

Temporary pond ecosystem functioning shifts mediated by the exotic red swamp crayfish (*Procambarus clarkii*): a mesocosm study

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Abstract Temporary ponds, acknowledged for their conservation value, are colonized by the invasive crayfish *Procambarus clarkii*. We have tested the consequences of this colonization for the ecosystem under two contrasted scenarios: one single individual arrival or three individuals arrival. We recreated the temporary pond ecosystem in 1 m² tanks to investigate the impact of the two crayfish densities. We studied the macrophyte community composition and abundance, chlorophyll *a* and total suspended solids

concentrations, and the diversity and functional composition of micro-crustacean and macro-invertebrate communities. We observed a reduction of macrophyte biomass in experimental crayfish mesocosms in comparison with control tanks, nearly 80 and 40% less in 3 and 1 crayfish/m² tanks, respectively. The macrophyte community shifted, followed by a filamentous algae development, an increase of bare sediment and turbidity in crayfish tanks. The macro-invertebrate community suffered a richness loss of 28 and 22%, in 3 and 1 crayfish/m² tanks, respectively. Functionally, macro-invertebrate diversity reduction most strongly affected the grazer, detritivore and predator trophic groups. Microcrustaceans seemed not to be affected by the introduction of the crayfish. The introduction of the crayfish greatly altered the ecosystem structure and subsequently the ecosystem functioning.

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Introduction

Temporary ponds are singular aquatic ecosystems that host a unique fauna and flora with remarkable evolutionary strategies to survive drought periods and contribute notably to regional diversity (Williams, 2005). These ecosystems are threatened worldwide due to habitat destruction, salinization and hydrological

changes resulting from climate change and water management (Oertli et al., 2005). More specifically, Mediterranean temporary ponds are recognized in international treaties for their biodiversity, for example, by the European Union's Habitat Directive (Natura code 3170, 92/43/CEE, 21 May 1992) and the Ramsar Convention (Resolution VIII 33). In arid or semi-arid areas such as the Mediterranean basin, these ecosystems are vital for numerous aquatic plants, vertebrates and invertebrates (Grillas et al., 2004; Gómez-Rodríguez et al., 2009; Díaz-Paniagua et al., 2010; Céréghino et al., 2012; Rhazi et al., 2012). Biological communities and ecosystem functioning in temporary ponds are mainly controlled by abiotic factors such as natural flooding events and water mineralization (Williams, 2005), so the arrival in the pond of a large omnivorous predator such as the red swamp crayfish (*Procambarus clarkii*, Girard) should represent a new and severe biological stressor for the communities.

Procambarus clarkii is a crayfish species native to North America, but nowadays it occurs in the aquatic ecosystems of 35 countries across all continents except Australia and Antarctic, and is the most widely introduced crayfish in the world (Holdich, 2002). Its biological traits make of it a species with high invasive potential (Holdich, 2002). Moreover, it attains high population densities, both in its area of origin and in invaded areas, being the largest aquatic invertebrate, along with other crayfish species. This exotic crayfish is responsible for several forms of impact at different levels, on ecosystem services, community structure and food webs, nutrient fluxes, physical structure and functioning of the ecosystem (see for review Gherardi & Acquistapace, 2007; Savini et al., 2010; Lodge et al., 2012). Ecosystem functionality is directly linked to species identity and abundance (Hooper et al., 2005; Cardinale et al., 2006; Duffy et al., 2007; Cardinale et al., 2009); therefore, a strong alteration of ecosystem functioning and a reduction of diversity is expected as a result of the introduction of *P. clarkii*.

In this work, we have studied experimentally in tanks the impact of red swamp crayfish colonization on Mediterranean temporary ponds. This ecosystem can be mimicked quite well with mesocosms (Blaustein & Schwartz, 2001). In the area of study, the Rhône delta (southern France), the crayfish actually colonizes temporary ponds (Meineri et al., 2014; Rodríguez-Pérez et al., 2014), and we aimed to test the effect of

two different crayfish densities (1 and 3 crayfish/m²) on the structure and functioning of the ecosystem.

We predicted a strong impact of the crayfish on macrophytes. This modification would facilitate the phytoplankton development, thus changing the primary production, and it also would increase turbidity. We also predicted that direct predation by the crayfish along with macrophyte reduction would cause a shift in the invertebrate assemblage. We hypothesized that a reduction of invertebrate diversity would occur, at both dimensions: horizontal (reduction of taxonomic richness within a trophic level) and vertical (reduction of trophic levels).

Materials and methods

Geographical context

The Rhône delta (~ 145,000 ha, Southern France) is a wetland internationally and nationally protected as a UNESCO Biosphere Reserve, Ramsar site and Natural Park. It hosts a wealth of different wetland types: saltpans, lagoons, rice fields, marshes and temporary ponds. A general increasing salinity gradient runs from the northern part to the southern part. The area has a Mediterranean climatic regime with total rainfall of ca. 600 mm and a total evapo-transpiration rate exceeding this quantity. In the Mediterranean climatic area, rainfall shows wide inter-annual variations affecting the wetlands hydro-period starting date and duration patterns. Those wetlands that maintain a natural flooding regime (i.e. temporary ponds) are inundated with the autumnal and early winter rains and they usually dry up in spring and early summer.

Experimental setup

This experiment was conducted in the experimental facilities of the Tour du Valat research centre, located within the boundaries of the Camargue Natural Park. During the first 2 weeks of October 2010, we gathered the top sediment layer (~ 5-cm depth), from a temporary wetland located within the Tour du Valat's Natural Reserve (Otero & Bailey, 2003), making a total surface of nearly 30 m². The sediment was pooled and mixed with the aid of a shovel, and then it was transferred into 30 plastic tanks (100 × 100 × 66 cm) in one layer of approximately 5-cm depth. We left the tanks open to

allow filling by autumnal rains, but the rainfall during autumn 2010 was insufficient (rainfall of 109.7 mm between 15 October and 30 November) to reach a height of 30 cm (300 l. approx), and we completed the water volume in the tanks, from the beginning of December 2010, with local non-chlorinated tap water. The water came from an irrigation channel and was conditioned for human consumption by filtration and UV treatment. Tanks were refilled with the same tap water when necessary to maintain the water level throughout the experiment.

