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Effects of pond age and a commercial substrate (the water cleanser™) on natural productivity, bacterial abundance, nutrient concentrations, and growth and survival of MARRON (*CHERAX CAINII* Austin, 2002) in semi-intensive pond culture

Anthony J. Cole*, Smita S. Tulsankar, Benjamin J. Saunders, Ravi Fotedar

Curtin Aquatic Research Laboratories, School of Molecular and Life Science, Faculty of Science and Engineering, Curtin University, 1 Turner Avenue, Technology Park, Bentley, Perth, Western Australia 6102, Australia

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ABSTRACT

The effects of a commercial substrate (The Water Cleanser™) (TWC[1]), and pond age, on bacterial abundance, nutrient concentrations, natural productivity and marron production, were investigated on a commercial marron (*Cherax cainii*) farm. The farm had 21 ponds, categorised as: 10 new ponds (2 juvenile, 4 grow-out, and 4 brooder; 5 with TWC, 5 without) and 11 old ponds (5 juvenile, 4 grow-out, and 2 brooder; 5 with TWC, 6 without). At the end of 7 months, TWC had no significant effects on the concentration of total ammonia nitrogen (TAN), nitrite nitrogen (NO_2-N) and nitrate nitrogen (NO_3-N), but reduced the concentration of orthophosphate. The initial concentrations of NO_2-N, NO_3-N after 18 weeks, and orthophosphate after 12 weeks, were significantly higher in old ponds. The phytoplankton abundance, after 6 weeks and 18 weeks, and bacterial abundance after 6 weeks were significantly higher in old ponds. Water quality and natural productivity in old and new ponds showed temporal fluctuations. There were no significant effects of TWC on phytoplankton or zooplankton abundance and diversity. In old ponds, there was a significant increase in juvenile marron final biomass of 26.3% with TWC, attributed to higher survival rate. The results suggest that TWC can be used to improve juvenile marron production.

1. Introduction

Marron (*Cherax cainii* Austin, 2002) are an omnivorous, polytrophic, parastacid crayfish and a target of fisheries and aquaculture in Australia (Molony and Bird, 2005; O'Brien, 1995; Rouse and Kartamulia, 1992). Water quality is of key importance in all forms of aquaculture, including marron farming. Excessive dissolved concentrations of ammonia, nitrite and nitrates can have physiological impacts on crayfish (Jensen, 1996). Nutrient-rich waters also stimulate growth of filamentous algae, which are an unlikely food source for crayfish and can greatly hinder crayfish mobility (Ulikowski et al., 2015). However, marron nutrition is partly dependent on phytoplankton growth, which is limited by nitrogen and phosphorous (Wallen, 1979). Zooplankton are also an important food source for crayfish, especially for juveniles (Jones, 1995). Seasonality can greatly affect the abundance and species composition of phytoplankton and zooplankton communities, partly due to fluctuations in solar radiation and temperature (Canovas et al., 1996; Armitage et al., 1973). The current trial was conducted from October to April; comprising of warmer and variable temperatures in October and November (spring), high temperatures with low rainfall and a long photoperiod in January (summer), and cooler and variable temperatures in March and April (autumn), showing seasonal changes in the water quality and development of natural productivity.

The age of ponds affects water quality, including nutrient concentrations, and plankton communities; impacting on food availability, and growth of cultured animals (Correia et al., 2002; Zimba et al., 2003). Newer ponds may take time to become 'established' and develop microbial and invertebrate populations of high nutritional value (Allan et al., 1995). The age of ponds may also affect plankton composition, for example Zimba et al. (2003) found that older channel catfish ponds were dominated by cyanobacteria, compared to newer ponds which had higher numbers of centric diatoms and green algae. Growth of algae communities, associated with periphyton, can be encouraged via the

* Corresponding author.
E-mail address: anthony.j.cole@postgrad.curtin.edu.au (A.J. Cole).
[1] TWC = The Water Cleanser (substrate).

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addition of substrates (Santhana Kumar et al., 2017).

Substrates are used in aquaculture systems to provide sites for the development of biofilm, a microbial consortium associated with a matrix of extracellular polymeric substances bound to submerged surfaces (Pandey et al., 2014), that has a potential to improve water quality, and crustacean survival and growth (Khatoon et al., 2007; Otoshi et al., 2006; Viau et al., 2012). Various substrates may be used as habitat for heterotrophic bacteria, including duckweed (*Lemna minor)* (Ardiansyah and Fotedar, 2016). Heterotrophic bacteria may aid in bioremediation of nitrogenous wastes and phosphates (Li and Boyd, 2016), and may enhance mineralization of organic matter, releasing ammonium into the water (Amin et al., 2012), but require a carbon source for certain functions, including denitrification (Hamlin et al., 2008). Concomitantly, addition of a carbon source may increase bacterial diversity (Hu et al., 2017). Carbon addition can change the predominant bacteria to *Bacillus* sp. over *Vibrio* sp., while use of a substrate (Aquamat®) has been shown to reduce *Vibrio* sp. counts in prawn ponds (Zhao et al., 2012; Santhana Kumar et al., 2017). Research suggests that attached biofilm, periphyton or particulate organic matter on artificial substrates can provide an additional food source for prawns and crayfish (Otoshi et al., 2006; Moss and Moss, 2004; Viau et al., 2012). Substrates have been used to encourage growth of primarily cyanobacteria and microalgae, including diatoms, to promote bioremediation and animal growth (Khatoon et al., 2007).

