle dry weight). *LTS*, low trace element supplementation, *MTS*, medium trace element supplementation, *HTS*, high trace element supplementation

respectively. Phylogeny assignment of quality reads obtained 15 phyla, 78 families and 126 genera. Near about saturation rarefaction plot revealed that each sample was sequenced at enough depth to capture most of the microbial diversity (Fig. 2A). Among the alpha diversity indices (observed species, Shannon, Chao1), Chao1 diversity was found significantly higher in LTS group, compared to MTS and HTS. Bray-Curtis beta-dispersion on basis of relative abundance (weighted UniFrac) revealed distinct clustering of samples; PERMANOVA identified significant ($P = 0.0233$) impacts of trace element on gut microbiota.

Relative and Significantly Abundant Bacterial Communities

At phylum level, the overall relative abundance was almost similar for all treatment groups and 12 samples with same dominant phyla, Proteobacteria. Higher abundance of *Tenericutes* was only observed for the LTS group, compared to all other treatments (Fig. 3A). At genus level, *Vibrio* was the most abundant bacteria in all samples, followed *by Aeromonas*, *Candidatus bacilloplasma* and *C. hepatoplasma* (Fig. 3B). The number of shared and unshared genus was found higher in LTS group, followed by HTS, MTS and CTL, respectively (Fig. 3C). Differential abundance at 0.05 level of significance revealed that *Vibrio*, *C. Bacilloplasma* and *Aeromonas* in CTL, LTS and MTS were replaced by *Citrobacter*, *Acinetobacter and Pseudomonas* in the HTS treatment (Fig. 3D).

Discussion

This is the first study that analyzes the effects of trace element supplementation on plankton and growth, health indices and intestinal flora of marron cultured in the same body of water under laboratory conditions. Maintaining favourable water quality conditions allowed for optimum plankton growth in the tanks, similar to autumn season [14]. Also, maintaining plankton density at a static density throughout the experiment by regular harvesting and addition prevented the results from being influenced by various phases of phytoplankton growth or nutrient influx due to plankton crashes. The constant input of nitrogen and P from marron feed, waste and senescence of phytoplankton would have helped to promote growth of phytoplankton, as nitrogen and P can both frequently be limiting to primary productivity [45]. However, fluctuations in TAN are common in aquaculture systems [46], as nutrient concentrations build up until bacterial populations can be established to oxidize ammonia and nitrite.

Phytoplankton require P to build biomolecules such as proteins and nucleic acids [13], which are essential for their growth. Addition of P resulted in an increase in reactive phosphate in the HTS treatment, which yielded the highest density of plankton [11, 47]. Similarly, higher fertilizer inputs resulted in increased primary productivity and tilapia (*Oreochromis niloticus*) production in ponds [48]. In our study, enhanced phytoplankton growth was triggered by the medium and high concentration supplementation of trace elements. The HTS tanks had the highest plankton density, due to higher phosphate and Si levels, and thus providing greater quantities of sustenance for the zooplankton community. Potassium,

