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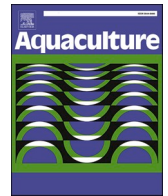
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# Integrating *Gammarus insensibilis* in biofloc systems: A sustainable approach to nutrient enrichment and waste valorisation in aquaculture

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## ABSTRACT

This research addresses the cultivation of *Gammarus insensibilis* in biofloc systems. The aim of the work was to valorise and to bioremediate aquaculture effluents while enhancing the nutritional value of bioflocs through the introduction of amphipods, which can be grown in close aquaculture systems. Two experimental diets (aquaculture waste detritus and commercial fish feed) were tested for amphipods in triplicated biofloc systems, against three control tanks without biofloc. The experiment was conducted over a six-week period at a temperature of 20 °C in the absence of light. The water quality, the survival, lipid profile and fatty acid composition of the amphipods and the bioflocs produced were studied. Significant differences were observed in the water quality between the treatment groups and the control, since nitrifying bacteria were present in the bioflocs formed in the treatment tanks. Indeed, 100 % mortality was reached in the control tanks from the second week of the experiment. The fish pellets diet and the pellet-based biofloc resulted in a significantly higher total lipid content than the detritus diet and biofloc. However, no differences were found in the essential long-chain polyunsaturated fatty acids (LC-PUFAs) content (ARA, EPA and DHA) between the commercial pellets and the detritus-based biofloc, thus justifying the enrichment of the biofloc with amphipods. Indeed, the wild amphipods displayed higher levels of saturated fatty acids and omega-6 PUFAs compared to the experimental groups, which demonstrated higher omega-3 PUFAs content, particularly DHA. The study highlights the nutritional advantages of pellet-based diets for amphipods, including improved survival and lipid content, while also emphasising the enhanced nutritional profile of the enriched detritus-based biofloc. We suggest that the integration of aquaculture detritus with supplemental aquafeed in BFT systems will support both the biofloculation process and the nutritional needs of the amphipods, thereby creating a sustainable and efficient cycle of waste valorisation and live food production. Further research is required in order to study the complete life cycle of amphipods and their reproductive capacity in these systems. A lower water temperature and the inoculation of microalgae should be considered in order to achieve higher survival rates and PUFAs content.

## 1. Introduction

Biofloc technology (BFT) was originated in the 1970s (Emerenciano et al., 2021) and has been widely adopted due to its environmental and economic benefits (Zimmermann et al., 2023). This technology is based on zero-water exchange systems with the formation of bioflocs in the culture medium where uneaten food, excess inorganic nutrients and faeces aggregate along with microorganisms (bacteria, microalgae such as diatoms, protozoa...) (Khanjani et al., 2024b). This aggregation is enabled by a matrix of extracellular polymeric substances secreted by

the microorganisms (Hargreaves, 2013). By maintaining a high carbon/nitrogen ratio (Hargreaves, 2013) through the addition of a carbon source (molasses, glycerol, flours; Zhao et al. (2016) to the culture medium, the colonies of chemoautotrophic bacteria present in the bioflocs assimilate the ammonium excreted by the cultured species during the nitrification process, transforming it into nitrate (Khanjani et al., 2022). Heterotrophic bacteria can also directly assimilate the ammonium into bacterial biomass. Additionally, microalgae contribute to nitrogen absorption during photosynthesis (Ebeling et al., 2006). BFT systems have been identified as a promising technology for sustainable

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food production, promoting low-trophic-level species cultivation, polyculture, and the development of a circular economy model (Khanjani et al., 2024c). The feeding efficiency and waste management are enhanced in these systems, as they have the capacity to increase nutrient utilisation and minimize environmental impact, achieved through reduced water and fertilizer use (Knowler et al., 2020; Zimmermann et al., 2023). Thus, the need for water exchange is minimized, which in turn reduces pumping costs, prevents fluctuations in temperature and reduces the energy requirement for heating (McCusker et al., 2023). In BFT systems, food requirements are also reduced, and an increase in the survival and growth rates of cultivated species has been observed (Khanjani and Sharifinia, 2024). Bioflocs present a favourable nutritional profile (Crab et al., 2012) and their consumption have been demonstrated to enhance growth and fortify the immune system of cultivated species by increasing the activity of their digestive enzymes, resulting in an increase in the feed conversion rate (Xu and Pan, 2013). BFT is primarily employed in the cultivation of penaeids (Crab et al., 2010b) but numerous studies have applied this technology to the cultivation of filter feeders, detritivores and even some herbivorous or omnivorous finfish, which are able to feed directly on particulate organic matter (Crab et al., 2012; Das and Mandal, 2021; Dinda et al., 2020). These species are tolerant to changes in the concentrations of dissolved oxygen in the water and nitrogenous compounds and can withstand high culture densities and high concentrations of suspended solids (El-Sayed, 2021; Emerenciano et al., 2021).

Despite all these advantages, bioflocs have been observed to present low proportions of long-chain polyunsaturated fatty acids (LC-PUFAs) (Crab et al., 2010a), maybe linked with the absence of PUFAs in bacteria (Zhukova and Kharlamenko, 1999), and some essential amino acids (EAA) such as methionine (Abbaszadeh et al., 2022). In this sense, several studies have demonstrated that the incorporation of live food, such as diatoms, rotifers, *Artemia* or copepods, into BFT can enhance the nutritional value of the bioflocs, as well as the growth and survival rates of the cultivated species (Abbaszadeh et al., 2022; Brito et al., 2016; de Andrade et al., 2021). Biofloc enrichment with amphipods has been addressed in only one previous study (Promthale et al., 2021). These authors reported a high survival rate of amphipods in the BFT conditions and a high potential for the enriched bioflocs to replace fishmeal. Amphipods cultivation interest has increased in the last years due to their high-quality nutritional profile (Baeza-Rojano et al., 2014; Guerra-García et al., 2016; Jiménez-Prada et al., 2018) adequate for feeding high market value fishes or cephalopods (Baeza-Rojano et al., 2010; Baeza-Rojano et al., 2013; Suontama et al., 2007). Indeed, the Spanish Institute of Oceanography (IEO) holds a patent for the methodology of cultivation of the common octopus paralarvae (Tur et al., 2020). This patent delineates particular zootechnical conditions and the utilisation of amphipods as an alternative live prey to crustacean zoeae, and is currently being utilised by the company “Nueva Pescanova” for the cultivation of *Octopus vulgaris*. However, the upscaling of the systems requires increasing amounts of live food, being a bottleneck for the development of these species aquaculture (García-Fernández, 2022). The marine amphipod *Gammarus insensibilis* Stock, 1966 has been highlighted as a promising species for being intensively cultured due to its adequate nutritional profile, large body size and high natural densities (Jiménez-Prada et al., 2020). Moreover, as a detritivorous species, *G. insensibilis* can feed on aquaculture sludge and wastes (Castilla-Gavilán et al., 2023).