Oil-based substrates may provide both a habitat and carbon source, as hydrocarbon. > 100 species of heterotrophic bacteria, including *Pseudomonas* sp. and *Bacillus* sp. are known to utilize hydrocarbons as a carbon source (Hu et al., 2017). Oil-based substrates may have multiple repercussions on the pond environment, such as improved water quality, enhanced ecology, improved animal health, and changes in bacterial composition. However, limited evidence based research has been conducted using oil-based substrates, especially in marron aquaculture.

The substrate used in this trial is an oil and wax based product known commercially as The Water Cleanser™, which acts as a substrate for the growth of heterotrophic bacteria and biofilm. TWC is thought to provide habitat to harbour bacteria, by providing a large surface area and a carbon source, maintaining populations of beneficial bacteria a prolonged period of time (Marine Easy Clean, 2015). TWC is also a potential biofilter for nutrient removal. Attached biofilm may provide an additional food source for marron, as microbial productivity is known to contribute as food for crayfish growth (Morrissy et al., 1984). TWC may promote the growth of heterotrophic bacteria, certain species of which are beneficial to marron health (Ambas et al., 2013). By providing a carbon source, TWC would benefit the growth of *Bacillus* species already present in aquaculture ponds. This field trial aimed to investigate the effects of a commercial substrate and pond age on water quality, natural productivity and marron production on a commercial marron farm.

2. Materials and methods

2.1. Location

A field trial was conducted near Manjimup, Western Australia at a commercial marron farm (34°18′75″ S, 116°06′61″ E). All laboratory based analytical work was conducted at the Curtin Aquatic Research Laboratories (CARL), Perth, Bentley, WA.

2.2. Study design

Marron were stocked in ponds from May 2016, and harvested in May 2017 by farmer. Twenty-one ponds were selected for the study, and categorised according to life stage, pond age, and presence of The Water Cleanser™ (TWC), as: 10 new ponds (2 juvenile, 4 grow-out, and 4 brooder; 5 with TWC, 5 without) and 11 old ponds (5 juvenile, 4 grow-out, and 2 brooder; 5 with TWC, 6 without). Ponds were treated to the same commercial marron farming practices. Ten kilograms of TWC, supplied by Marine Easy Clean Pty Ltd., were added to treatment ponds, as 10 × 1 kg rectangular blocks, and dispersed evenly around the ponds. Blocks were designed to stand upright at the bottom of the ponds, as per the supplier's suggestion.

2.3. Animals

Marron were stocked as juveniles, monosex grow out 40–90 g, monosex grow out 95–130 g and brooders in May–June 2016. Adults were removed from brooder ponds following juvenile production in summer-autumn. Approximately 3500 juvenile marron were stocked in juvenile ponds, while 150–155 kg of 1 + marron were stocked in grow out ponds. Final biomass (kg per pond) was calculated for juvenile and grow out ponds at harvest in May–June 2017. Juvenile survival rate was estimated by counting the numbers stocked, calculating the average weight at harvest and estimating the number harvested.

2.4. Sampling and analysis

2.4.1. Water quality

Sampling was carried out every six weeks, in spring (October and November), summer (January), and autumn (March and April), over seven months. Ponds had a shallow gradient, approximate depth of 1.5 m and area of 1005 to 1325 m^2.

During sampling, pH, temperature and dissolved oxygen (DO) were taken with an Ecoscan pH meter 5 (Eutech), and Oxyguard Handy Polaris meter respectively in all ponds. Turbidity was measured using a secchi disc, with clear ponds given a turbidity reading of 150 cm (approximate pond depth). 100 mL of water was taken from each ponds for nutrient concentration analysis. The concentrations of total ammonia nitrogen (TAN), NO_2-N, NO_3-N and orthophosphate were analysed using Permachem reagents from HACH® and a Skalar colorimeter autoanalyser (Downs et al., 2008). All measurements of water quality were in accordance with standard methods for the examination of water and wastewater (APHA, 1998).

2.4.2. Bacteria

During daylight, 1.5 mL of pond water was collected in sterilized Eppendorf tubes for bacteria analysis. Samples were kept on ice during transportation, and were analysed for Colony Forming Units (CFU) within 24 h of sampling. A 50 μL sample of pond water was directly cultured onto standard plate count agar (Oxoid, U.K.) using aseptic techniques. This dilution factor was adequate to attain plates with 30 to 300 colonies. After incubation for 48 h at 30 °C, colonies were counted and expressed as CFU/mL as previously described (Leonard et al., 2000); calculated by multiplying the colony count by the dilution factor. Colonies were identified according to colony morphology to estimate diversity and *Bacillus* sp. count. *Bacillus* sp. were distinguished by colony morphology. At the end of trial, 3 samples of TWC were collected from 3 randomly selected ponds and analysed for bacterial species identification by Matrix-assisted laser desorption ionization time-flight mass spectrometry (MALDI-TOF MS), by the Department of Agriculture (Western Australia).

2.4.3. Natural productivity

Phytoplankton were sampled with a fine plankton net and concentrated from 500 mL of pond water into 100 mL plastic containers. Zooplankton samples were sampled with a coarse plankton net of 60 μm, and concentrated from 15 L of pond water into 100 mL containers. Phytoplankton samples were preserved with 2–3% Acid Lugol's Iodine within 24 h of sampling. Samples were then inverted several times, and a sub-sample was counted using a haemocytometer at 400× magnification. Identification was carried out to genus level, and species richness calculated, as number of genera per sample. Zooplankton

samples were preserved in 70% Ethanol within 24 h of sampling. Number and species present in 1 mL sub-samples were counted in a petri dish at 20× magnification. Identification was carried out to family level, and species richness calculated, as number of families per sample. The presence of dinoflagellates, and water bug count per 100 mL sample, was also measured. Plankton were identified with reference to Ingram et al. (1997). Phytoplankton and zooplankton abundance were calculated by using equations adapted from Ingram et al. (1997) and Nugroho and Fotedar (2013):

Phytoplankton Abundance (cells/L) = ((No. ×1000/(volume of grid (0.1 mm^3) × No.of grid squares counted)* (Conc.Vol. /1 mL))/Tot. Vol.

