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How much waste can the amphipod *Gammarus insensibilis* remove from aquaculture effluents? A first step toward IMTA

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ABSTRACT

In recent years marine amphipods have been highlighted as an alternative live feed resource for Integrated Multi-Trophic Aquaculture (IMTA). *Gammarus insensibilis* Stock, 1966 is a native amphipod from the Mediterranean Sea and the north Atlantic Ocean and it is highly abundant in southern Spain marsh ponds. Its high potential for being intensively reared has been demonstrated in previous studies, as this species can feed on detritus and presents an interesting nutritional profile. In the present work, *G. insensibilis* specimens were maintained in closed batches at three different densities (100, 500 and 1000 ind L^{-1}) and fed on dry or wet detritus (uneaten food and faeces from a fish RAS effluent) in order to assess for the first time the amount of wastes that they were able to remove. In parallel, a trial was performed to study the effect of dissolved inorganic nitrogen compounds (highly abundant in aquaculture effluents) on their survival, and the median lethal concentrations (LC_{50}) in 96 h were calculated. Amphipods in all experiments and conditions showed promising survival rates higher than 80%. No advantages were observed when gammarids were maintained at high densities due to the significantly higher ammonium concentrations in these treatments, having an effect on intake but allowing sub-lethal conditions. Indeed, *Gammarus insensibilis* showed the highest tolerance for nitrate (1308 mg N-NO_3^- L^{-1}), followed by nitrite (39.77 mg N-NO_2^- L^{-1}) and ammonium (33.23 mg N-NH_4^+ L^{-1}). Individuals at low densities removed significantly higher amounts of detritus, between 154.98 and 169.78 mg (dry weight detritus) per g (wet weight amphipods) and day. No differences were observed between removal rate of dry or wet detritus. Thus, authors recommend the use of wet detritus for a better handling and up-scaled trials in open or RAS systems equipped with a biofilter in order to avoid high ammonium concentrations impacting on intake.

1. Introduction

Global population demand for animal protein is estimated to double before 2050 (Elferink and Schierhorn, 2016; Tripathi et al., 2019). Concerned by the environmental crisis and attracted by the healthy lifestyle trends, consumers are conscious of the need of reducing meat consumption and the benefits of including essential fatty acids in their diet. Today, the aquaculture industry produces more than half of the fish and seafood used for human consumption (FAO, 2022) and plays a fundamental role in responding to this need for proteins and essential fatty acids. For this reason, the European Commission has activated several strategies (EU missions “Restore our Ocean and Waters” or “Green Deal”, Atlantic Action Plan, Blue Growth strategy) aiming intensification of the aquaculture production in an environmentally sustainable way (Alexander et al., 2015). It promotes the research for alternative live feed organisms and the progress in Integrated Multi-Trophic Aquaculture (IMTA). These two topics are at the core of the present work.

Integrated Multi-Trophic Aquaculture systems are composed of two or more functional groups that are trophically connected by nutrient flows (Dunbar et al., 2020), allowing groups of the lower trophic levels to feed on the wastes generated by those on the upper levels. Thus, IMTA tries to mitigate two of the most important issues in aquaculture: the efficient use of water and the environmental impact of its effluents (Troell et al., 2009) that are highly rich in organic particles and dissolved nutrients from undigested fish feed and faeces.

During the last decade, there is also an increasing interest in the potential use of marine amphipods as an alternative live feed resource for IMTA (Fernandez-Gonzalez et al., 2018; Guerra-García et al., 2016; Woods, 2009). Amphipods are the most diverse crustaceans group in

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terms of lifestyle, habitat, size and feeding behaviour (Guerra-García et al., 2014). They show a high abundance, great species richness and a widespread global distribution, playing an important role in the ecology of rocky habitats and sandy bottoms (De-La-Ossa-Carretero et al., 2010). They are an important part of the food chain energy exchange, as they are a resource for many predators, being a link between primary and secondary producers and upper trophic level animals, such as fish, birds or mammals. Amphipods exhibit fast growth rates and can live at high densities (Ashton, 2006). Several researches have shown that amphipods are characterized by high levels of proteins and omega-3 and omega-6 polyunsaturated fatty acids (Baeza-Rojano et al., 2014; Jiménez-Prada et al., 2018) and that they are an optimal source of live feed for cephalopods (Baeza-Rojano et al., 2010; Baeza-Rojano et al., 2013b). As they are opportunistic feeders being able to feed on detritus, their culture in IMTA for effluents bioremediation has also been highlighted in previous studies (Guerra-García et al., 2016; Jiménez-Prada et al., 2020). Indeed, Jiménez-Prada et al. (2020) revealed the potential of the marine amphipod *Gammarus insensibilis* Stock, 1966 from Southern Spain marsh ponds, as a promising species for being intensively cultured due to its adequate nutritional profile, large body size and high natural densities.