With this background, we suggest that *G. insensibilis* has the potential for utilisation as a live food source and could be cultivated intensively in BFT systems, thereby enriching bioflocs nutritional value. Moreover, their ability to feed on detritus would permit the valorisation of aquaculture wastes and the bioremediation of the effluents (Castilla-Gavilán et al., 2023). In the present study, *G. insensibilis*, fed with commercial aquafeed or detritus from aquaculture effluents, has been cultivated in BFT systems. The water quality, the survival rate and the nutritional profile of the amphipods as well as the biofloc produced were analysed.

## 2. Materials and methods

Specimens of *G. insensibilis* were sampled in March 2023 from a natural marsh pond at the IFAPA Centre “El Toruño” (El Puerto de Santa María, Cadiz Bay, Spain). Amphipods on their original substrate (*Ulva* sp. thalli, where they mainly inhabit) were transported to IFAPA facilities and transferred into a 400 L tank to allow their acclimation during a week prior experimentation. This tank was connected to a recirculation system (RAS) equipped with a cooling, mechanical filter, protein skimmer, ultraviolet lights and biofilter (Castilla-Gavilán et al., 2023). They were illuminated ( $1.5\text{--}1.7 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) with fluorescent tubes in a 8:16 h (light:darkness) to acclimate amphipods to the darkness, according to Promthale et al. (2021). The seawater was maintained at the same conditions than in the natural environment,  $17^\circ\text{C}$  and  $38 \text{ g L}^{-1}$  salinity, and was renewed daily in continuous water flow.

Prior to the experiment, 2700 adults of *G. insensibilis* were sampled from the tank, devoid of algae and placed in 9 culture units (300 specimens per culture unit) filled with 25 L of aerated and mixed seawater. Three hundred additional “wild” specimens were sampled and stored at  $-80^\circ\text{C}$  for lipid profile analysis and comparison with the specimens used in the experiment. The culture units were maintained in darkness without any water exchange, following Promthale et al. (2021). An artificial substrate for amphipods was provided in all culture units (plastic mesh, following Castilla-Gavilán et al., 2023). Amphipods were starved for 24 h to standardize their nutritional conditions. Two experimental treatments were tested against a control, at  $20^\circ\text{C}$  (Promthale et al., 2021), in triplicate culture units. In the first treatment, *G. insensibilis* specimens were fed with grounded sea bream commercial pellets ( $0.2 \text{ g day}^{-1}$ ). In the second treatment, the feed was waste detritus ( $1.2 \text{ g day}^{-1}$ ) obtained through the cleaning of the filters of a Senegalese sole RAS unit and consisted primarily of fish faeces and uneaten fish feed pellets. The quantity of food was determined by considering the organic matter percentage of each type, with detritus containing six times less organic matter than pellets. Three samples of each food type were also stored at  $-80^\circ\text{C}$  for lipid profile analyses.

Between three and four times a week (20 sampling days in total), three water samples were taken from the tanks and filtered on GF/C fiberglass filters that were treated following Castilla-Gavilán et al. (2023), in order to determine total suspended solids in the water and the organic matter content of these solids. Water was then analysed: dissolved nitrate and nitrite concentrations were sequentially quantified by the reduction of  $\text{NO}_3^-$  to  $\text{NO}_2^-$  with vanadium ( $\text{VCl}_3$ ) following the method of García-Robledo et al. (2014). Ammonium concentration was determined by the indophenol-blue method (Aminot et al., 1997), and carbon concentration was then adjusted to a 16:1C:N ratio through the addition of corn flour (Promthale et al., 2021). The control treatment was also fed with detritus, and water was also sampled daily for monitoring purposes and ammonium concentration analyses, but no carbon source was added in order to avoid the bio-flocculation process. Water parameters (mean dissolved oxygen, temperature, salinity and pH) were checked daily in all tanks and were found to be  $6.1 \pm 0.04 \text{ mg L}^{-1}$  DO,  $21.1 \pm 0.33^\circ\text{C}$ ,  $40.1 \pm 4.14 \text{ g L}^{-1}$  salinity (typical of marsh environments), and  $8.1 \pm 0.09 \text{ pH}$ . The experiment lasted six weeks except for the control tanks, since 100 % mortality was observed from the second week. At the end of the experiment, surviving amphipods and the sludge (bioflocs) produced in the 6 experimental culture units were sampled, amphipods were counted and all samples were stored at  $-80^\circ\text{C}$  prior to biochemical analysis.

All the freeze samples were freeze-dried and grounded in to a fine powder. Ashes, moisture, total lipids and fatty acids profile were analysed. Total lipids (TL) and fatty acids (FA) profile methods were those previously described by Hachero-Cruzado et al. (2014). Briefly, TL were extracted from powder following the methodology of Folch et al. (1957) with slight modification by Christie and Han (2012). To extract FA, total lipid extracts were subjected to acid-catalysed transmethylation. Fatty

acids methyl esters (FAME) were separated and quantified by gas chromatography and the identified FA were compared to the authentic standards (FAME Mix C37) and well-characterized fish oil (Menhaden Oil, SUPELCO, USA) [Christie and Han \(2012\)](#).

Mean and standard deviation (SD) were calculated for all data. Statistical analyses and plots were performed using RStudio software. Survival was analysed through a one-way ANOVA test between amphipods in both treatments and the control. Differences in the inorganic nitrogen concentration of the different treatments were tested with two-way ANOVAs following an experimental design with orthogonal factors: *treatment*, a fixed factor with three levels (control vs pellets vs detritus) and *time*, a fixed factor with twenty levels (sampling days). Differences in TL between amphipods in the different treatments and between produced bioflocs and diets were also studied through one-way ANOVAs. Where ANOVAs indicated significant differences ( $p < 0.05$ ), the source of the differences was identified using a Tukey test. Prior to ANOVAs, the normality was tested with Shapiro-Wilk test ( $p > 0.05$ ) and the homogeneity of variances was tested with Levene test ( $p > 0.05$ ). If variances remained heterogeneous even after data transformation, untransformed data were still analysed, as ANOVA is a robust statistical test and is relatively unaffected by the heterogeneity of variances ([Underwood, 1997](#)). In such cases, the level of significance was reduced to  $<0.01$  to avoid type I error. Additionally, principal component analyses (PCA) were conducted to FA matrixes for the ordination of the samples.