Zooplankton Abundance (ind. /L) = (No. ×(Conc.Vol. /Sub. Vol.))/Tot. Vol.

where Tot. Vol. = Total volume of water (L) collected from the pond, Conc. Vol. = Volume of water (mL) containing concentrated plankton after sieving, Sub. Vol. = Sub-sample of water (mL) from concentrated volume in which plankton is counted, and No. = Mean number of cells or individuals counted.

2.5. Statistical analysis

All data were expressed as mean ± standard error. Data was analysed between treatment and pond age with a multivariate two way analysis of variance (ANOVA) and independent *t*-tests. Least significant difference (LSD) post-hoc tests were used for multiple mean comparisons when the *p* value showed significant differences. For data which were not normally distributed Kruskal-Wallis and Mann Whitney tests were used. Finally, Pearson correlations were carried out to determine relations between water quality, productivity, and bacteria counts. All computations were done with SPSS version 23.0 and a p value of < 0.05 was deemed significant.

3. Results

3.1. Water quality

All water quality parameters were within the optimal range for marron (Morrissy et al., 1984; Morrissy, 1990; Environment Protection Water Quality Policy, 2003), except TAN (Table 1). The maximum pH was higher than the optimum for *Cherax quadricarinatus* (Villarreal and Peláez, 1999). Turbidity ranged from 20 to 150 cm (clear). There was no significant difference in water quality grand means between ponds with and without TWC over the course of the trial. However, pond age had a significant influence on water quality, bacterial abundance and phytoplankton grand means (Table 2).

There was no significant difference in pH, temperature, DO, or turbidity of ponds with and without TWC. The DO level was significantly higher in new ponds after 12 to 24 weeks. The pH and DO level were significantly higher in brooder ponds after 6 weeks, while life stage had no significant effect on the physico chemical parameters at all other times. Temperature was highest after 12 weeks (summer); with a mean of 24.60 °C in old ponds and 24.81 °C in new ponds (Fig. 1). Turbidity was significantly lower in old ponds than new ponds, at 85.83 cm and 136.33 cm respectively, after 6 weeks. Pond age had no significant effect on turbidity for the remainder of the trial. No data were available for physico-chemical parameters in October.

Table 1
Optimum ranges for water quality compared to observed range for all ponds throughout the study.

Parameter	Optimum	Observed range
Temperature (°C)	11–30[1]	20.5–29.3
pH	6.5–9.0[4]	7.25–9.19
DO[a] (mg/L)	> 5[2]	> 6.3
TAN[b] (mg/L)	< 0.01[3]	< 0.3
NO_2-N (mg/L)	< 1.0[3]	< 0.089
NO_3-N (mg/L)	< 10.0[3]	< 2.0

[1] Morrissy, 1990.
[2] Morrissy et al., 1984.
[3] Environment Protection (Water Quality) Policy, 2003.
[4] Villarreal and Peláez, 1999.
[a] Dissolved oxygen.
[b] Total ammonia nitrogen.

3.2. Nutrient concentrations

Concentrations of TAN, NO_2-N, NO_3-N and total nitrogen were not significantly affected by TWC. After 24 weeks (April), significantly lower concentrations of orthophosphate were present in old ponds with TWC (Table 3). Meanwhile, pond age had a significant effect on all nutrient concentrations except TAN (Fig. 2), and Total Nitrogen. Seasonally, TAN concentration peaked after 6 weeks, while all nitrogenous waste levels fell after 12 weeks (summer), coinciding with an increase in bacteria and plankton abundance. The concentrations of Total Nitrogen were lowest after 12 and 24 weeks. No TAN data could be obtained after 18 weeks.

3.3. Natural productivity

The Water Cleanser™ had no significant effect on the phytoplankton abundance and species richness. Meanwhile, both the abundance and species richness of phytoplankton were significantly higher in old ponds than new ponds after 6 weeks (spring) and 18 weeks (autumn). Phytoplankton abundance ranged from 500,000 to 14 500,000 cells/L. Species found during the study included *Closterium* sp., *Chlamydomonas* sp., *Scenedesmus* sp., *Monoraphidium* sp., *Spirogyra* sp., *Volvox* sp., *Pediastrum* sp., *Tetraedron* sp., *Chlorella* sp., *Pandorina* sp., *Euglena* sp., *Navicula* sp., *Aulacoseira* sp., unidentified dinoflagellates, and unidentified centric diatoms. Phytoplankton species richness was highest in the warmer seasons, after 6 weeks (spring) and 12 weeks (summer), while zooplankton species richness was highest after 18 weeks (autumn). Phytoplankton and zooplankton abundance were highest after 12 weeks (summer) (Fig. 3). Zooplankton abundance ranged from 3 to

Table 2
Grand means and standard errors for water quality between old and new ponds ($n = 55$, $n = 50$ respectively).