Despite all this previous research, there is a lack of studies adressing the bioremediation capacity of these organisms. As pointed by Lamprianidou et al. (2015), the evaluation of IMTA as an effluent bioremediation method needs the knowledge of waste nutrients removal rates by the extractive species (amphipods in this case) and the ratio of feed supply to extractive organisms required for this removal. Thus, the main objective of this work was to assess the bioremediation capacity of *G. insensibilis* fed on fish wastes by quantifying the amount of detritus that the species is able to remove when reared at different densities. Strengths and weaknesses of this species culture are discussed, comparing with other detritivorous species currently used on IMTA, such as sea urchins, sea cucumbers or polychaetes (Grosso et al., 2021; Orr et al., 2014; Yousefi-Garakouei et al., 2019).

Moreover, taking into account the dissolved inorganic nitrogen compounds issues in aquaculture effluents (Ahmad et al., 2021) and the role of IMTA systems on their transformation (Abreu et al., 2011; Troell et al., 2009), the present study also evaluated the toxic effects of these compounds on *G. insensibilis* survival through the calculation of median lethal concentrations (LC_{50}) of ammonia-N, nitrate-N and nitrite-N, as already performed for other amphipods species (Alonso and Camargo, 2006; Camargo et al., 2005; Kohn et al., 1994) and other extractive species (i.e. crustaceans, equinodermes) used on IMTA (Barbieri et al., 2016; Basuyaux and Mathieu, 1999; Frias-Espericueta et al., 2000).

2. Materials and methods

2.1. Experimental conditions

All *G. insensibilis* used for the following experiments were sampled from a natural marsh pond at the IFAPA Centre “El Toruño” (El Puerto de Santa Maria, Cadiz Bay, Spain). Amphipods on their original subtract (*Ulva* sp. thalli, where they mainly inhabit) were transported to IFAPA facilities and transferred into a 400 L tank to allow their acclimation prior experimentation. This tank was connected to a recirculation system (RAS) equipped with a cooling, mechanical filter, protein skimmer, ultraviolet lights and biofilter, with seawater maintained at 17 °C and 38 g L^{-1} salinity under a 12 h/12 h (light/dark) photoperiod. During the acclimation period, the water was renewed daily in continuous water flow. Prior each experiment, adults of *G. insensibilis* were sampled from the tank, devoid of algae and starved for 24 h to standardize their nutritional conditions (Alexander et al., 2015).

Six experiments were carried out between February and May 2022:

2.2. Gut content assay

To explore visually the intake of *G. insensibilis* and the adequacy of fish waste as food for them, a first preliminary assay was carried out, in which the gut content of the amphipods fed on two different diets (dry or wet diet) were compared. For this purpose, 12 batches of 50 specimens were randomly distributed in 12 flasks filled with 50 ml of seawater (density = 1000 ind L^{-1}) under the same water conditions described above. Twelve other flasks devoid of animals were used as a negative control. As proposed by Baeza-Rojano et al. (2013a), all flasks were provided with a 70 × 50 mm plastic mesh as an artificial substratum (7 × 2 mm pore diameter). All treatments and controls were fed ad libitum on the same amount of food.

The diet was composed of the waste detritus obtained through the cleaning of the meagre (*Argyrosomus regius*) and Senegalese sole (*Solea senegalensis*) culture tanks at IFAPA facilities and consisted primarily of fish faeces and uneaten fish feed pellets. Detritus was abundantly rinsed with fresh water and distilled water to remove salt, filtered through a 200 μm mesh to remove the excess of water and divided into aliquots of approximately 300 mg. Dry diet was used for a better handling, avoiding biases linked to a variable water content in the samples. Aware that these would not be the real conditions in a large-scale culture and that another bias could be a rapid solubility in the water of the dry detritus, the experiment was also conducted using wet detritus. Aliquots destined for the “dry diet” were frozen at −20 °C, freeze-dried, dry weighted and distributed in the corresponding 12 flasks (6 flasks with gammarids and 6 control flasks). Dry weight (DW) data was also used to establish a conversion factor, in order to estimate dry mass from wet mass and vice versa (Rice et al., 2012). The aliquots used for the “wet diet” were directly distributed in the corresponding 12 flasks.