Until 11 April 2011, the tanks were left undisturbed to allow the development of macrophyte and micro-invertebrate (zooplankton and benthic invertebrates) communities. Tanks were kept uncovered throughout the whole experiment to allow the colonization of mesocosms by flying macro-invertebrates and amphibians. We randomly assigned each tank to one of the crayfish density treatments (1 or 3 crayfish/tank, hereafter D1 and D3, respectively) or alternatively to control treatment (without crayfish, hereafter C), making a total of 10 replicates for each treatment. We chose the highest experimental density (D3) with the aim of stocking a reasonable density based on visual estimations in a temporary marsh in the area (5–6 ind/m²) and on published densities (Nyström et al., 1996, 1999; Gherardi & Acquistapace, 2007; Klose & Cooper, 2012), but downgrading it to take into account the possibility that confinement might enhance the impact of the crayfish. We randomly introduced crayfish individuals in the tanks with a total carapace length (measured from the tip of the rostrum to the telson) of ca. 4.5–5.5 cm, the most frequent size class in the nearby temporary marsh at the time of capture, regardless of gender proportions. We used juvenile individuals to avoid the occurrence of reproduction events in the tank resulting from the introduction of gravid females. We monitored weekly the survival of crayfish in the tanks, and only seven individuals died during the first weeks of the experiment. We replaced them by newly captured individuals from the same marsh. We always introduced individuals of the most frequent size class occurring in the nearby marsh, in order to include individuals from the same cohort, the largest introduced individual being about 7 cm total carapace size. We provided the crayfish with shelter by adding in each tank the same number of PVC tubes (5-cm diameter and 30-cm length) as the number of introduced crayfish.

Sampling methods

Before the introduction of the crayfish, we sampled macrophyte biomass and cover percentages by species, chlorophyll *a* concentration, total suspended solid (TSS), and invertebrate communities, in order to have a baseline status for comparison with subsequent samplings.

To sample macrophyte biomass, we formed a grid of cells of 10 × 10 cm with strings and we randomly assigned one grid cell to each sampling date, the same cell for the 30 mesocosms at each date. We excluded all the border grid cells to avoid any border effect on the macrophytes. We used a 9.5-cm diameter PVC corer for sampling (~1% surface of the tank). Once sampled, we rinsed macrophytes with tap water to clean them of sediment and to detach invertebrates from plants, and then dried the plants for 24 h at 80°C before weighing them with a precision scale (Mettler-ToledoTM AG245; precision 0.00001 g). We used the same sediment cores to assess chironomid larvae abundance when the sampling was coincident with the invertebrate sampling date. Macrophyte species abundance was assessed by estimating the cover percentage for each occurring species using the string grid. We also recorded the percentage of uncovered sediment.

For TSS and chlorophyll *a* concentration (mg/l) measurements, we filtered a 1.5 l sample at each sampling date through a 64 µm mesh to measure TSS and chlorophyll *a* concentration (mg/l). The samples were stocked for less than 7 days until processed in dark and cold (4–5°C). Chlorophyll *a* was extracted following the methanol extracting method (Talling & Drives, 1963). TSS and chlorophyll *a* concentration estimations were done with a spectrophotometer.

Micro-invertebrates (cladocerans, copepods and ostracods) were sampled with a 1 l beaker from 10 points spread throughout the mesocosms. The subsamples were filtered through a 64-µm mesh size net, pooled into one single sample per mesocosm (6.6% of total volume), and then fixed with ethanol (70%) and stocked until they were sorted. We counted and identified cladocerans, copepods and ostracods to species level in most cases, with the aid of a stereomicroscope, and with a microscope when necessary, and taxonomic keys (Ghetti & McKenzie, 1981; Alonso, 1996; Meisch, 2000). When the sample was too concentrated for any of the taxa, we counted all the other less abundant taxa and then we

concentrated the sample to a 10 ml volume and took three 1.5 ml subsamples. We averaged the abundance of the given taxa for the whole sample with the three subsamples.

We sampled macro-invertebrates with a hand dip net (frame 21×16 cm, mesh size 0.5 mm), sweeping from the right side to the left side and back (one stroke), and sweeping up and down in the water column, for a total of seven strokes, covering the whole surface without extracting the net for the whole sequence. We repeated the same sweeping sequence throughout the whole surface from the front to the rear of the tank. We counted and identified immediately all the individuals to the highest taxonomic resolution that we could achieve using a stereomicroscope when necessary at the nearby laboratory, and once identified we released the macro-invertebrates back into their original mesocosm. In order to keep animals alive so as to avoid any impact on the community, we did not dissect the invertebrates; therefore, taxonomic resolution for certain taxa was not very precise. For identification, we used the taxonomic keys of Friday (1988), Nieser et al. (1994) and Tachet et al. (2000).

We sampled macrophyte abundance, TSS and chlorophyll *a* every 2 weeks. In order to minimize the destructive effect on macrophytes of dip net sweeping, we only sampled invertebrates every 4 weeks. We started sampling in the 15th week of 2011 (11–17 April, hereafter w0), and we finished in the 27th week (4–10 July, hereafter w12). Hereafter, the weeks of 17th (April), 19th, 21st (May), 23rd and 25th (June) are denoted as w2, w4, w6, w8 and w10, respectively.