Parameter	Old	New
pH	8.19 ± 0.05	8.30 ± 0.04
Temp (°C)	23.3 ± 0.30	23.2 ± 0.24
DO (mg/L)	7.92 ± 0.12[a]	8.46 ± 0.14[b]
Turbidity (cm)	73.53 ± 5.90[a]	97.78 ± 6.26[b]
NH_3-N (mg/L)	0.056 ± 0.009	0.054 ± 0.008
NO_2-N (mg/L)	0.006 ± 0.002[a]	0.003 ± 0.001[b]
NO_3-N (mg/L)	0.441 ± 0.056[a]	0.296 ± 0.028[b]
Orthophosphate (mg/L)	0.35 ± 0.03	0.36 ± 0.06
HPC (CFU/mL) (×10^3)	3.02 ± 0.48[a]	1.78 ± 0.31[b]
Est. bacteria diversity	6.95 ± 0.50	6.16 ± 0.41
Phyto abundance (cells/mL)	4.33 ± 0.48[a]	2.31 ± 0.23[b]
Phyto diversity	6.26 ± 0.39[a]	4.94 ± 0.35[b]
Zoo abundance (cells/mL)	99.7 ± 14.9	69.8 ± 10.8
Zoo diversity	2.25 ± 0.088	2.43 ± 0.118

Superscript letters [a, b] indicate a significant difference ($p < .05$).

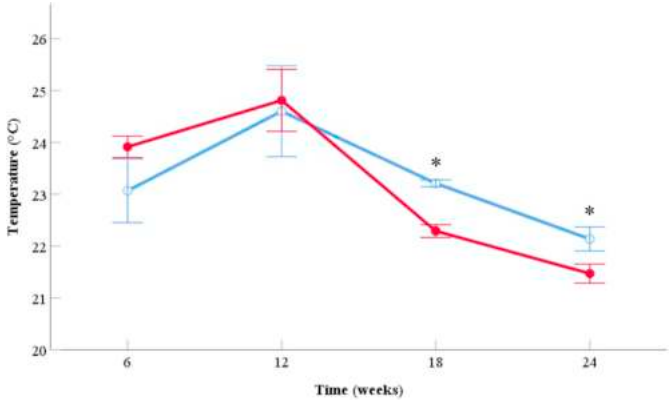

Fig. 1. Variation of temperature from spring (6 weeks) to autumn (24 weeks), according to old ponds (open circles) and new ponds (filled circles), with mean and standard error (old ponds, $n = 11$; new ponds, $n = 10$). An asterisk represents a significant difference between pond age ($p < .05$).

160 individuals/L and was not significantly affected by TWC or age of ponds, though was higher in the brooder life stage than juvenile or grow-out after 24 weeks (autumn). Phytoplankton species richness was higher than in juvenile ponds than brooder ponds after 6 weeks (spring), while in the first sampling, zooplankton species richness was higher in grow out ponds than brooder ponds, at 7.78 and 4.63 genera respectively. There was a significant interaction between substrate type and life stage, where zooplankton species richness was higher in ponds with substrate in juvenile and grow-out ponds, but higher in ponds without substrate in brooder ponds, after 18 weeks (autumn). In ponds with TWC; zooplankton species richness was higher in new ponds after 6 weeks (spring), while phytoplankton species richness was higher in old ponds after 18 weeks (autumn). Zooplankton species richness was also higher in new ponds after 24 weeks, in ponds without TWC. The abundance of certain species appeared to be somewhat seasonal, e.g. Cladoceran abundance was greatest in spring. Zooplankton species found include rotifers; *Branchionus* sp. and *Keratella* sp.; Calanoida and Cyclopoida copepods (adult and nauplii); and Cladocerans *Daphnia* sp. and *Moina* species. Insecta (Orders Hempitera and Coleoptera) were commonly found.

No strong correlation ($R > 0.70$) was present between phytoplankton and zooplankton abundance. There was a significant negative correlation between phytoplankton abundance and zooplankton species richness in old ponds in November ($p = .005$, $R = -0.749$). There were no strong correlations ($R > 0.70$) found between any nutrient concentrations and plankton parameters, or between the final biomass and survival rate of juvenile marron.

3.4. Bacteria

The heterotrophic plate count (HPC), *Bacillus* sp. count and estimated colony diversity are summarized in Table 4. Life stage had no significant effect on bacterial abundance, while estimated colony diversity was higher in juvenile ponds than grow-out and brooder ponds, in the first sampling. Biofilm was observed on TWC surface after six weeks. After 24 weeks, no bacteria data could be obtained. At the end of trial, TWC samples from three randomly selected ponds were analysed, using MALDI-TOF MS, and the following bacteria species were found: *Aeromonas eucrenophila, Pseudomonas brassicacearum, Bacillus cereus, Bacillus thuringienis, Pseudomonas anguilliseptica, Pseudomonas extremorientalis, Rheineimera soli, Aeromonas bestarium, Exiguobacterium sp., Pseudomonas cedrina, Aeromonas veronii,* and *Bacillus vietnamensis.*

No strong correlations were found between HPC and nutrient concentrations. In old ponds the concentration of orthophosphate was positively correlated with estimated colony diversity in January ($p = .009$, $R = 0.743$). A two-way ANOVA found no significant interaction effects between pond age and substrate.

3.5. Marron growth and survival

Final biomass was calculated for grow-out and juvenile ponds at harvest. No growth data was available for brooder ponds. In juvenile old ponds, final biomass was significantly higher with TWC (Fig. 3). There was a mean increase of approximately 26%. Final biomass was not significantly different between old and new ponds, or between ponds with and without substrate irrespective of pond age.

Survival rate of juvenile marron was not significantly different between ponds with or without substrate in old or new ponds, irrespective of pond age, or between old and new ponds (Fig. 4). However, survival rate was approximately 20% higher in old ponds with substrate than without (Fig. 5).