Water parameters (temperature, dissolved oxygen, pH and salinity) were checked twice a day and pure oxygen was daily injected into the lid covered flasks to avoid low dissolved oxygen concentrations due to amphipods metabolism and detritus degradation. After 48 h from the beginning of the experiment, survival was assessed and 50% of alive amphipods were fixed in 70% ethanol prior to the diet study.

At least, 10 specimens from each replicate were observed, thus accounting a total of 60 specimens per diet type. Gut content was analysed following the method proposed by Bello and Cabrera (1999) modified by Guerra-García and Tierno de Figueroa (2009) for its use on amphipods. Gammarids samples of each diet type were introduced in vials with Hertwig's liquid (270 g of chloral hydrate, 19 ml of chloridric acid 1 N, 60 ml of glycerine and 150 ml of distilled water; Guerra-García et al., 2014). As *G. insensibilis* has a relatively thick cuticle and a considerable size, samples had to be heated in an oven at 65 °C for at least 12 h. After this procedure, they were mounted on slides, observed and photographed under a stereomicroscope equipped with a Leica DFC420 camera. Total digestive tract length and the area occupied by the content were determined from photographs (Fig. 1) using ImageJ software (Schindelin et al., 2015; Schindelin et al., 2012). The gut content of the individuals observed was also extracted for study under a microscope equipped with a Leica DF450C camera to determine its nature. Gut content of some *G. insensibilis* sampled from the natural marsh pond were also analysed to compare diet components and percentage of occupied area in their digestive tract with those of the specimens used in the present study.

2.3. Effect of amphipods density and diet type on feed intake

Once we verified that *G. insensibilis* was able to feed on fish waste, the consumption was evaluated by performing two consecutive experiments (with a dry diet and with a wet diet respectively). Three different densities of gammarids were tested: 100 individuals L^{-1} (low density), 500 ind L^{-1} (medium density) and 1000 ind L^{-1} (high density). For each

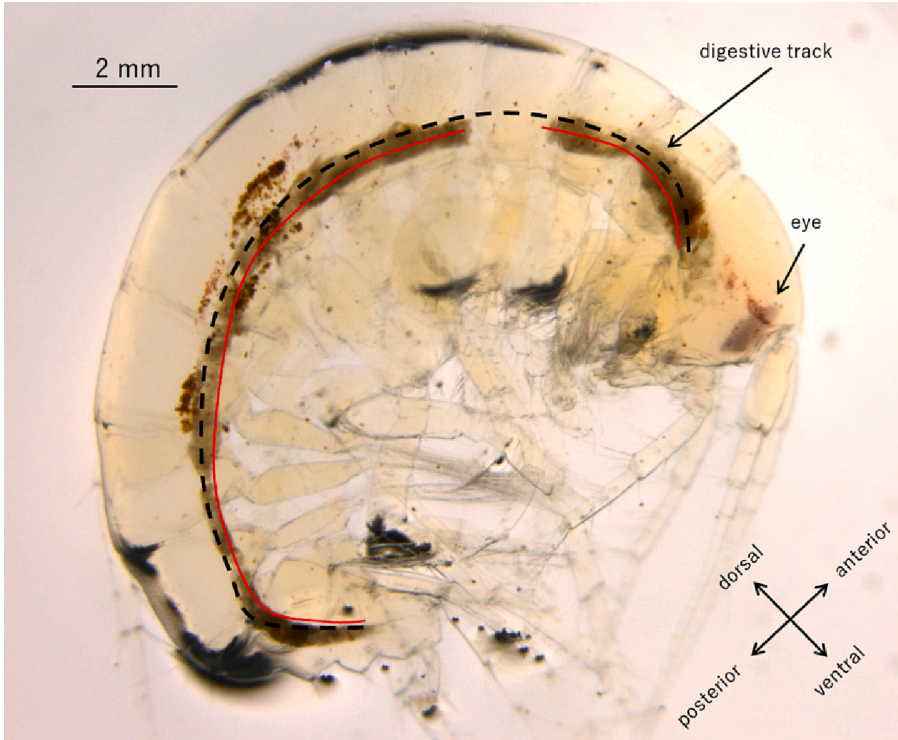

Fig. 1. Right lateral view of *G. insensibilis* observed under a stereomicroscope (x10). Black dotted line represents total digestive track length. Red line represents the digestive track occupied area. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

experiment, six batches of 5, 25 or 50 specimens were randomly distributed in 18 flasks filled with 50 ml of seawater. Six other flasks devoid of animals were used as a negative control. The experiments were run following the same methodology described above during 48 h.