Data analysis

Macrophytes, total suspended solids (TSS) and chlorophyll a concentration

We analysed macrophyte biomass variation following a linear mixed model with a split-plot design for repeated measurements, where Treatment (3 levels: Control, D1 and D3) and Date (7 levels: w0, w2, w4, w6, w8, w10 and w12) were fixed factors and Tank Id. was a random factor nested in Treatment. We tested for temporal autocorrelation significance incorporating different autocorrelation structures to the models (first-order autoregressive, compound symmetry, a general correlation structure and autoregressive-

moving average) and selecting on the basis of the log-likelihood ratio criteria. We validated the models by examining graphically residuals distribution (Bolker et al., 2009). When the interaction between Date and Treatment was significant, we did pairwise comparisons between Treatment levels for each sampling date, by the means of Welch's test for groups with unequal variance, adjusting the $\alpha = 0.05$ by the False Discovery Rate (FDR) for multiple tests (Garcia, 2003, 2004).

For the analysis of the plant community changes, we used the % of the surface covered for each species as variable, including in the database the % of bare sediment and the % of coverage of filamentous algae, transformed with a logit transformation (Warton & Hui, 2010). We analysed the dataset with a Principal Response Curve analysis (PRC). We tested the significance of the PRC diagram by Monte Carlo permutation, with 999 permutations (Van den Brink & Braak, 1999). When the general test was significant, we also tested the significance of the treatment effect at each date with a Monte Carlo permutation test by date.

For the analysis of the TSS and chlorophyll *a* concentration, we followed the same procedure as described above for the biomass of macrophytes. Chlorophyll *a* concentration was $\log(x + 1)$ transformed before the analysis.

Micro- and macro-invertebrates

We analysed the effects on alpha diversity, measured as taxonomic richness, for micro- and macro-invertebrates separately because we used different sampling techniques for each invertebrate group. We did not use any other alpha diversity metric because we used different taxonomic resolutions within each invertebrate taxon (Magurran, 2003). We used Generalized Linear Mixed Models (GLMMs, (McCullagh & Nelder, 1989) with the same linear model design as above, and with log link function and Poisson error distribution. In this case, Date factor had only four levels (w0, w4, w8 and w12). Fixed factor significance was assessed by means of the deviance test (Zuur et al., 2009). When the interaction Treatment \times Date was significant, we also did a pairwise comparison (see macrophytes section for further details). Model validity was checked by visual examination of residual plots (Bolker et al., 2009).

Micro- and macro-invertebrate communities shifts were also analysed by using the PRC technique, after having applied a Hellinger transformation to abundances to reduce the effect of the overrepresented taxa (Legendre & Gallagher, 2001). In the case of macro-invertebrate dataset, all the coleoptera larvae, identified up to genus, were included in the analysis as different taxa from adult individuals, identified up to species level in most cases. On the basis of PRC analysis, results showing slight initial differences between treatments and trends, and the more obvious differences between treatments at the end of the experiment, we also performed a permutation MANOVA analysis (Anderson, 2001), taking advantage of its greater sensitivity, of the macro-invertebrate community dataset. We analysed independently the community data before the crayfish introduction (w0) and at the end of the experiment (w12), the factor Treatment being the sole independent variable. We used Bray-Curtis dissimilarities index on abundances after the application of square-root transformation, with 999 permutations to calculate p values. When Treatment factor was significant, we performed pairwise comparisons between the three factor levels (C, D1 and D3) by repeating the permutation MANOVA and adjusting the level of $\alpha = 0.05$ by FDR correction.

We analysed the trophic guilds diversity of the macro-invertebrates. We estimated the functional feeding guild composition, assigning to each taxon its contribution to the total score for a given guild (i.e. Chironomini larvae: 0.3 contribution to ‘active filter-feeders’ guild and 0.7 contribution to ‘detritivore’ guild for each individual) to calculate the richness within each guild, and added up all the specific contributions. For the assessment of the abundances for each functional group, we multiplied the species valence by the total number of individuals of the given taxon in the sample, finally adding up all the scores from different taxa within the sample of the same feeding guild. We extracted the valences for each taxon from Moog (1995). We transformed the trophic guild composition matrix with $\log(x + 0.01)$ before applying a PRC analysis (see above for further details).

For analysis, we used the following packages of R software (R Development Core Team, 2012): nlme 3.1-103 (Pinheiro et al., 2012), lme4 0.999375-42 (Bates et al., 2011), ‘car’ (Fox & Weisberg, 2011) and Vegan 2.0-3 (Oksanen, 2011).

Results

Communities description

We found five macrophyte species (*Tolypella hispanica*, *Chara aspera*, *Ranunculus peltatus*, *Zannichellia pedunculata* and *Callitriche truncata*), none of them exclusive to any treatment and evenly present in all the tanks. *Tolypella hispanica* biomass peaked in w0 (up to 90% cover), declining in w2 and disappearing in May (w4). *Chara aspera* was by far the most abundant macrophyte species, reaching a cover percentage over 90%, notably by the end of June (w10). *Ranunculus peltatus*, a floating macrophyte, reached its maximum abundance in May (w4 and w6) with recorded cover percentages of up to 50%. Both *Z. pedunculata* and *C. truncata* were always minor species in terms of coverage with peak abundances of about 10% of cover surface. From w10 (end June), mats of filamentous algae developed, sometimes reaching 100% of cover.

We identified 70 invertebrate taxa (see Online Appendix 1); 14 were micro-invertebrates (copepods, ostracods and cladocerans), and 56 macro-invertebrates. 22 taxa of macro-invertebrates occurred in less than 10% of samples per sampling date (found only once or twice). Only 3 taxa, *Gerris* sp. (Heteroptera), *Hydroglyphus pusillus* and *Helophorus grandis* (Coleoptera), occurred in more than 90% of the samples at a given date. All the taxa occurring exclusively in a given treatment were only found once or twice. All the micro-invertebrate taxa were present in every treatment, except harpacticoid found just once. Mediterranean Tree Frog (*Hyla meridionalis*) tadpoles occurred in all the tanks.