4. Discussion

The Water Cleanser™ had a significant effect on the juvenile marron, because this is a more critical stage of the crayfish life cycle, where mortality can be high (Ghanawi and Saoud, 2012). Artificial substrates have previously improved survival rate in various prawn cultures including *Penaeus monodon* tank culture (Khatoon et al., 2007), and *Litopenaeus vannamei* pond culture (Santhana Kumar et al., 2017); both attributed to improved water quality. Viau et al. (2012) have shown that biofilm attached to artificial substrate can improve survival and contribute to growth of *C. quadricarinatus* (redclaw crayfish) juveniles, by providing a complementary food source, as well as improving water quality, which is essential for good survival. Jones et al. (2002) found that another species in the *Cherax* genus, *Cherax destructor*, consumes biofilm attached to artificial substrate. TWC also provided biofilm, containing bacteria, protozoans, nematodes, and microalgae including diatoms. Signs of biofilm utilization were present in both juvenile and grow out ponds. In *C. quadricarinatus* culture, analysis of stomach contents found that they consumed various periphyton found in biofilm, including ciliates, rotifers and nematodes (Viau et al., 2012), which can be nutritionally important (Da Silva et al., 2008). Better nutrition, provided by good natural productivity, can help to improve juvenile crayfish survival (Ghanawi and Saoud, 2012).

Table 3
Orthophosphate in old and new ponds with and without TWC.

Orthophosphate	Control		TWC		Overall	
	Old	New	Old	New	Old	New
0 weeks (Oct)	–	–	–	–	0.21 ± 0.06	0.27 ± 0.04
6 weeks (Nov)	0.09 ± 0.02	0.36 ± 0.18	0.19 ± 0.06	0.10 ± 0.04	0.13 ± 0.03	0.23 ± 0.10
12 weeks (Jan)	0.57 ± 0.07^{m}	0.12 ± 0.06^{n}	0.42 ± 0.03	0.24 ± 0.15	0.50 ± 0.05^{m}	0.18 ± 0.08^{n}
18 weeks (Mar)	0.51 ± 0.08	0.48 ± 0.07	0.65 ± 0.13	0.39 ± 0.07	0.57 ± 0.07	0.44 ± 0.05
24 weeks (Apr)	^{a}0.43 ± 0.22	1.12 ± 0.24	^{b}0.27 ± 0.24 ^{m}	0.45 ± 0.24^{n}	0.36 ± 0.04	0.79 ± 0.25

Superscript $^{a, b}$ indicate a significance between ponds with and without TWC; superscript $^{m, n}$ indicate a significant difference between old and new ponds ($p < .05$).

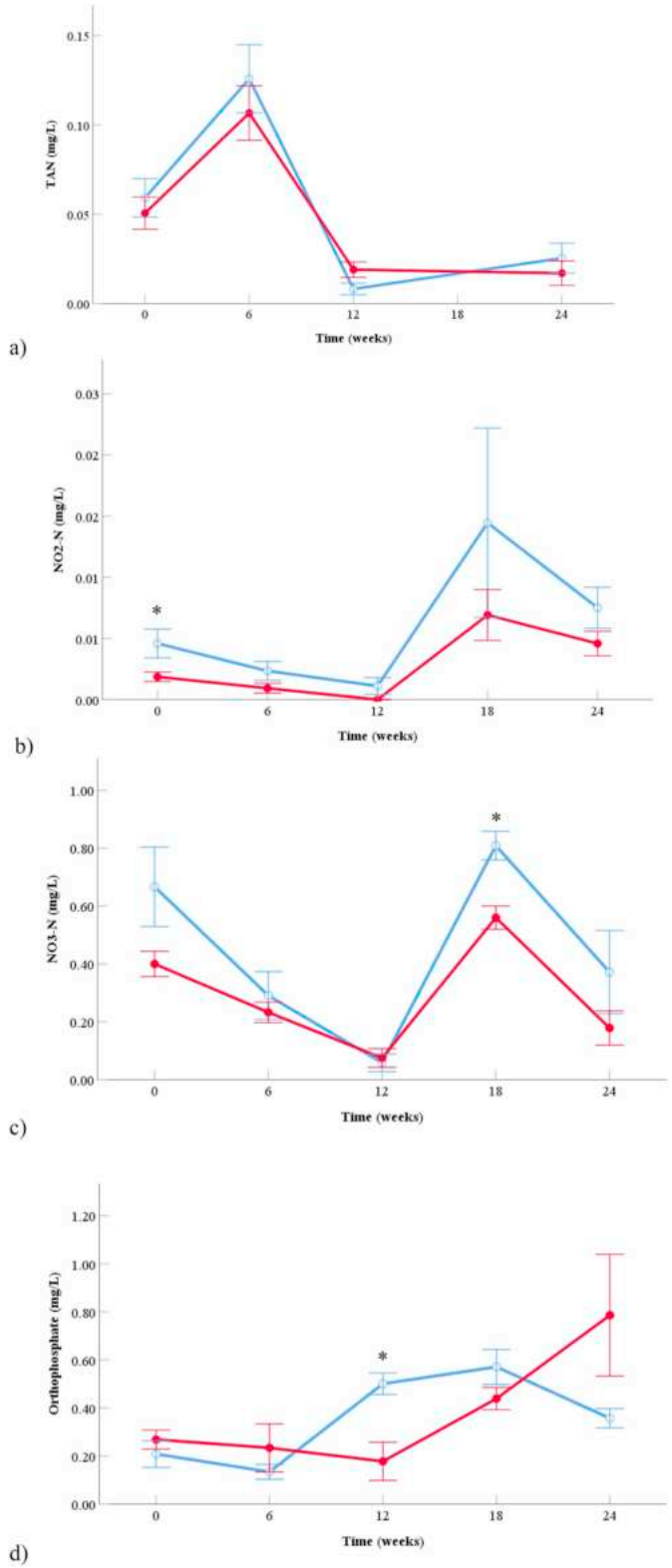

Fig. 2. Variation in concentrations of a) TAN (Total Ammonia Nitrogen), b) NO_2-N, c) NO_3-N and d) Orthophosphate, comparing old (open circles) and new (filled circles) ponds over time. No TAN data was available at 18 weeks. An asterisk represents a significant difference between pond age ($p < .05$).