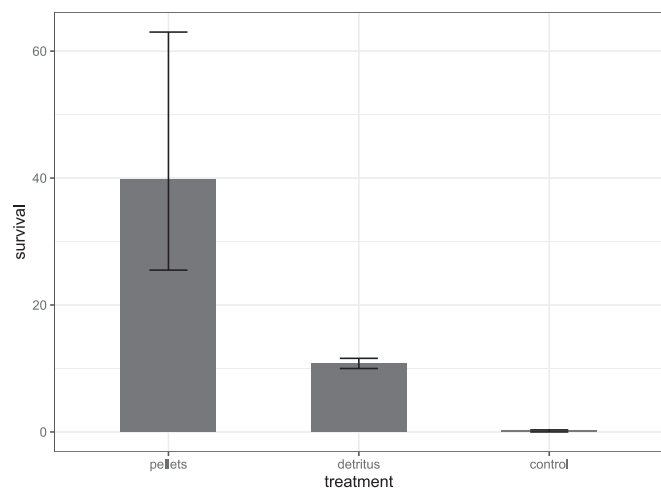
### 3. Results

#### 3.1. Survival rate

Survival was significantly higher in the tanks fed with pellets than in those fed with detritus (one-way ANOVA;  $df = 2$ ;  $SS = 0.28$ ;  $MS = 0.14$ ;  $F$  value = 7.95;  $p$  value = 0.0206; [Fig. 1](#)). No amphipods were observed in the control tanks from the ninth day.

#### 3.2. Water quality

Regarding inorganic nitrogen concentrations, statistics are summarized in the [Table 1](#). Ammonium ( $N-NH_4^+$ ) levels were significantly higher in the control tanks than in the other treatments ([Fig. 2](#)), with no differences observed between the latter ([Table 1](#)). Nitrite ( $N-NO_2^-$ ) and nitrate ( $N-NO_3^-$ ) concentrations were in general low ([Fig. 3](#)), and no differences were found between tanks fed with pellets or detritus ([Table 1](#)).



**Fig. 1.** Mean amphipods survival rates (%) in the different treatments. Bars represent the maximum and minimum survival rates.

**Table 1**

Results of the two-way ANOVAs for ammonium, nitrite and nitrate concentrations.

Source of variation	df	SS	MS	F value	P value
<b>Ammonium</b>					
Treatment (T)	2	11.48	5.74	15.33	0.000
Time (t)	19	18.40	0.97	2.59	0.002
T: t	21	1.80	0.09	0.23	0.999
Residuals	86	32.20	0.37		
Levene Test	42			4.10	0.343
Tukey Test				control > detritus	0.000
				control > pellets	0.000
				detritus = pellets	0.997
<b>Nitrite</b>					
Treatment	1	0.00	0.00	0.06	0.805
time	4	0.00	0.00	5.07	0.006
T: t	4	0.00	0.00	1.04	0.410
Residuals	20	0.00	0.00		
Levene Test	9			1.58	0.189
<b>Nitrate</b>					
Treatment	1	0.01	0.01	3.12	0.093
time	4	0.14	0.04	9.73	0.000
T: t	4	0.04	0.01	2.38	0.086
Residuals	20	0.07	0.00		
Levene Test	9			1.37	0.266

#### 3.3. Nutritional profile

The [Fig. 4](#) illustrates the organic matter (OM) and the total lipids (TL) content of the samples. Significant differences were observed in TL content between the experimental diets and the bioflocs (one-way ANOVA;  $df = 4$ ,  $SS = 228.68$ ,  $MS = 57.17$ ,  $F$  value = 42.14,  $p$  value  $<0.001$ ). The pellets diet exhibited the significantly highest TL content (Tukey test;  $p$  values  $<0.001$ ) and the biofloc from the pellets treatment presented a significantly higher content than the biofloc from the detritus treatment (Tukey test;  $p$  value = 0.047). Concerning amphipods, no differences were found between them (one-way ANOVA;  $df = 2$ ,  $SS = 14.38$ ,  $MS = 7.19$ ,  $F$  value = 1.65,  $p$  value = 0.268). Differences in the OM content were only found between the diets and the bioflocs (one-way ANOVA;  $df = 3$ ;  $SS = 6447$ ;  $MS = 2148.9$ ;  $F$  value = 210.1;  $p$  value  $<0.001$ ). The pellets presented the highest content (Tukey test;  $p$  values  $<0.001$ ), followed by the pellet-based biofloc, which presented a significantly higher content than the two other treatments (Tukey test;  $p$  values  $<0.001$ ). No differences were found between detritus-based diet and biofloc (Tukey test;  $p$  value = 0.6509).

The fatty acid profile (in percentage) of the diets and bioflocs analysed and the one-way ANOVAs are summarized in the [Table 2](#). The isoC<sub>16</sub> fatty acid, which is characteristic of bacteria ([Tanaka et al., 2014](#)), was analysed among the SFA. While the pellets presented no evidence of this FA, the detritus diet and the different bioflocs did ([Table 2](#)), thereby corroborating the presence of biofloc-forming bacteria. Major FA were the SFA 16:0 (PA), the MUFAs 18:1n7 (cis-VA) and 18:1n9 (OA), the omega-6 PUFA 18:2n6 (LA) and the omega-3 PUFA 22:6n3 (DHA). The pellet-based diet presented the significantly highest level of OA (Tukey test;  $p$  value  $<0.001$ ). However, the pellet-based biofloc did not show the same proportion of OA, and displayed a significantly lower content than the detritus-based biofloc (Tukey test;  $p$  value = 0.008) or no differences with the detritus-based diet (Tukey test;  $p$  value = 0.392). The pellet-based biofloc had the significantly highest level of cis-VA (Tukey test;  $p$  value  $\leq 0.01$ ), while the pellets presented the lower content ([Table 2](#)). These findings support the presence of biofloc-forming bacteria, as cis-VA is a product of many heterotrophic bacterial species ([Gillan and Sandstrom, 1985](#)).

In general, the detritus-based diet presented the significantly highest content of SFA and the lowest MUFAs content. The pellet-based diet exhibited the significantly highest content of omega-3 and omega-6