After 24 h, mortality was checked and any dead specimens were removed. A 50% water exchange was carried out and the water being removed was filtered on pre-weighed GF/C fibreglass filters that were rinsed with distilled water, stored at −20 °C and freeze-dried. Water samples were also stored at −20 °C. After 48 h, alive amphipods in each flask were sampled, rinsed with distilled water to remove salt, stored at −20 °C and freeze-dried. Seawater was filtered on pre-weighed GF/C fibreglass filters. Water samples and filters were treated and stored as explained above.

All filters and amphipods samples were dry weighted prior to muffle furnace combustion at 500 °C for 4 h to determine ash weight contents and ashes free dry weight (AFDW) of uneaten food. At the same time, 12 detritus samples of the same WW were also combusted to determine the organic matter content on their initial conditions. Daily Food Consumption (DFC) rates were calculated according to Gergs and Rothhaupt (2008) and following the equation:

$$\mathrm{DFC}\left(\mathrm{mg_{food}\ g^{-1}{}_{amphipod}\ hour^{-1}}\right) = (\mathrm{Fg{-}Fr})/(\mathrm{W\ x\ t}).$$

where Fg was the dry weight (DW) in mg of the given food and Fr was the DW (mg) of the remaining food. W was the DW (g) of the amphipods and t was the time in hours.

Chemical analyses were carried out on water samples in order to assess water quality for reared amphipods. Nitrate and nitrite concentrations were sequentially quantified by the reduction of NO_3^- to NO_2^- with vanadium (VCl_3) following the method of García-Robledo et al. (2014). Ammonia concentration was determined by the indophenol-blue method (Aminot et al., 1997) and results were then correlated with DFC in order to identify a possible toxic effect of this compound on the intake.

2.4. Toxic effects of inorganic nitrogen on G. insensibilis survival

Three additional experiments were performed at different times in order to investigate ammonia, nitrite and nitrate median lethal concentrations (LC_{50}) for *G. insensibilis*. Each one of these toxicity tests were carried out in 20 replicated flasks under the same water parameters as in the experiments described above. Three different concentrations of each inorganic nitrogen compound were tested on the animals (5 batches of 10 gammarids per treatment) against a control (5 batches of 10 gammarids in clean seawater).

The test solutions were prepared using ammonium chloride, sodium nitrate and sodium nitrite. Following the methodologies of previous studies on marine amphipods and other marine or freshwater invertebrates (Alonso and Camargo, 2006; Basuyaux and Mathieu, 1999; Kohn et al., 1994; Soucek and Dickinson, 2012; Valencia-Castañeda et al., 2018), the concentrations tested were of 90, 120 and 150 mg $NH_4Cl\ L^{-1}$, 7000, 9000 and 11,000 mg $NaNO_3\ L^{-1}$ and 150, 300 and 500 mg $NaNO_2\ L^{-1}$.

Specimens in all treatments and controls were starved and provided a plastic mesh as artificial subtract (same mesh size as described above). Mortality and water parameters (temperature, dissolved oxygen, pH, salinity) were checked daily. A 100% water exchange was also carried out every day in order to maintain nitrogen compounds concentration. The experiments lasted 96 h. Median lethal concentration was determined using the probit method (Finney, 1971).

2.5. Data analyses

Mean and standard error (SE) were calculated for all data. Statistical analyses were performed using RStudio software.

Differences in survival among “dry diet”, “wet diet” and “marsh ponds” gammarids were analysed by a two samples *t*-test and gut content by a one-way ANOVA.