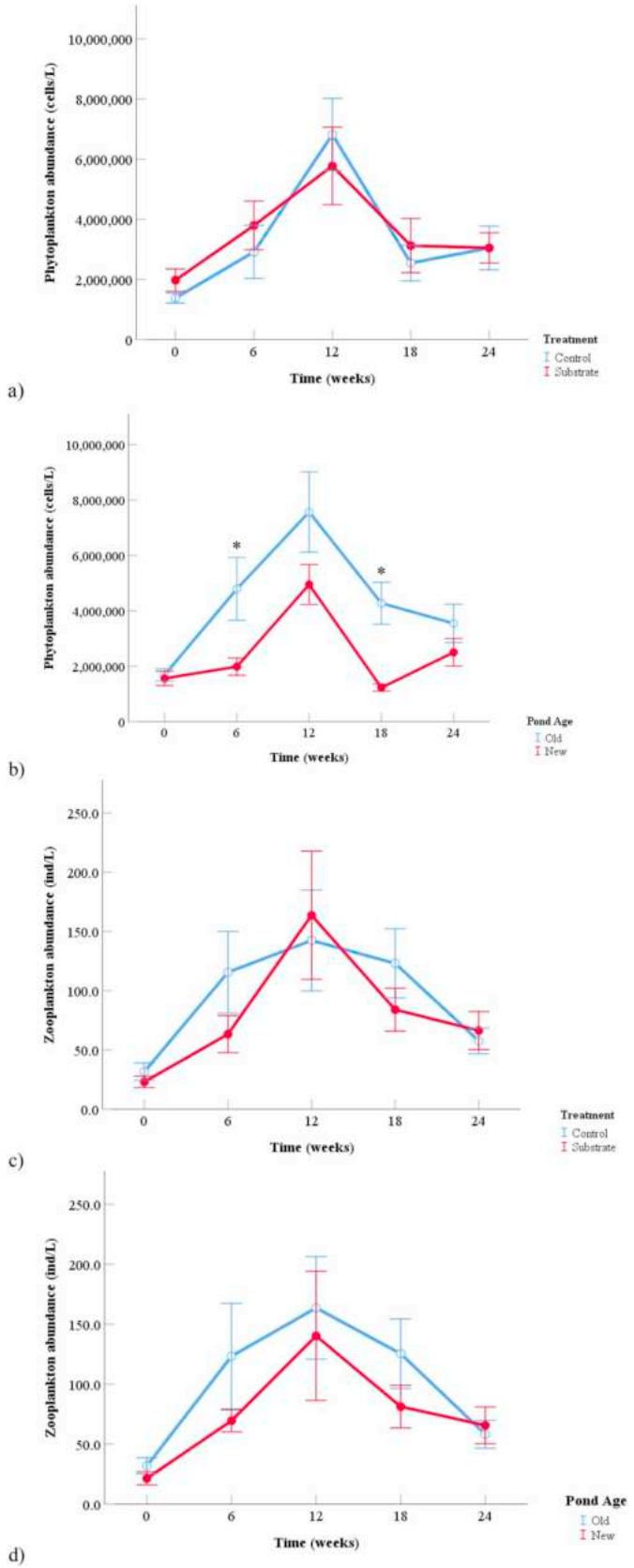

Fig. 3. Fluctuation of phytoplankton (a, b) and zooplankton (c, d) abundance (no. / L), comparing with and without substrate (control), and pond age. An asterisk represents a significant difference between pond age ($p < .05$).

Detritus and zooplankton are important food items due to their nutritional composition and availability (Browne et al., 1992; Jones, 1995). In the present study substrate had no significant effect on zooplankton abundance, however. Uddin et al. (2009) found similar results

Table 4
The Heterotrophic Plate count (HPC) ($\times 10^3$) and *Bacillus* sp. count as CFU/mL and estimated diversity between old and new ponds with and without TWC.

Variable	Control		TWC		Overall old	New
HPC (CFU/mL)	Old	New	Old	New		
1st Sampling (Oct)	3.58 ± 3.03	1.36 ± 0.62	3.23 ± 2.26	1.93 ± 1.69	3.40 ± 1.75	2.45 ± 0.92
2nd (Nov)	2.35 ± 0.90	1.20 ± 0.23	3.05 ± 0.88	1.42 ± 0.17	2.64 ± 0.62^{m}	1.30 ± 0.14^{n}
3rd (Jan)	6.23 ± 1.30	3.94 ± 1.70	3.47 ± 1.10	3.21 ± 1.00	4.98 ± 0.93	3.58 ± 0.94
4th (Mar)	1.17 ± 0.18	1.02 ± 0.19	1.24 ± 0.30	0.74 ± 0.16	1.20 ± 0.16	0.88 ± 0.12
Bacillus sp.						
1st (Oct)	< 20.0	40.0	20.0	< 20.0	20.00	40.0
2nd (Nov)	< 20.0	20.0	20.0	< 20.0	20.0	20.0
3rd (Jan)	36.7 ± 15.0	16.0 ± 4.0	48.0 ± 30.1	24.0 ± 9.80	41.8 ± 15.1	20.0 ± 5.2
4th (Mar)	^{a}50.0 ± 10.0	20.0 ± 12.7	^{b}120.0 ± 26.1^{m}	20.0 ± 9.43^{n}	81.8 ± 16.5^{m}	20.0 ± 9.4^{n}
Estimated colony diversity						
1st (Oct)	3.8 ± 0.5	3.6 ± 0.7	3.0 ± 0.7	2.5 ± 0.3	3.4 ± 0.4	3.3 ± 0.4
2nd (Nov)	4.7 ± 0.5	4.9 ± 0.4	4.6 ± 0.3	5.0 ± 0.3	4.7 ± 0.3	4.9 ± 0.2
3rd (Jan)	^{a}10.7 ± 0.6^{m}	7.4 ± 0.7^{n}	^{b}8.0 ± 0.3	9.4 ± 1.4	9.5 ± 0.6	8.4 ± 0.8
4th (Mar)	^{a}10.8 ± 0.9^{m}	7.4 ± 0.8^{n}	^{b}8.0 ± 0.8	9.4 ± 0.5	9.6 ± 0.7	8.4 ± 0.5