Macrophytes, chlorophyll *a* and total suspended solids

Ten weeks after the introduction of the crayfish, there was a strong reduction in macrophyte abundance in comparison with C mesocosms, attaining D3 mesocosms of 84% in w10 and 79% in w12. Reduction in D1 tanks reached 45% in w10 and 29% in w12 (see Fig. 1). Treatment and Date factors interaction was highly significant, and none of the autocorrelation structures tested significantly improved the model (Table 1). All the treatments differed significantly for macrophyte biomass in w10, C being the highest and

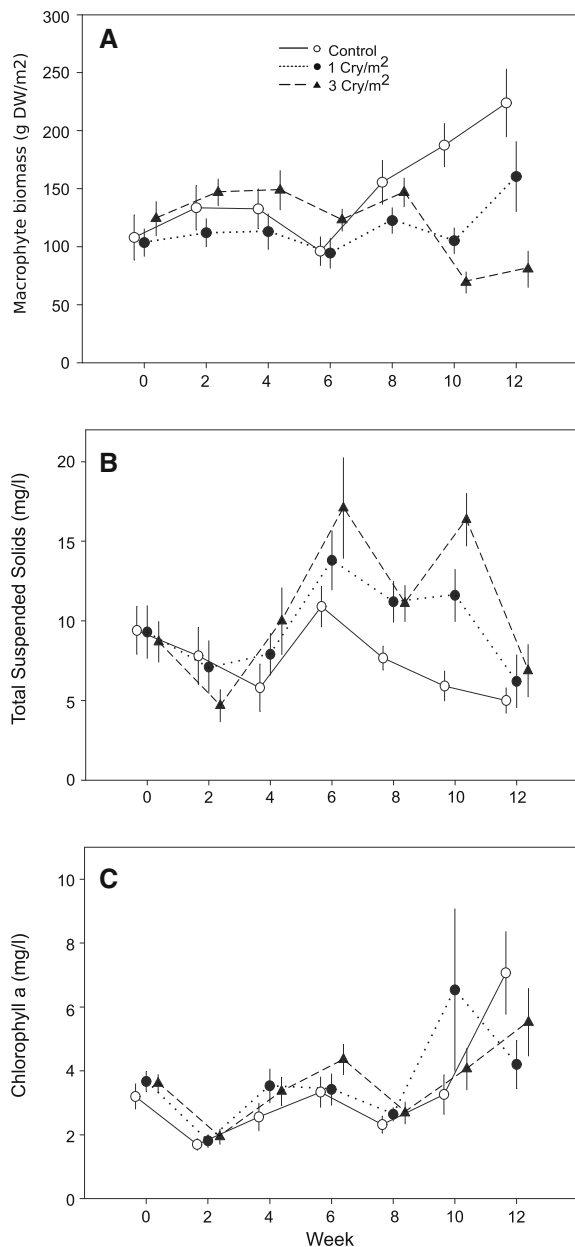


Fig. 1 **A** Macrophyte biomass plot; **B** total suspended solids plot; **C** chlorophyll *a* concentration plot. *Solid lines* (control), *dotted lines* (1 crayfish/m²) and *dashed lines* (3 crayfish/m²) show the trend of the measurement. *Symbols* correspond to mean values and *bars* to standard error. *Abscissa axis* shows the sampling weeks from the beginning (w0) until the end of the experiment (w12)

D3 the lowest biomass (Table 2). In w12 D3 tanks had significantly less biomass than D1 and C tanks (Table 2).

Table 1 Mixed model results for fixed factors, Treatment and Week and their interaction analysing their effects on macrophyte biomass (Macrophytes), totals suspended solids (TSS) and chlorophyll *a* concentration (Chl *a*)

	Df num	Df den	<i>F</i>	<i>P</i>
Macrophytes				
Treat	2	27	20.95	<0.001
Week	6	162	4.13	<0.001
Treat × Week	12	162	10.43	<0.001
TSS				
Treat	2	27	6.5	0.005
Week	6	161	13.39	<0.001
Treat × Week	12	161	3.68	<0.001
Chl <i>a</i>				
Treat	2	27	0.62	0.45
Week	6	162	33.83	<0.001
Treat × Week	12	162	0.51	0.9

There was a lower abundance of *C. aspera*, the most abundant species, and a greater development of filamentous algae and more uncovered sediments in D3 tanks than in other treatment tanks at the end of the experiment (Fig. 2). The mean cover percentage for *C. aspera* was 50% in D3 at w12, but it reached 89 and 92% in D1 and C, respectively (Fig. 2A2). On the other hand, filamentous algae attained a mean cover of 84% of the tanks' surface area in D3 mesocosms, 45% in D1 tanks and less than a quarter (23%) in C tanks (Fig. 2A1). The total surface area of bare sediment increased in D3 mesocosms. Neither *C. truncata* nor *Z. pedunculata* abundances differed significantly among treatments. Permutation test on the PRC diagram showed a highly significant ordination (Fig. 2; RDA1 $F_1 = 70.68$, $P = 0.005$). Overall, Treatment variable explained 11.13% of the total variance and Date explained 61.44%, with the remaining 27.1% of non-explained variance. Partial permutation by date test showed a significant effect of Treatment variable from early June (w8 $F_2 = 3.23$, $P = 0.01$, 19.3% variance explained; w10 $F_2 = 8.66$, $P = 0.005$, 39.1% variance explained; w12 $F_2 = 8.10$, $P = 0.005$, 37.5% variance explained). No effect was detected at previous dates.