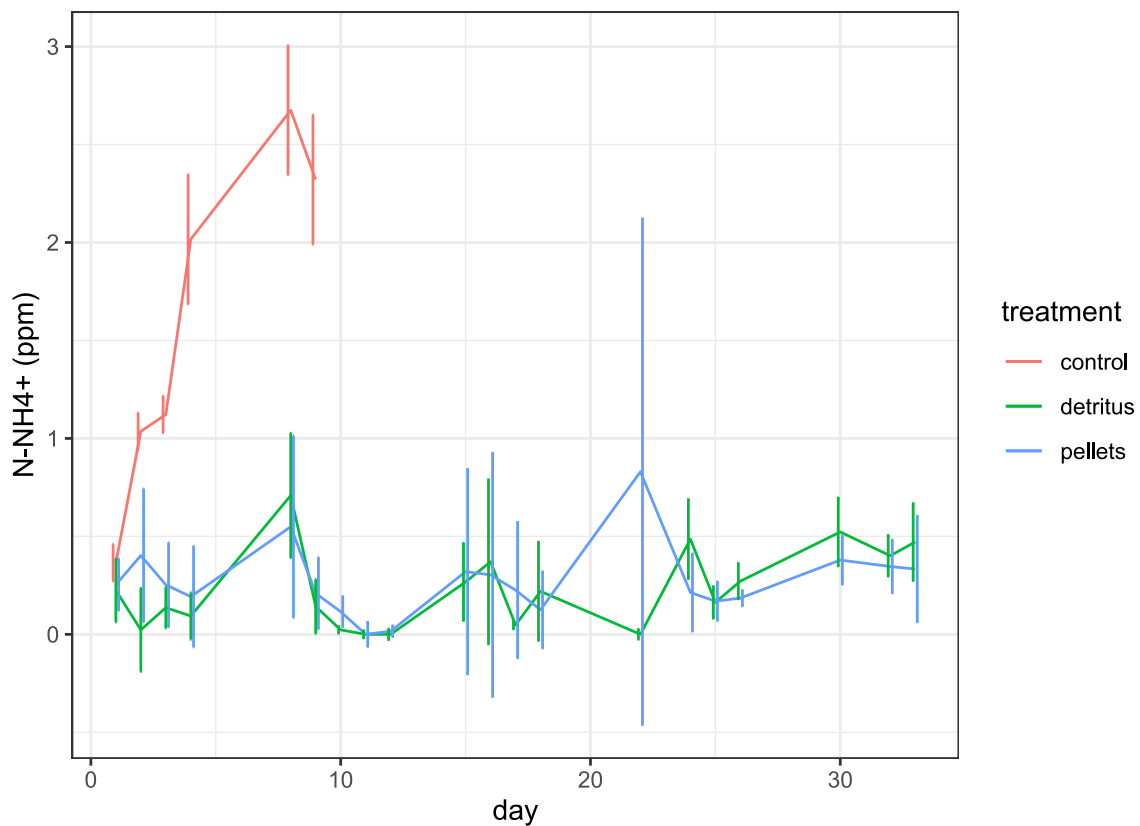


Fig. 2. Ammonium concentration (mean ± SD) in the different culture units during the experiment.

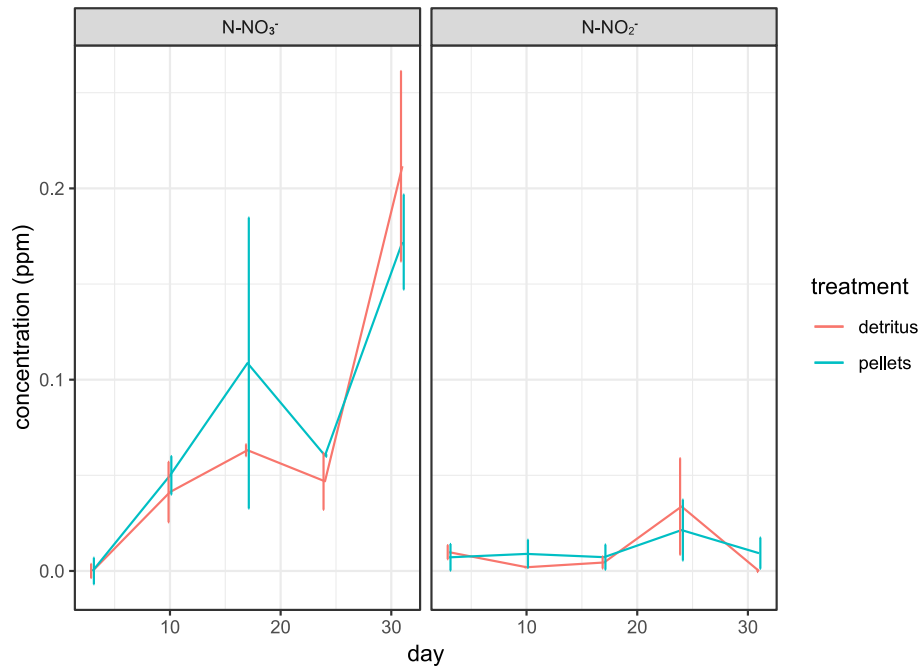


Fig. 3. Nitrate and nitrite concentrations (mean ± SD) in the different culture units during the experiment.

PUFAs (Table 2). However, the biofloc generated in this treatment presented the lowest proportion of PUFAs, showing no significant differences with the detritus-based diet (Tukey test;  $p$  value = 0.990) and a significantly lower content than the detritus-based biofloc (Tukey test;  $p$  value = 0.010).

The results of the PCA based on diets and bioflocs were also in

agreement with the previous results and identified OA, cis-VA, LA and PA as major contributors to the total variance. The first principal component explained 57% of the total variance and the second principal component explained 34.5%. PC1 correlated positively with the fatty acids OA ( $r = 0.950$ ,  $n = 20$ ,  $p < 0.005$ ) and LA ( $r = 0.925$ ,  $n = 20$ ,  $p < 0.005$ ), and negatively with cis-VA ( $r = -0.756$ ,  $n = 20$ ,  $p < 0.005$ ). PC2



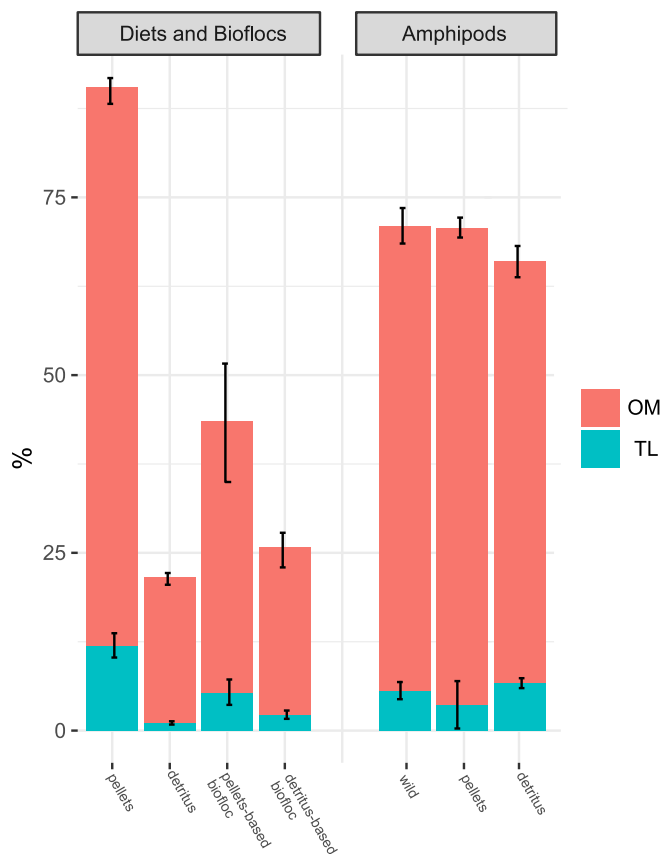


Fig. 4. Organic matter (OM) and total lipids (TL) content (%) in the two diets tested (pellets and detritus), in the bioflocs sampled from the different treatments at the end of the experiment, and in the amphipods in both treatments and those sampled from the wild (mean  $\pm$  SD).

correlated positively with PA ( $r = 0.902$ ,  $n = 20$ ,  $p < 0.005$ ) and negatively with cis-VA ( $r = -0.603$ ,  $n = 20$ ,  $p < 0.005$ ). The axes clearly separate the diets from the bioflocs, and differentiate the bioflocs based on their PA and cis-VA content (Fig. 5).