Superscript $^{a, b}$ indicate a significant difference between ponds with and without TWC; superscript $^{m, n}$ indicate significant difference between old and new ponds ($p < .05$).

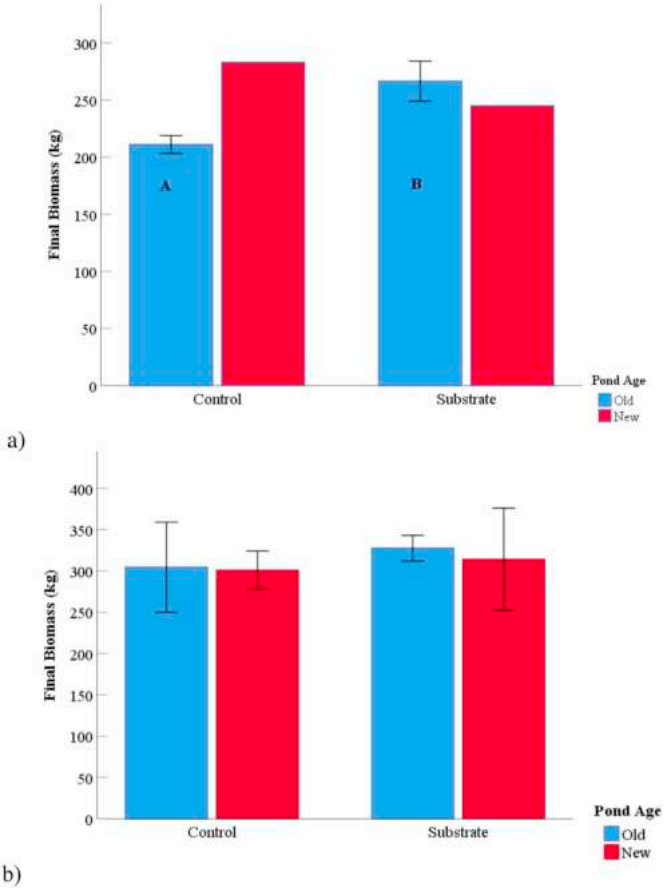

Fig. 4. Mean final biomass for juvenile (a) and grow-out (b) ponds, between old and new ponds with substrate and without substrate (control). A and B represent significant differences ponds with and without TWC ($p < .05$). Where no standard error bar is present n = 1.

in the zooplankton populations of tilapia (*Oreochromis niloticus*) and freshwater prawn (*Macrobrachium rosenbergii*) polyculture; where there were no significant differences between Rotifera and Crustacea numbers in ponds with and without bamboo substrate with attached periphyton. Phytoplankton composition varied however, where ponds with bamboo substrate had higher numbers of Bacillariophyceae, Chlorophyceae and Cyanophyceae than control ponds (Uddin et al., 2009).

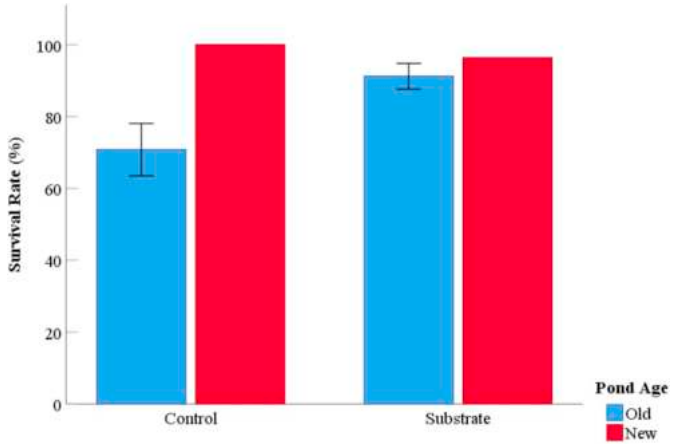

Fig. 5. Survival rate in juvenile ponds between old and new ponds with and without substrate (control). Where no standard error bar is present n = 1.

Natural productivity generally increases in the warmer months (Armitage et al., 1973; O'Brien and de Noyelles Jr, 1974; Affan et al., 2005). The population of Calanoida copepods (*Skistodiaptomus pallidus*) in a large freshwater pond in Kansas, U.S. was found to be more abundant in spring and autumn, and much lower in winter (Armitage et al., 1973). O'Brien and de Noyelles Jr (1974) found a high level of chlorophyll *a,* indicating high phytoplankton abundance, in late summer, while Affan et al. (2005) found the lowest phytoplankton abundance in winter. In the current trial the highest abundance of phytoplankton and zooplankton was also in summer. While a relationship clearly exists between phytoplankton and zooplankton, no strong correlation was found between phytoplankton and zooplankton abundance in this study, as in another study (Armitage et al., 1973). McKnight et al. (1990) showed that increased zooplankton grazing can cause a decrease in phytoplankton abundance, however. In the current trial, higher zooplankton diversity was related to lower phytoplankton abundance, possibly showing enhanced grazing by certain species, such as rotifers (*Keratella* sp.). The increase in phytoplankton in summer in the current trial would have depleted the ammonia and nitrate levels, while the increased concentrations of orthophosphate in summer may have helped to trigger the increase in phytoplankton growth (O'Brien and de Noyelles Jr, 1974).