Experimental D3 and D1 tanks were more turbid than C tanks (Fig. 1). Treatment and Date factors interaction was highly significant (Table 1), and it was significantly

Table 2 Post-hoc test comparing Treatment levels by means of Welch's test for macrophyte biomass, total suspended solids (TSS) and macro-invertebrate taxonomic richness

		Macrophytes			TSS			Macro-invertebrates			
		<i>t</i>	df	<i>P</i>	<i>t</i>	df	<i>P</i>	<i>t</i>	df	<i>P</i>	
Significant <i>P</i> value was fixed at $\alpha = 0.05$ but corrected with the False Discovery Rate yielding a α -corrected values of 0.038 for macrophytes and macro-invertebrates variables and 0.045 for total suspended solids variable. C, D1 and D3 denoted control, 1 and 3 crayfish/m ² treatments, respectively. W0, w2, w4, w6, w8, w10 and w12 denoted the week where samples were taken in 2011, the 15th, 17th, 19th, 21st, 23rd, 25th and 27th weeks, respectively. According to corrected α values, the significant comparisons are emboldened in the table	W0	C vs D1	0.21	14.92	0.84	0.04	17.83	0.76	0	16.02	1
	W0	C vs D3	0.41	17.66	0.69	0.35	17.54	0.72	-2.44	17.83	0.03
	W0	D1 vs D3	0.28	16.57	0.78	0.29	16.90	0.77	-2.88	16.84	0.01
	W2	C vs D1	0.97	15.13	0.35	0.29	17.90	0.77	-	-	-
	W2	C vs D3	0.44	15.94	0.67	1.51	14.22	0.15	-	-	-
	W2	D1 vs D3	-0.66	17.86	0.52	1.24	14.90	0.24	-	-	-
	W4	C vs D	0.87	17.79	0.4	-1.06	17.71	0.30	0.14	14.51	0.89
	W4	C vs D3	0.27	17.69	0.79	-1.64	16.24	0.12	-0.13	16.78	0.90
	W4	D1 vs D3	-0.52	17.03	0.61	-0.85	15.09	0.41	-0.34	16.91	0.74
	W6	C vs D1	0.12	17.96	0.90	-1.29	15.91	0.21	-	-	-
	W6	C vs D3	-0.0001	17.70	1	-1.82	11.82	0.09	-	-	-
	W6	D1 vs D3	-0.13	17.46	0.9	-0.90	14.53	0.38	-	-	-
W8	C vs D1	1.81	14.54	0.09	-1.94	15.62	0.07	1.70	17.32	0.11	
W8	C vs D3	1.76	17.43	0.1	-2.40	15.64	0.03	2.14	17.99	0.05	
W8	D1 vs D3	0.19	16.16	0.85	-0.38	18	0.7	0.19	17.15	0.85	
W10	C vs D1	3.60	14.76	0.002	-3.62	12.02	0.004	-	-	-	
W10	C vs D3	6.83	13.98	<0.001	-5.63	11.51	<0.001	-	-	-	
W10	D1 vs D3	4.42	17.85	<0.001	-1.85	17.83	0.08	-	-	-	
W12	C vs D1	1.52	17.98	0.14	-0.65	12.97	0.53	1.82	15.38	0.09	
W12	C vs D3	5.15	14.97	<0.001	-1.04	11.71	0.32	3.36	14.2	0.004	
W12	D1 vs D3	3.21	14.70	0.006	-0.3	16.97	0.77	1.99	17.72	0.06	

improved with a general correlation structure (Log-Likelihood ratio = 43.683, $P = 0.003$). Pairwise comparisons showed the as significant difference between D3 and C in w8, and between C and D1 and D3 in w10; TSS mean value was always the lowest in C treatment (Table 2).

The model, improved with general correlation structure (log-likelihood ratio = 60.044, $P = <0.0001$), yielded non-significant as the interaction of Treatment and Date factors for chlorophyll *a* concentration. Treatment factor was not significant (Table 1; Fig. 1).

Micro-invertebrate and macro-invertebrate

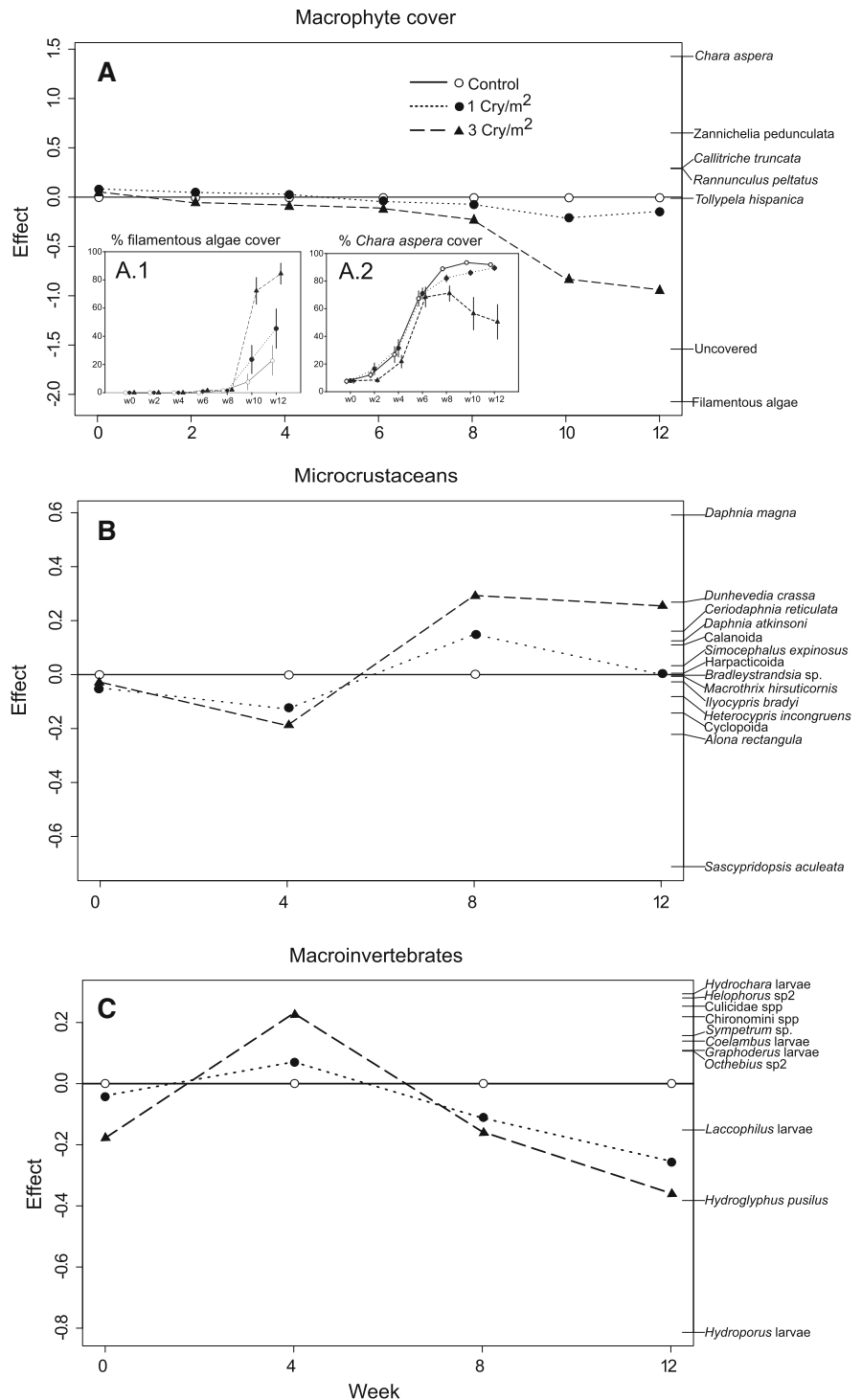
We did not find any evidence of crayfish impact on the micro-invertebrate assemblage. PRC diagram (Fig. 2) shows that the deviations of D1 and D3 from C values were not sufficiently wide to conclude that a negative impact occurred, and the ordination was not significant ($F_1 = 4.01$, $P = 0.27$). In addition, the analysis of taxonomic richness also showed non-significant results for the interaction Treatment \times Date, or for Treatment