The fatty acid composition of the wild amphipods was compared with that of the amphipods used in the experiment (fed with pellets or detritus). Table 3 shows results (in %) and one-way ANOVAs. The major fatty acids were: the saturated fatty acids (SFA) 16:0 (palmitic acid; PA) and 18:0 (stearic acid; SA); the monounsaturated fatty acids (MUFAs) 16:1n7 (palmitoleic acid, POA), 18:1n9 (oleic acid, OA) and 18:1n7 (cis-vaccenic acid; cis-VA); and the polyunsaturated fatty acids (PUFAs) 18:2n6 (linoleic acid, LA), 20:4n6 (arachidonic acid, ARA), 20:5n3 (eicosapentaenoic acid, EPA) and 22:6n3 (docosahexaenoic acid, DHA). Only total SFA composition showed significant differences between the amphipods, with the wild animals displaying a higher concentration than those used in the experiment (Tukey test;  $p$  value  $< 0.001$ ). This was mainly attributed to a higher individual PA content (Table 2). Concerning MUFAs, wild amphipods presented a significantly higher content of POA while amphipods fed with pellets showed a higher content of OA (Table 2). Wild amphipods also showed the highest content of omega-6 PUFAs due to the higher LA and ARA levels, while the amphipods used in the experiment presented higher contents of omega-3 DHA. The principal differentiating factors between the two feeding regimes were the significantly higher levels of PA (Tukey test;  $p$  value = 0.012) and OA (Tukey test;  $p$  value  $< 0.001$ ) observed in the pellets group.

Principal components analyses (PCA) were consistent with the results shown above. The first principal component explained 57 % of the total variance and the second principal component explained 23 %.

Principal component 1 (PC1) correlated positively with the fatty acids DHA ( $r = 0.854$ ,  $n = 9$ ,  $p < 0.005$ ) and cis-VA ( $r = 0.705$ ,  $n = 9$ ,  $p < 0.025$ ), and negatively with PA ( $r = -0.958$ ,  $n = 9$ ,  $p < 0.005$ ), EPA ( $r = -0.695$ ,  $n = 9$ ,  $p < 0.025$ ), ARA ( $r = -0.934$ ,  $n = 9$ ,  $p < 0.025$ ) and LA ( $r = -0.718$ ,  $n = 9$ ,  $p < 0.025$ ). PC2 correlated positively with OA ( $r = 0.821$ ,  $n = 9$ ,  $p < 0.005$ ). Axes clearly separated samples by treatments (wild, pellets or detritus) (Fig. 6).

## 4. Discussion

### 4.1. Survival rate

There are limited reports on the amphipods culture. Nevertheless, the survival rate observed in this study aligns with the findings of Jiménez-Prada et al. (2020), who reported values of approximately 40 % over a 21-days experiment. Similarly, Ribes-Navarro et al. (2022) observed comparable survival rates of *Gammarus locusta* when water at 20 °C and/or diets rich in short-chain PUFAs and SFAs were utilised in their experiment. However, their results indicated that lower temperatures (5–15 °C) and diets rich in LC-PUFAs were associated with enhanced survival rates.

### 4.2. Water quality

No statistically significant differences were found in water quality parameters among the treatments in this study. The results on nutrients concentration in the treatments tanks, when compared to the control, confirmed the effect of the carbon source on the colonisation of heterotrophic bacteria and the flocculation process (Crab, 2010; Hargreaves, 2013; Khanjani et al., 2024a): the concentration of ammonium was significantly lower, while nitrate cumulates in the treatment tanks, thus indicating that nitrifying bacteria were present in the biofloc and being consistent with previous works (Abbaszadeh et al., 2022; Brito et al., 2016).

### 4.3. Nutritional profile

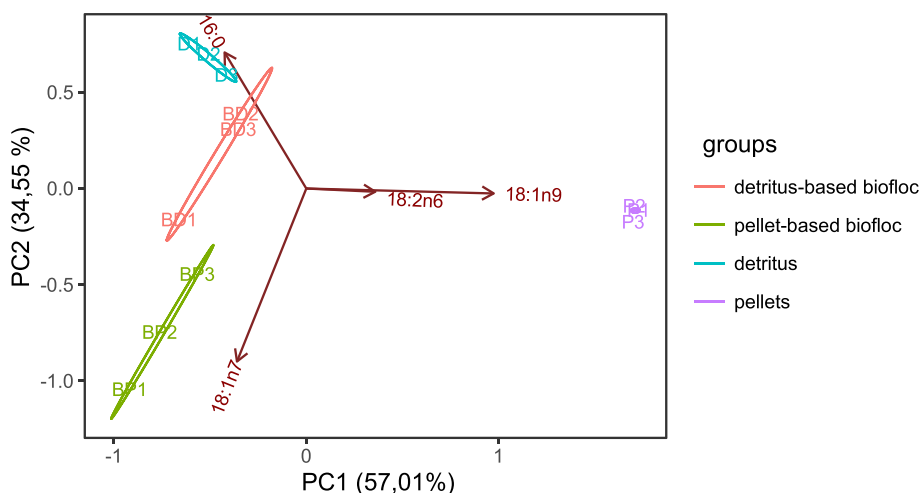
The flocculation success was also corroborated by the lipid profile of the bioflocs, which exhibited major fatty acids including PA, cis-VA, OA and LA, as already observed in previous work (Anand et al., 2014). Essential LC-PUFAs were not among the major FA. However, the present study revealed clearly higher amounts of ARA (0.8 %), EPA (3.1 %) and DHA (5.5 %) than Anand et al. (2014), who found only 0.4 % ARA and did not detect DHA or EPA in their biofloc. These values were similar to the FA profile of the commercial aquafeed used in the present study (pellets), which is formulated to meet the nutritional needs of fish (see Table 2: no differences were found in the ARA, EPA and DHA content between the pellets and the detritus-based biofloc). This justifies the enrichment of the biofloc with amphipods. Indeed, amphipods showed a profile rich in EPA, DHA, LA and ARA, as also demonstrated by Jiménez-Prada et al. (2018). The gammarids from both treatments showed the same amount of TL as the wild specimens, yet demonstrated a reduction in ARA and EPA, which was counterbalanced by a higher amount of DHA. Interestingly, Ribes-Navarro et al. (2021) have demonstrated that gammarid amphipods are devoid of desaturase genes that are complementary to the elongases needed for the biosynthesis of LC-PUFAs. Consequently, they are unable to synthesise these compounds de novo. Similarly to the study conducted by Promthale et al. (2021), our experiment was performed in darkness. Thus, it's unlikely that these compounds were acquired through the diet (phytoplankton). It has been proposed that the high percentages of DHA detected in gammarids, even if fed with diets lacking LC-PUFAs, are the result of an accumulation of previous diets (Ribes-Navarro et al., 2022), which could explain the results observed in the present study. These selective retention mechanisms would enable them to survive during periods of limited or no bioavailability of these nutrients (Ribes-Navarro et al., 2022).