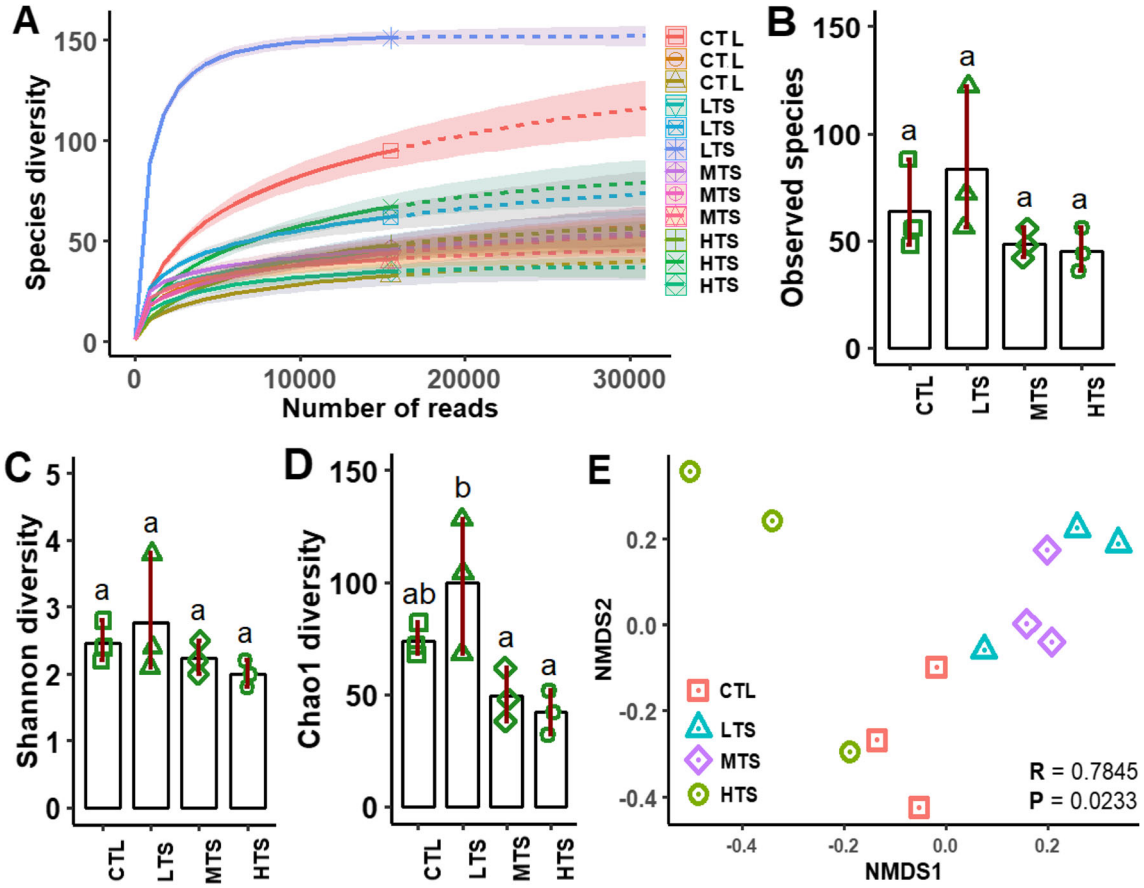

Fig. 2 Alpha-beta diversity of microbial communities in the marron hindgut at the conclusion of the experiment. (A) Rarefaction curve in terms of sequence depth; (B) observed species; (C) Shannon diversity; (D) Chao1 diversity; (E) Beta ordination plot showing clustering of samples based on relative abundance of OTUs. Abbreviations: CTL, control tanks; LTS, low trace elements; MTS, medium trace elements; HTS- High trace element supplementation

sodium and chloride were also added as part of chemical compounds of Mn, Si and P, which are essential nutrients for plankton growth, and may have improved marron growth and health mediated through planktons in HTS tanks [9, 49–51]. The added P may have been utilized by the phytoplankton, periphyton grown on tank edges and eventually by zooplankton or be returned as detritus on the tank bottom [5]. The quantity of P and Si added in the HTS treatment tanks was high enough to trigger plankton density, while this response was much reduced in the LTS and MTS tanks. On P addition in three different concentrations (0.2, 0.1 and 0.05 $g*m^{-3}$), Shrestha [47] observed no significant effect of different P levels of chlorophyll-α in tilapia ponds and also higher P addition did not increase the tilapia yield.

The phosphate concentration increased over the experiment time, likely due to the marron feed, plankton senescence and marron waste. The plankton biomass and species composition are regulated by the availability of nutrients, while most planktons are limited by P and nitrogen, and diatoms are often limited by silica [5, 13]. The diatom presence was more abundant only in HTS (supplementary data #1), and similar results of increase in diatoms on Si enrichment was observed by Nwankwegu [12]. Addition of trace elements, such as Mn, to water may enrich phytoplankton, rotifers and other zooplankton that feed on them, and in turn improve the diet of cultured animals such as marron [52].

Improved growth rate and health indices of marron in HTS can be associated to the higher plankton density; the plankton may have provided a food source in a fresh form or in the form of detritus, improving the marron nutrition. Natural productivity plays an important role in the production of white leg shrimp (*Litopenaeus vannamei*) and yabbies (*C. albidus*) [17, 53]. A study on red claw (*C. quadricarinatus*) juveniles showed the greatest increase in weight (%) and highest harvest mean weight in tanks with the use of zooplankton [54]. Comparatively, in a diet of zooplankton, unidentified bacteria and the macrophyte fed to juvenile red swamp crayfish (*Procambarus clarkii*), the higher growth rate was achieved with zooplankton [55]. It is likely that the marron grown in HTS obtained added nutrition from the high plankton density therein, although it is unclear whether they obtained nutrition from the phytoplankton, zooplankton or detritus. Past research has shown that zooplankton are an important food source for juvenile crayfish, while plant matter from phytoplankton and