The concentration of orthophosphate was significantly reduced in ponds with TWC after 24 weeks. A study in intensive shrimp (*Penaeus paulensis*) culture has shown the effectiveness of biofilm, attached to

PVC tubes, in reducing ammonium and phosphate levels (Thompson et al., 2002). TWC had no significant effect on concentrations of nitrogen metabolites in marron ponds though, partly due to low nutrient concentrations. Fotedar (2004) reported ammonia and nitrite levels of 0.5 mg/L or less in semi-intensive marron ponds in Jurien Bay, Western Australia. In systems with higher nutrient loading, substrates have been used to improve water quality. A substrate (Aquamat) combined with sand sediment has resulted in low TAN and orthophosphate levels in shrimp (*L. vannamei*) tanks (Bratvold and Browdy, 2001). Phosphorous in water can be oxidised to produce energy for microbes, and used for their growth and metabolism (Lananan et al., 2014). Phosphorous may then be converted into cellular polyphosphate and protein. Meanwhile, the application of an artificial substrate (Aquamat) into semi-intensive shrimp (*L. vannamei*) ponds has resulted in a reduction of 12% in the concentration of nitrogen in effluent, attributed to attached heterotrophic bacterial and algal biomass (Santhana Kumar et al., 2017). TWC may provide habitat for primarily heterotrophic bacteria and microorganisms rather than algae (Personal observations).

Biofilm containing heterotrophic bacteria was found attached to TWC surface in marron ponds. This could be the main cause for the higher *Bacillus* sp. count found in pond water with substrate. Another study has found a variety of heterotrophic bacteria, including *Bacillus* sp. and *Pseudomonas* sp., on duckweed substrate (Ardiansyah and Fotedar, 2016). Application of *Bacillus* sp. is generally used to increase *Bacillus* sp. counts in aquaculture water (Zokaeifar et al., 2014), however TWC maintains the *Bacillus* sp. population without further addition, by providing a habitat and carbon source, as hydrocarbon. *Bacillus* sp. have been shown to reduce concentrations of orthophosphate and nitrogen in aquaculture water (Wang et al., 2005; Lalloo et al., 2007; Xie et al., 2013). TWC with attached *Bacillus* sp. could affect marron via ingestion of biofilm or by altering the bacterial composition of water, potentially improving physiological condition of marron (Ambas et al., 2013). Addition of *Bacillus* sp. in water has been shown to increase survival of the Indian white shrimp (*Penaeus indicus*) in tanks and earthen ponds (Ziaei-Nejad et al., 2006). Of the species found on TWC surface; *Bacillus thuringienis* helps to maintain eco equilibrium and inhibits the proliferation of harmful organisms (Zhou et al., 2009), while *B. cereus* is a common and beneficial probiotic in aquatic animals (Deng et al., 2014; Hong et al., 2005; Balcázar et al., 2006). *Aeromonas* sp. and *Pseudomonas* sp. are common indigenous bacteria in aquatic animals, sometimes in association with animals, and ubiquitous in nature. *Pseudomonas* sp. are commonly used as probiotics, while others are pathogenic (Balcázar et al., 2006).

No discernible effect was present on bacterial abundance in the water column, largely due to the high variation in bacterial abundance. Schveitzer et al. (2013) also found no significant differences in microbial activity between tanks with and without substrate. Bacterial abundance in marron ponds was highest in summer, coinciding with a low concentration of nitrogen. The optimum temperature for denitrification, for nitrifying bacteria and for several *Bacillus* strains, is between 25 °C and 35 °C (Song et al., 2011; Hargreaves, 1998). Phosphorous also limited the growth of certain species, as inferred by the significant correlation between orthophosphate and estimated bacterial diversity. Phosphorous is often a limiting element in microbial growth (Kirchman, 2012).

Fertilization of newly-dug freshwater prawn ponds has been suggested by Correia et al. (2002), due to low biomass of microbial and macroinvertebrate populations. Fertilization is uncommon in marron aquaculture though, due to the risk of eutrophication leading to algal blooms (Comm. with farmers). Nutrient concentrations, turbidity and dissolved oxygen levels were lower in new marron pond, resulting in lower phytoplankton abundance, bacterial abundance, *Bacillus* sp. count, and estimated bacterial diversity. In a study on channel catfish (*Ictalurus punctatus*) ponds, Zimba et al. (2003) also found that older ponds had significantly higher nitrogen and phosphorous concentrations. Pond age had no discernible effect on zooplankton abundance, but significantly higher species richness was present in new marron ponds. Abu Hena and Hishamuddin (2014) found similar results, recording no differences in zooplankton abundance between old and new ponds, and higher diversity and evenness in new ponds. Macroinvertebrate populations were more established in older ponds.

5. Conclusions

The trial demonstrated the significant effects of pond age on water quality, natural productivity and bacterial abundance. The Water Cleanser™ significantly improved the growth rate of juvenile marron in old ponds, attributed to a high survival rate, and to an extent to biofilm attached to the substrate providing a complementary food source. Additionally, there was higher abundance of *Bacillus* sp. in ponds with TWC. Effects on water quality were limited however, with a reduction in the concentration of orthophosphate only, in the final sampling. TWC may be applied to pond culture with no detrimental effects to natural productivity or marron, however more research is needed to determine its effects on plankton and microbial ecology, and marron health and physiology.

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