**Table 2**

Fatty acids composition (mean % ± SD) of the diets used in the experiment (i.e. pellets, detritus) and the bioflocs sampled from these two treatments at the end of the experiment.

Fatty acid	pellets	detritus	Biofloc pellets	Biofloc detritus	P value	Tukey
<b>SFA</b>						
14:0	1.44 ± 0.00	3.81 ± 0.13	1.57 ± 0.44	2.64 ± 0.16	0.000**	d > all; bd > bp; p = bp
15:0	0.24 ± 0.01	0.78 ± 0.01	0.66 ± 0.19	0.60 ± 0.05	0.017*	d > p; p = bp = bd; d = bp = bd
16:0	16.43 ± 0.03	38.21 ± 2.12	22.06 ± 0.92	26.78 ± 1.37	0.000***	d > bd > bp > p
iso-C <sub>16</sub>	0.00 ± 0.00	0.84 ± 0.05	1.48 ± 0.32	0.79 ± 0.15	0.000**	bp > bd > d > p; d = bd
17:0	0.33 ± 0.00	1.09 ± 0.16	1.31 ± 0.26	0.48 ± 0.31	0.000**	d = bp > p; p = bd; bp > bd
18:0	4.87 ± 0.09	8.49 ± 0.06	8.22 ± 1.09	10.37 ± 1.03	0.000**	bd > bp > p; d = bd
20:0	0.36 ± 0.00	0.64 ± 0.06	0.82 ± 0.09	0.70 ± 0.03	0.000***	bp > bd = d > p
22:0	0.29 ± 0.02	0.65 ± 0.07	1.47 ± 0.36	1.19 ± 0.22	0.000***	p < bp; p = d = bd; bp = d = bd
24:0	0.19 ± 0.00	0.76 ± 0.08	1.08 ± 0.27	1.16 ± 0.16	0.000***	bd = bp = d > p
∑ SFA	24.14 ± 0.15	55.28 ± 2.46	38.68 ± 1.01	44.71 ± 2.05	0.000***	d > bd > bp > p
<b>MUFAs</b>						
16:1n5	0.03 ± 0.03	0.02 ± 0.02	0.36 ± 0.19	0.39 ± 0.23	0.044	
16:1n7	3.68 ± 0.04	3.21 ± 0.13	8.16 ± 2.14	5.83 ± 1.65	0.001**	bp > p = d; bp = bd
18:1n5	0.16 ± 0.00	0.06 ± 0.01	0.58 ± 0.06	0.41 ± 0.23	0.000***	bp = bd > d; d = p; bp > p; bd = p
18:1n7	3.31 ± 0.09	4.73 ± 0.09	18.99 ± 5.55	8.01 ± 4.27	0.000***	bp > p = d = bd
18:1n9	30.67 ± 0.03	9.59 ± 0.71	8.30 ± 0.87	10.67 ± 1.64	0.000***	p > all; bd > bp; d = bp; d = bd
20:1n9	2.30 ± 0.01	2.38 ± 0.12	0.73 ± 0.31	2.42 ± 0.33	0.000***	bp < p = d = bd
22:1n11	1.22 ± 0.01	2.30 ± 0.06	0.87 ± 0.41	3.06 ± 0.45	0.003**	bd > bp; bd = p = d; bp = p = d
24:1	0.51 ± 0.01	1.88 ± 0.06	1.02 ± 0.23	2.39 ± 0.28	0.000***	p = bp; p = d = bd; d > p; bd > bp
∑ MUFAs	41.89 ± 0.09	24.16 ± 0.84	39.01 ± 2.72	33.19 ± 3.98	0.000***	p = bp > bd > d
<b>n-6 PUFAs</b>						
18:2n6	14.93 ± 0.01	4.15 ± 0.21	3.96 ± 0.33	5.04 ± 0.23	0.000***	p > bd > bp > d
20:4n6	0.85 ± 0.00	0.41 ± 0.12	0.68 ± 0.33	0.80 ± 0.24	0.440	
∑ n-6 PUFAs	15.78 ± 0.02	4.56 ± 0.34	4.64 ± 0.24	5.84 ± 0.17	0.000***	p > all; d = bp; bd > d; bd > bp
<b>n-3 PUFAs</b>						
18:3n3	2.36 ± 0.01	0.48 ± 0.02	0.32 ± 0.25	0.37 ± 0.12	0.000***	p > d = bp = bd
20:5n3	3.68 ± 0.00	2.28 ± 0.15	1.87 ± 0.59	3.11 ± 0.90	0.002**	p > bp; p = d = bd; bp = d = bd
22:5n3	0.94 ± 0.00	0.46 ± 0.01	0.21 ± 0.03	0.48 ± 0.08	0.000**	p > all; bd > bp; bd = d
22:6n3	6.73 ± 0.03	3.76 ± 0.18	3.92 ± 1.12	5.54 ± 1.66	0.010*	p > d = bp; d = bp = bd; p = bd
∑ n-3 PUFAs	13.72 ± 0.04	6.99 ± 0.37	6.31 ± 1.73	9.50 ± 2.63	0.000***	p > all; bd > bp; d = bd; d = bp
∑ PUFAs	29.49 ± 0.03	11.56 ± 0.70	10.95 ± 1.87	15.34 ± 2.77	0.000***	p > all; bd > bp; d = bd; d = bp

p = pellet-based diet; d = detritus-based diet; bp = pellet-based biofloc; bd = detritus-based biofloc; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acid.



**Fig. 5.** Principal component analysis (PCA) plot based on FA composition (in % of FA) of experimental diets (i.e. pellets or detritus) and biofloc sampled from the experimental tanks. Only major FA are shown. 16:0 = PA, 18:1n7 = cis-VA, 18:1n9 = OA, 18:2n6 = LA. D = detritus; P = pellets; BD = detritus-based biofloc; BP = pellet-based biofloc.