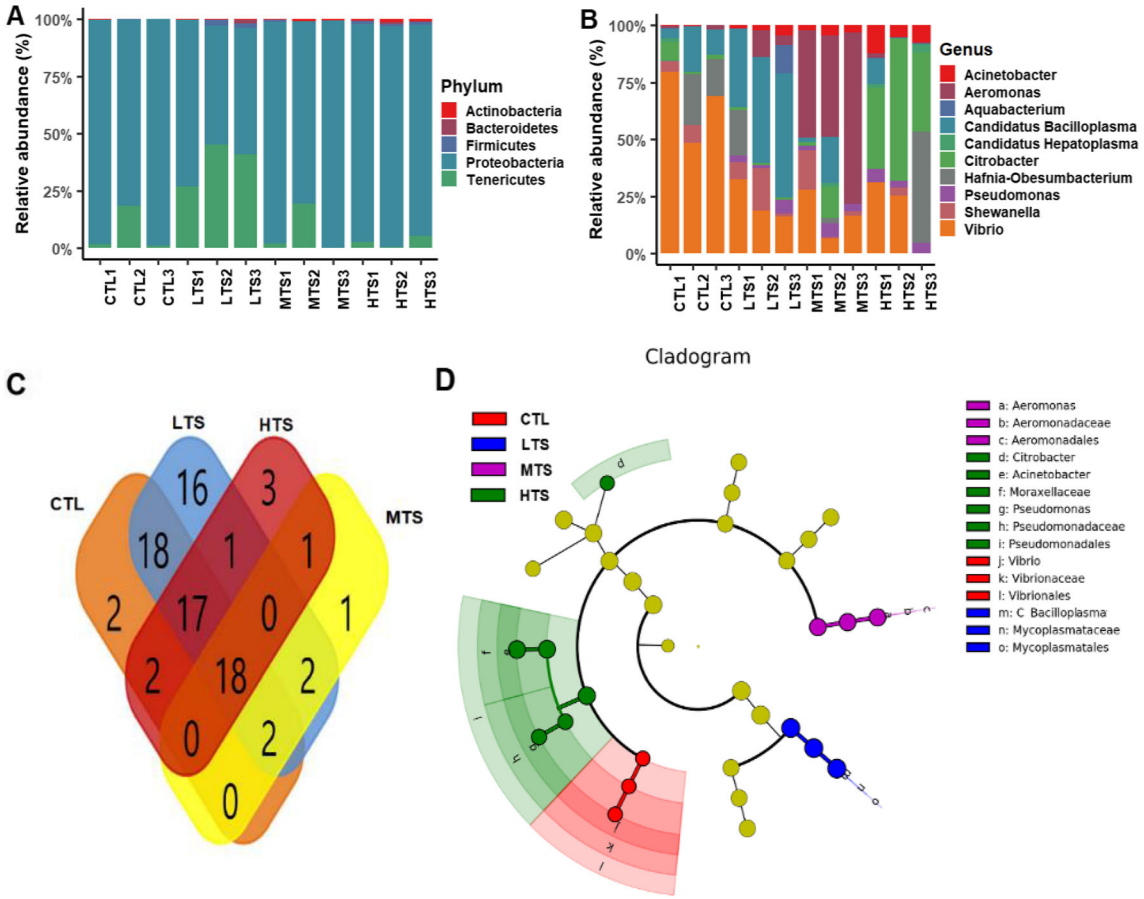

Fig. 3 Relative and differential abundance of microbial communities in four different groups of marron at the conclusion of the experiment. (A) Relative abundance at phylum level; (B) relative abundance at genus level; (C) number of shared and unshared genus in different treatments; (D) statistically significant bacteria at various taxa level in four different treatments. Abbreviations: CTL, control tanks; LTS, low trace elements; MTS, medium trace elements; HTS- high trace element supplementation

macrophytes may be less important [55]. However, plant material may provide nutritional elements not available in animal matter such as carotenoids [17]. Copepods, cladocerans and rotifers present in the tank water may have improved the health and growth of marron, as formulated feed often lacks important nutrients that the natural feeds contain [56]. Juvenile marron is thought to be poor filter feeders, so any phytoplankton probably would have been of value after settling on the cage bottom. Adult copepods or cladocerans may have been actively caught by the marron with their chelae or picked up off the cage bottom, while smaller zooplankton such as rotifers and copepod nauplii may have only been ingested as part of the detritus.