factor itself (Fig. 3; Treatment: $\chi^2 = 0.076$ df = 1 $P = 0.9$. Date: $\chi^2 = 31.76$ df = 3 $P = <0.0001$. Treatment \times Date: $\chi^2 = 1.61$ df = 6 $P = 0.9$).

Crayfish introduction had a negative impact on macro-invertebrate taxonomic richness and from w8, both D3 and D1 treatments had less richness than C (Fig. 3; Treatment: $\chi^2 = 3.52$ df = 1 $P = 0.2$. Date: $\chi^2 = 25.82$ df = 3 $P = <0.0001$. Treatment \times Date: $\chi^2 = 12.84$ df = 6 $P = 0.045$). Pairwise comparisons showed that taxonomic richness was the highest in D3 tanks at the beginning of the experiment, and at the end of the experiment (w12), D3 treatment mesocosms had significantly fewer taxa (28% less) than C (see Table 2). Although the differences between D1 and C tanks were not significant, there were 22% fewer taxa on average in D1 mesocosms than in C in w12.

Crayfish had also a negative effect on the macro-invertebrate community, and PRC plot (Fig. 2) showed a differentiation in D3 and D1 trends compared with C. Although the permutation test did not show a significant result for PRC ordination ($F_1 = 3.37$ $P = 0.21$), the analysis of the Bray-Curtis

Fig. 2 **A** Macrophyte cover percentage (logit transformed) Principal Response Curve (PRC) plot. Uncovered indicates bare sediment. **A.1** Cover percentage trend of the filamentous algae throughout the experiment in each treatment. **A.2** *Chara aspera* cover percentage in each treatment throughout the experiment. **B** Microcrustacean abundance (Hellinger transformed) PRC plot. **C** Macroinvertebrate abundance (Hellinger transformed) PRC plot. Only the taxa with an effect greater than ± 0.1 are plotted. *Solid lines* (control), *dotted lines* (1 crayfish/m²) and *dashed lines* (3 crayfishes/m²) show the trend of the measurement. *Abscissa axis* shows the sampling weeks from the beginning (w0) until the end of the experiment (w12)



distances between treatments at w0 and w12 showed a significant effect of Treatment factor at the end of the experiment (w0 pseudo $F_2 = 0.65$, $P = 0.8$; w12

pseudo $F_2 = 1.72$, $P = 0.03$). Only the pairwise difference between C and D3 was significant at w12 (C vs D1 pseudo $F_1 = 1.20$, $P = 0.3$; C vs D3 pseudo

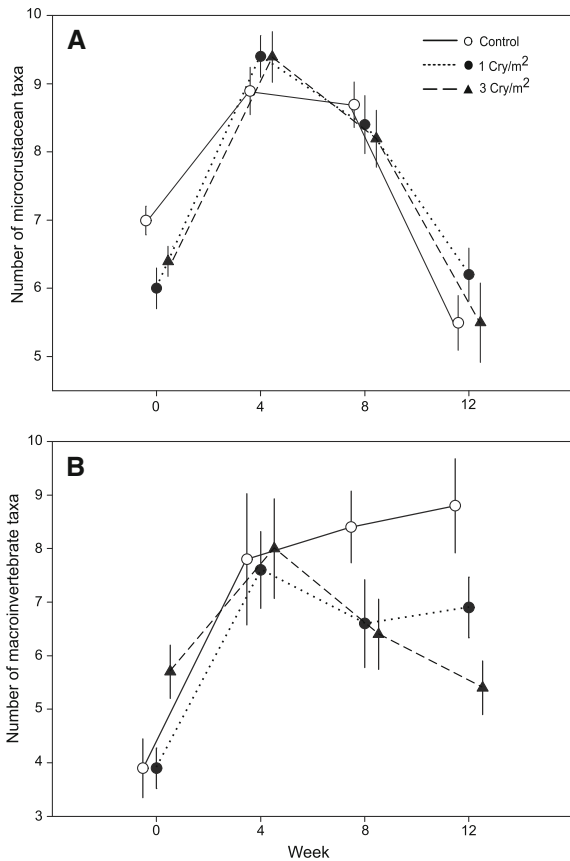


Fig. 3 **A** Micro-crustacean assemblage taxonomic richness plot. **B** Macro-invertebrate assembly taxonomic richness graph. Solid lines (control), dotted lines (1 crayfish/m²) and dashed lines (3 crayfish/m²) show the trend of the measurement. Symbols correspond to mean values and bars to standard error. Abscissa axis shows the sampling weeks from the beginning (w0) until the end of the experiment (w12)

$F_1 = 3.15$, $P = 0.001$; D1 vs D3 pseudo $F_1 = 0.79$, $P = 0.6$).