The detritus-based biofloc presented a higher TL content and a more nutritious FA profile than the detritus-based diet, promoted by the presence of the microorganism assemblage and the amphipods. Moreover, this biofloc showed higher percentages of PA, OA and LA than the pellet-based biofloc. This is probably due to the higher mortality rate in

this treatment, with fewer individuals being sampled for biochemical analyses and further enriching the biofloc. It is important to note that these are relative results and that absolute values of these FA were similar in both treatments (data not shown), since the pellet-based biofloc showed a significantly higher amount of TL. However, the

**Table 3**Fatty acids composition (mean %  $\pm$  SD) of *G. insensibilis* from salt marshes and those used in the experiment, fed with fish pellets or detritus.

Fatty acid	wild	pellets	detritus	P value	Tukey
<b>SFA</b>					
14:0	1.46 $\pm$ 0.12	0.75 $\pm$ 0.15	0.63 $\pm$ 0.17	0.000***	w > p = d
15:0	0.32 $\pm$ 0.27	0.41 $\pm$ 0.02	0.47 $\pm$ 0.02	0.538	
16:0	20.40 $\pm$ 0.14	16.44 $\pm$ 0.37	15.47 $\pm$ 0.26	0.000***	w > p > d
16:0i	0.18 $\pm$ 0.03	0.90 $\pm$ 0.03	0.79 $\pm$ 0.39	0.057	
17:0	0.12 $\pm$ 0.01	1.03 $\pm$ 0.04	1.22 $\pm$ 0.05	0.000***	w < p < d
18:0	3.46 $\pm$ 0.12	3.83 $\pm$ 0.15	4.39 $\pm$ 0.15	0.000***	w < p < d
22:0	0.30 $\pm$ 0.26	0.37 $\pm$ 0.16	0.70 $\pm$ 0.06	0.073	
$\Sigma$ SFA	26.23 $\pm$ 0.51	23.74 $\pm$ 0.42	23.67 $\pm$ 0.29	0.000	w > p = d
<b>MUFAs</b>					
16:1n7	5.97 $\pm$ 0.28	3.67 $\pm$ 0.29	4.16 $\pm$ 1.42	0.036*	w > p = d
18:1n9	15.89 $\pm$ 0.27	22.17 $\pm$ 0.85	16.14 $\pm$ 0.95	0.000***	p > w = d
18:1n7	4.30 $\pm$ 0.16	6.48 $\pm$ 1.25	8.35 $\pm$ 3.17	0.115	
18:1n5	0.19 $\pm$ 0.01	0.65 $\pm$ 0.28	0.77 $\pm$ 0.39	0.092	
20:1n9	0.80 $\pm$ 0.03	1.22 $\pm$ 0.03	0.79 $\pm$ 0.06	0.000***	p > w = d
20:1n7	0.33 $\pm$ 0.04	0.52 $\pm$ 0.11	0.57 $\pm$ 0.26	0.243	
$\Sigma$ MUFAs	27.47 $\pm$ 0.63	34.70 $\pm$ 2.55	30.77 $\pm$ 4.40	0.064	
<b>n-6 PUFAs</b>					
18:2n6	7.43 $\pm$ 0.09	6.77 $\pm$ 0.63	5.69 $\pm$ 0.69	0.021*	w > d; w = p; p = d
20:2n6	0.94 $\pm$ 0.02	0.85 $\pm$ 0.17	0.84 $\pm$ 0.18	0.636	
20:4n6	7.59 $\pm$ 0.12	4.90 $\pm$ 0.52	5.49 $\pm$ 1.23	0.013*	w > p = d
22:5n6	0.55 $\pm$ 0.01	0.77 $\pm$ 0.15	0.64 $\pm$ 0.08	0.084	
$\Sigma$ n-6 PUFAs	15.51 $\pm$ 0.22	13.29 $\pm$ 1.44	12.66 $\pm$ 2.13	0.040*	w > d; w = p; p = d
<b>n-3 PUFAs</b>					
18:3n3	0.75 $\pm$ 0.04	0.37 $\pm$ 0.32	0.19 $\pm$ 0.34	0.108	
20:5n3	15.25 $\pm$ 0.28	11.37 $\pm$ 0.87	13.43 $\pm$ 3.34	0.136	
22:5n3	0.97 $\pm$ 0.03	0.56 $\pm$ 0.09	0.76 $\pm$ 0.15	0.008**	w > p; w = d; p = d
22:6n3	4.82 $\pm$ 0.00	9.47 $\pm$ 0.41	11.81 $\pm$ 2.17	0.002**	w < p = d
$\Sigma$ n-3 PUFAs	21.79 $\pm$ 0.26	21.77 $\pm$ 1.21	26.19 $\pm$ 4.83	0.176	
16:2n4	0.44 $\pm$ 0.48	0.05 $\pm$ 0.05	0.39 $\pm$ 0.07	0.261	
16:4n1	1.08 $\pm$ 0.47	0.87 $\pm$ 0.60	1.33 $\pm$ 0.30	0.526	
$\Sigma$ PUFAs	39.82 $\pm$ 1.12	35.98 $\pm$ 2.66	40.57 $\pm$ 6.36	0.386	

w = wild; p = pellets; d = detritus; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acid.

pellet-based biofloc did not present a better profile than the pellet-based diet, highlighting the nutritional value of the diet, but also pointing the interest of valorising aquaculture wastes in BFT systems (Das et al., 2023; Kala et al., 2023). Furthermore, the pellet-based biofloc exhibited a significantly higher percentage of isoC<sub>16</sub> and cis-VA, commonly