The SR and THC remained similar between treatments; however, these parameters are less associated with nutrition, and the results showed that all the treatment tanks had similar tank conditions. The HM (%) was significantly lower in MTS and HTS while Hid was significantly higher in HTS which suggests that marron from tanks MTS and HTS had good health condition as compared to CTL and LTS treatment tanks. The hepatopancreas is an important digestive gland and is used for storage of energy and nutrients [56] and maybe a good indicator of crayfish condition [57]. Higher plankton density in MTS and HTS may have increased the amount of nutrients and energy stored in the hepatopancreas reflecting an improvement in overall health of the juveniles.

The trace elements' presence in tank water was not similar to their supplemented concentrations; the aquatic environmental dynamics are complex; the presence of plankton or bacteria may have absorbed or converted the trace elements. Also the supplementation of Mn and P and their presence in tank water did not affect their concentrations in marron haemolymph; Cu concentration was higher in marron from treatment HTS. Freshwater crustaceans accumulate and store the trace elements such as Ca and Cu in haemolymph [58]. More than 50% of the whole body Cu load is stored in haemolymph [59]. It is an essential micronutrient and is an integral part of the respiratory pigment haemocyanin [60]. Haemocyanin maintenance requires the accumulation of Cu in relatively large quantities than trace levels, for its transport and storage within the body [61]. The Cu accumulation in decapod crustaceans is regulated only up to the physiological threshold levels [60].

The gut bacterial communities have been reported to play a key role in digestion and immunity of aquatic animals,

although the effects of dietary supplementation including probiotics, feed additives and protein sources on gut microbial communities of crayfish have been investigated [62, 63]. Different P levels had no effects on the alpha diversity measurements; however, the inclusion of different P levels on water influenced the gut microbial communities. Particularly, higher P level induced the growth of *Acinetobacter*, *Pseudomonas* and *Citrobacter*; the genera identified previously from marron gut [64] and widely reported as phosphate solubilizing bacteria in water [65, 66]. Bacteria can be transmitted from water to aquatic animals through feeding and the symbiotic correlation between water and gut microbiota of crayfish has been established in some studies [64, 67]. The phosphate solubilizing bacteria in the gut of marron in our study were likely sourced from the water in the culture tank. P inclusion also reduced the abundance for *Vibrio*, a genus commonly regarded as pathogenic for crayfish [68]. Though marron is a disease-free species [69], yet environmental pollution and habitat change constantly increase the chance of infections by emerging pathogen like *Vibrio*. Overall, different trace elements and plankton densities influenced marron growth, health and gut microbiota.

Conclusion

Trace elements influenced plankton growth with the addition of Si resulting in an increased diatom abundance. Increased plankton density was associated with improved juvenile marron growth, health indices and a more diverse gut microbiota. The individual roles of phytoplankton or zooplankton on marron growth and health cannot be isolated at this point. Feeding trials with phytoplankton or zooplankton separately may provide more insight on their overall effect on marron.

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Author Contribution Smita Sadanand Tulsankar: conceptualization, designing and set up of the experiment, day to day feeding, data collection, data analysis and writing of the manuscript. Md. Javed Foysal: marron gut microbiota analysis, writing and reviewing the manuscript. Anthony J. Cole: plankton analysis, writing, reviewing and editing the manuscript. Monique Marthe Gagnon: designing experiment, supervision, reviewing and editing the manuscript. Ravi K. Fotedar: conceptualization, supervision, methodology validation, reviewing and editing of the manuscript.

Data Availability The experimental data will be provided on request and the raw data for marron gut microbiota in FASTQ files has been deposited to National Centre for Biotechnology Information (NCBI) BioProject under the accession number PRJNA682157.

Code Availability Not applicable.

Declarations

Ethics Approval Animal ethics approval is not mandatory for the invertebrate animal studies at Curtin University, Australia. However, all the required protocols were followed while handling the animals, as per the guidelines of Animal Welfare Act, Western Australia and the Australian Code for the Care and Use of Animals for Scientific Purposes (NHMRC, 2013).

Consent for Publication Not applicable.

Competing Interests The authors declare no competing interests.

Submission Declaration The manuscript has not been published previously and accepted for publication elsewhere or it is not under consideration for publication elsewhere. The submitted manuscript has been approved by all authors.

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