Macro-invertebrate community trophic diversity also showed a significant shift after 12 weeks of crayfish presence (Fig. 4; RDA1 $F_1 = 14.38$ $P = 0.005$, Treatment factor explained 20.1% of the variance and Date only 2.9% of variance). In the by date RDA analysis, Treatment factor was always significant. The abundance of predatory invertebrates was favoured by crayfish presence (Fig. 4). Nevertheless, by the end of the experiment, this functional group showed a reduction in horizontal diversity and species richness within the predator's trophic guild. This species loss was more intense in D3 mesocosms (Fig. 4). Active filtrators, detritivore, grazers and

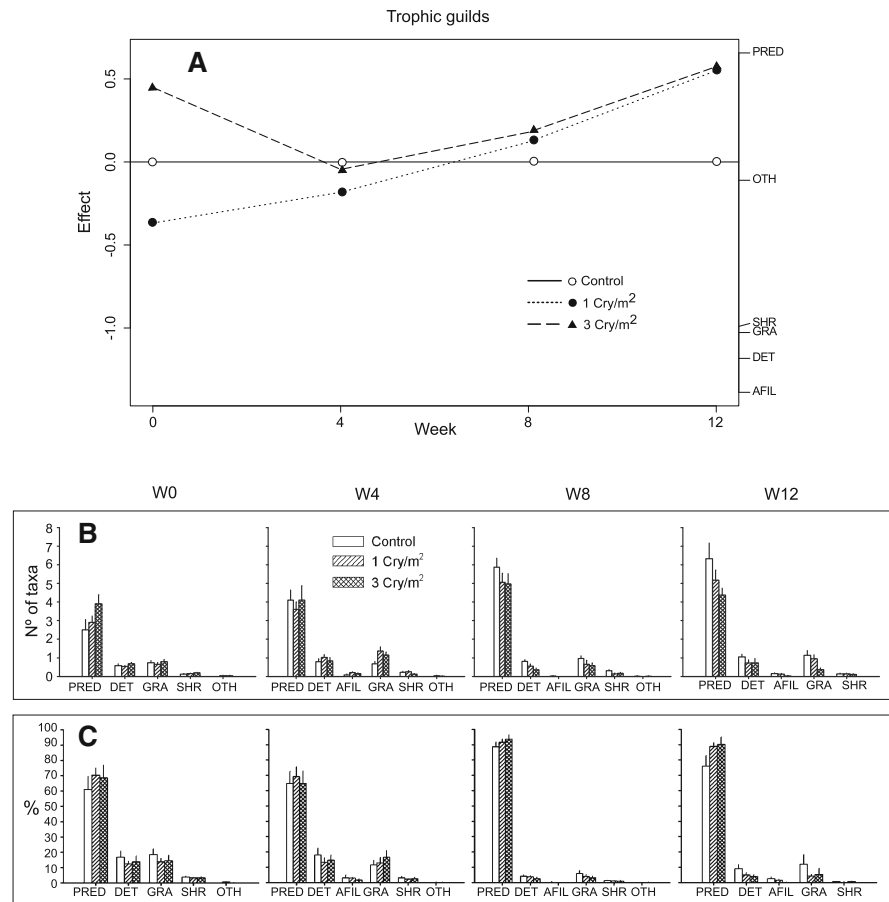
shredders were severely impacted by the crayfish, both in abundance terms and in horizontal diversity (Fig. 4).

Discussion

Conspicuous macrophyte reduction was attained rapidly, only 10 weeks after the introduction of the crayfish, in D3 and D1 tanks. This reduction would have certainly been even stronger with the introduction of adult crayfish in the tanks, because juveniles are known to have a more carnivorous diet than adults (Correia, 2002; Alcorlo et al., 2004; Bondar et al., 2005). Furthermore, in a natural pond, a more intensive impact on macrophytes is to be expected if adults colonized it just after flooding, as macrophyte shoots are more prone to crayfish consumption (Lodge, 1991; Anastácio et al., 2005). On the other hand, although temporary pond ecosystems are quite well mimicked in mesocosms (Blaustein & Schwartz, 2001), macrophyte reduction might be overestimated because of a confinement effect. It is worth noting that other works using mesocosms, and showing a very high crayfish impact on macrophyte density, used higher crayfish densities (i.e. 8 ind/m²) than we did (Lodge et al., 1994; Gherardi & Acquistapace, 2007), and even with just one single individual, reduction was also appreciable. Moreover, in HD tanks, we cannot exclude possible antagonistic behaviour that could reduce the activity of any of the crayfish in the tank, somehow reducing the impact. Nevertheless, Pintor et al. (2009) showed that at high densities, *Pacifastacus leniusculus* increased the number of antagonistic interactions, but did not reduce its foraging activity. The strong reduction of *Chara aspera*, the most abundant macrophyte, in D3 tanks from w10 (Fig. 2), was in agreement with the experimental evidence of Cronin (1998), showing that *P. clarkii* prefers plants highly branched and with a filamentous morphology, easier to handle, than thick broad leaf plants for consumption.