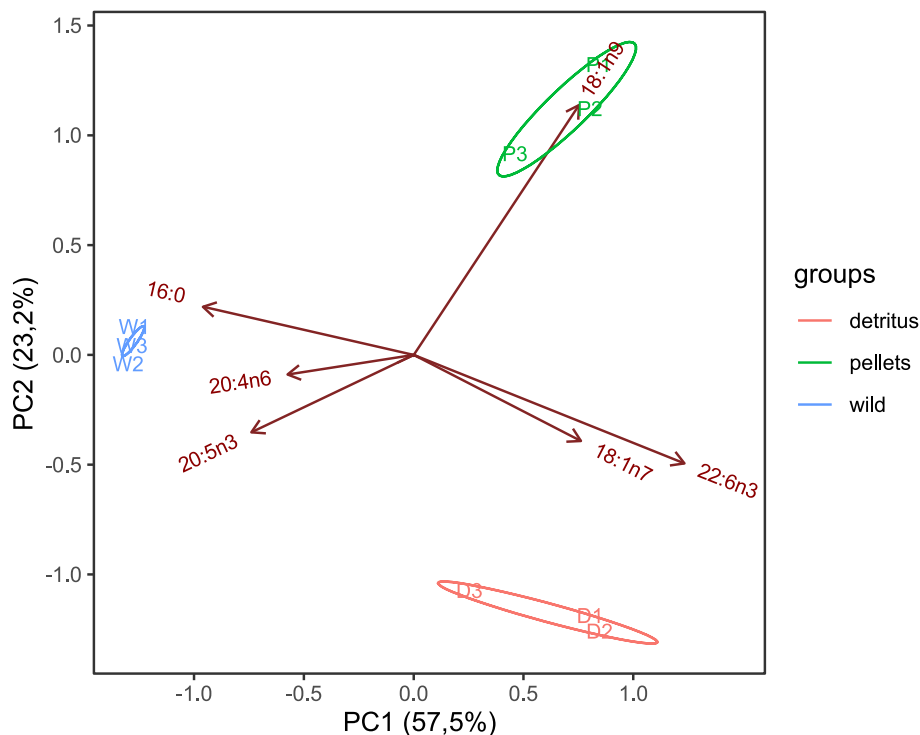
associated with bacteria (Rontani et al., 2003). This is attributed to the significantly higher content of OM in the pellets and pellet-based biofloc, which provides essential nutrients and facilitates the establishment of heterotrophic bacteria and the conversion of the OM into bacterial biomass (Zhukova and Kharlamenko, 1999). This demonstrates the advantage of the pellets treatment in terms of biofloculation capacity (Faust, 2014), in addition to the improved survival rates. It has been shown that the FA profile of amphipods as well as their survival rate are closely linked to the diet (Ribes-Navarro et al., 2022). Thus, the integration in BFT systems of aquaculture waste detritus with a supplementation of commercial aquafeed could potentially enhance the overall nutritional profile while enhancing the biofloculation process for the bioremediation of the aquaculture effluents (Castilla-Gavilán et al., 2023). Moreover, it will be suitable to carry out the experiment under light conditions and with the addition of diatoms or chlorophytes, which have been highlighted by other authors to improve the LC-PUFAs and EAA profiles (Khatoun et al., 2009; Martins et al., 2016), and water quality (Brito et al., 2016; de Andrade et al., 2021).

The interest in integrated BFT systems (a combination of integrated multi-trophic aquaculture, IMTA, and BFT) has increased in recent years (Borges et al., 2020; Carvalho et al., 2023; Legarda et al., 2021; Lima et al., 2021). In these systems, the residues of one fed species serve as a source of OM for bioflocs, which can be enriched with a second species, which in turn is fed to a third species. This approach aims to minimize the production of waste and the use of aquafeed. The present study shows that the effluent of a fish RAS can be valorised to feed the medium of a BFT system enriched with amphipods. Both bioflocs and amphipods could be further used as food, as their fatty acids profile would satisfy the requirements of various species of interest in aquaculture as are salmonids (Carr et al., 2023), marine crustaceans (González-Félix et al., 2002), sea bream (Ibeas et al., 1996) or turbot (Castell et al., 1994). The use of bioflocs to feed fish has been demonstrated to be an effective approach, as evidenced by the findings of several studies (Borges et al., 2020; Holanda et al., 2023; Legarda et al., 2021; Pinho et al., 2021; Poersch et al., 2021; Silva et al., 2022). Furthermore, amphipods have also been successfully employed as a dietary source for fish (Moren et al., 2006; Suontama et al., 2007) and cephalopods (Baeza-Rojano et al., 2010; Baeza-Rojano et al., 2013). Finally, the present study supports the growing interest in the use of amphipods in aquaculture, with a particular focus on BFT systems. It aims to promote further research in this topic, given that to date, BFT systems have been predominantly studied in mono-specific or multi-specific decapod crustaceans aquaculture (Bajracharya et al., 2024; Ekasari et al., 2014; Emerenciano et al., 2021; Galasso et al., 2024; Huang et al., 2023; Lima et al., 2021; Nguyen et al., 2024; Pérez de Jesús et al., 2024; Pérez-Velasco et al., 2023; Pimentel et al., 2024; Qiu et al., 2023; Reis et al., 2023). Few studies have been conducted on echinoderms (Chen et al., 2018a, 2018b) and only a single study has been carried out on amphipod crustaceans (Promthale et al., 2021).

## 5. Conclusion

This study demonstrates the potential of using *G. insensibilis* in BFT systems, focusing on its survival and nutritional profile, when fed with commercial aquafeed or detritus from aquaculture effluents. The findings reveal that *G. insensibilis* can be effectively cultivated in BFT systems, leveraging its ability to feed on detritus, which promotes biofloculation and enhances the nutrient profile of the biofloc. The presence of bioflocs enriched with amphipods significantly improves the overall fatty acid composition, particularly in essential LC-PUFAs such as EPA, DHA, and ARA. This enrichment addresses the inherent nutritional deficiencies of bioflocs, making them more suitable as food for high-value aquaculture species. The study highlights the dual benefits of this approach: enhancing the nutritional value of bioflocs while contributing to the bioremediation of aquaculture effluents. Furthermore, the use of close aquaculture systems reduces costs and maintain





**Fig. 6.** Principal component analysis (PCA) plot based on FA composition (in % of FA) of amphipods from the wild or fed with experimental diets (i.e. pellets or detritus). Only major FA are shown. 16:0 = PA, 20:4n6 = ARA, 20:5n3 = EPA, 18:1n9 = OA, 22:6n3 = DHA, 18:1n7 = cis-VA. D = detritus; P = pellets; W = wild. Three replicates were analysed, both in the wild and in each treatment (3 tanks per treatment). Each replicate consisted on a pool of amphipods.

isolation from natural environment. The integration of aquaculture detritus with supplemental aquafeed in BFT systems will support both the biofloculation process and the nutritional needs of the amphipods, thereby creating a sustainable and efficient cycle of waste valorisation and food production.

The use of *G. insensibilis* in BFT systems represents a promising strategy for improving the sustainability and productivity of aquaculture operations. By optimizing the use of organic waste and producing nutritionally rich bioflocs, this approach can significantly contribute to the development of a circular economy in aquaculture, reducing environmental impacts and enhancing the growth and health of cultivated species. Further research is needed in order to study the complete life cycle of amphipods and their reproductive capacity in these systems. A lower water temperature and the inoculation of microalgae should be considered in order to achieve higher survival rates and PUFAs content.

#### CRedit authorship contribution statement

**Marta Castilla-Gavilán:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **José Manuel Guerra-García:** Validation, Supervision, Methodology, Data curation. **Ismael Hachero-Cruzado:** Validation, Supervision, Methodology, Data curation, Conceptualization.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Data availability

Data will be made available on request.

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