Turbidity increased in crayfish tanks from the 6th week on (Fig. 1), except in July when the crayfish's activity probably decreased due to warming. Turbidity increase was facilitated not only by crayfish food-seeking and locomotive activity re-suspending sediments into the water column (Angeler et al., 2001), but also by the increase of bare sediment in the

Fig. 4 **A** Trophic guilds assemblage PRC plot. Trophic groups codes are: PRED Predators, OTH Others (omnivores, cannot be classified into other categories), SHR Shredders, GRA Grazers, DET Detritivores, AFIL Active Filtrators. *Solid lines* (control), *dotted lines* (1 crayfish/m²) and *dashed lines* (3 crayfish/m²) show the trend of the measurement. *Abscissa axis* shows the sampling weeks from the beginning (w0) until the end of the experiment (w12). **B** The taxonomic richness sorted by trophic groups for each sampling week. **C** The relative abundance of each trophic group for each sampling week in percentage. *Bars* show mean values and *error bars* standard error



crayfish tanks. In parallel, an increase in phytoplankton concentration was also to be expected due to crayfish activity (Rodríguez et al., 2003). Sediment re-suspension releases nutrients into the water column, increasing their availability for phytoplankton (Schaffer, 1998). Nevertheless, chlorophyll *a* concentration did not differ between treatments (see Table 1 and Fig. 1). In temporary ponds, when macrophytes decay by the end of the flooding period, nitrogen and phosphorus availability increases and phytoplankton density peaks (Naselli-Flores & Barone, 2012). Crayfish activity enhanced this process, but facilitated the growth of filamentous algae rather than phytoplankton. Furthermore, according to Rober et al. (2011), filamentous algae development is also enhanced by invertebrate grazer reduction. A similar reduction occurred in our experiment (Fig. 4), but in contrast, grazers were still abundant because *H. meridionalis* tadpoles were not numerically affected by the presence of the red swamp crayfish, even in the

D3 treatment (for further information, see Rodríguez-Pérez et al., 2014).

Crayfish did not affect micro-crustacean assemblage composition (Fig. 2) nor the richness (Fig. 3). On the other hand, the observed macro-invertebrate richness reduction was similar to the reduction found at Ventura River, with twice the crayfish density as in D3 tanks (Klose & Cooper, 2012). Our results agreed with published studies showing that crayfish in general, and the red swamp crayfish in particular, preferentially prey on slow moving large prey, grazers, shredders and detritivores being the most strongly affected guilds (Lodge et al., 1994; McCarthy et al., 2006; Correia & Anastácio, 2008; Klose & Cooper, 2012; Jackson et al., 2014). Probably, both direct predation and structural simplification of the habitat reduced the invertebrate community (Nyström et al., 1996; Srivastava, 2006; Jackson et al., 2014).

The impact of red swamp crayfish on temporary pond ecosystem functioning was diverse and complex

and involved different levels. Crayfish greatly simplified the habitat structure in the pond by the depletion of macrophytes. The subsequent increase of filamentous algae (for review of the role of macrophytes, see Jeppesen et al., 1998) promoted a shift in primary producers, and probably a change in primary productivity also occurred. Furthermore, the crayfish impacted macro-invertebrate diversity, thus modifying structurally and functionally the food web. This diversity loss was not so intense as to affect vertical diversity, but a significant reduction in horizontal diversity was observed in crayfish tanks, grazers, predators and detritivores being the most strongly affected groups. Predators seemed to have benefited from crayfish presence (Fig. 4), but only the most abundant taxa (*Hydroporus* larvae and *Hydroglyphus pusillus*, both small-sized coleoptera) increased, while other predators declined or disappeared in crayfish mesocosms (see Fig. 4), leading to a shift in predator guild composition and functionality. Richness and abundance of grazer and detritivore invertebrates declined (Fig. 4), but herbivory was more intense in crayfish mesocosms. It is also likely according to crayfish trophic studies (Correia, 2002; Alcorlo et al., 2004; Geiger et al., 2005; Jackson et al., 2014) that detritivory increased too, in contradiction with the evidence presented by Cardinale et al. (2006). We presume that all these food web modifications greatly altered the flux of energy and the stock of matter.

In the temporary pond ecosystem, dry and wet phases are linked by the organic matter left by one community to the following community occurring in the alternative phase, mutually feeding each other (Mozley, 1944; Williams, 2005). The mineralization of the organic matter by the microbial community makes it available to primary producers (Naselli-Flores & Barone, 2012). However, the red swamp crayfish strongly alters the decomposition dynamics. A significant part of the organic matter will no longer be available for the following phase because it is consumed by the crayfish and exported if the crayfish escape from the pond during the dry season, or sequestered in the case of the crayfish retreating within their galleries to survive the drought period.

Temporary ponds contribute largely to increase regional richness in diversity inventories, because they harbour species exclusively occurring in such a system (Boix et al., 2001; Waterkeyn et al., 2008; Díaz-Paniagua et al., 2010; Rhazi et al., 2012). In the case of

macro-invertebrates, abundance and richness are positively correlated to macrophyte beds in ponds (Della Bella et al., 2005). Our results show both a severe depletion of macrophyte abundance in crayfish tanks, and a marked reduction of macro-invertebrate richness caused by crayfish colonization, at rate of nearly 30% in D3 tanks and 20% for D1 tanks (Fig. 3B), pointing out a major conservation issue for real temporary ponds.

In conclusion, the colonization by the exotic red swamp crayfish can alter strongly the ecosystem structure and subsequently the ecosystem functioning of temporary ponds. Such a shift is density-dependent. Further work is needed to understand to what extent, the intensity of the perturbation depends on the time at what the crayfish colonization happens throughout the pond's wet period. In addition, we think that testing the resilience of the ecosystem after the introduction of the crayfish in the following wet phase would provide useful insights for the development of pond conservation programs, including the limitation, or the total eradication, whenever possible, of crayfish populations in invaded ponds.

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