For the feeding experiments, to determine whether survival, daily food consumption, organic matter content in the uneaten food and

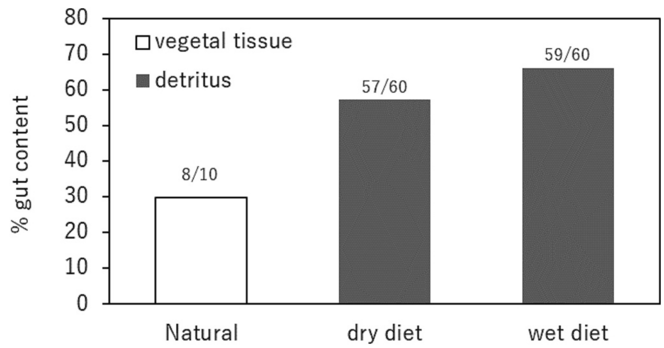

Fig. 2. Diet comparison in *G. insensibilis* from natural marsh ponds or from experiments fed on dry or wet diet (i.e. detritus). Each bar represents the total area occupied by the content in the whole digestive tract. Data are mean values of specimens analysed ($n = 6$). Numbers on the bar (n/N) indicate the number of specimens examined (N) and the number of those with some contents in the digestive tract (n).

nitrogen water content varied among diet type and density treatments, two-way ANOVAs were conducted with the following factors: "Diet", a fixed factor with two levels ("dry" or "wet") and "Density", a fixed factor with four levels ("low", "medium", "high" and "control") and orthogonal with the factor "Diet". Since total consumption was not related to the biomass of amphipods in each treatment, biomass was considered as a covariable and differences in total consumption tested by a two-ways ANCOVA.

Prior to ANOVAs or ANCOVA, the homogeneity of variances was tested with Levene test. Since proportions were compared, an arcsine transformation was carried out (Sokal and Rohlf, 1995). Posteriori multiple comparison Tukey tests were conducted in all pairwise between treatments when significant differences were found ($P < 0.05$).

For toxicity tests, the LC_{50} was determined by plotting probit transformed percent mortality against log concentration (Finney, 1971). For each nitrogen compound, linear regressions were established between concentration and mortality.

3. Results

3.1. Gut content assay

Survival rates were 83% for gammarids fed on the dry diet and 84.67% for those fed on the wet diet and no statistical differences were found between these values (t-test, $t = -0.6715$, $P = 0.5171$). The study of the gut contents of *G. insensibilis* (Fig. 2) shows significantly higher (one-way ANOVA, $F = 6.126$, $P = 0.0029$) occupation percentages of the whole digestive tract in animals feed on detritus compared to those sampled from their natural habitat (i.e. marsh ponds). No significant differences were found between dry or wet conditions ($P = 0.1685$). Animals in both treatments only presented detritus (Fig. 3A) in their digestive tract while animals from marsh ponds only presented vegetal tissue (Fig. 3B).

Table 1
Survival (mean percentage ± SE; n = 6) observed in both experiments (dry diet and wet diet) for the three experimental densities (low = 100 ind L^{-1}, medium = 500 ind L^{-1}, high = 1000 ind L^{-1}).

	Dry diet experiment	Wet diet experiment
Low density	86.7% ± 4.2	83.3% ± 6.1
Medium density	91.3% ± 2.8	92.7% ± 2.6
High density	90.7% ± 1.9	96.3% ± 1.4

3.2. Effect of amphipods density and diet type on survival and feed intake

In general, survival was always higher than 80% (Table 1) and no statistical differences were found between diets nor densities (Table 2).

Total consumptions (mean ± SE) for each experimental density and controls are illustrated in Fig. 4 and the result of the ANCOVA test is presented in Table 2. In both experiments, *G. insensibilis* removed detritus, as no significant consumption was observed in the controls. Total consumption was significantly higher in the three densities than in the control and no differences were found between densities. Significant differences were also found between dry and wet diets (Table 2). Concerning the removal rate or the daily food consumption (DFC), Fig. 5 illustrates the consumption of the given food (i.e. detritus expressed by mg of dry weight) per gram of gammarids (dry weight) and per hour). Maximal removal rate, 42.57 mg g^{-1} h^{-1}, was reached for gammarids fed on the dry diet at the low density. Two-way ANOVA evidenced significant differences between diets (Table 2). Moreover, gammarids in the low density treatments always showed significantly higher DFC values (Table 2) than gammarids at medium or high densities. A qualitative difference was also observed between densities in both experimental diets: detritus was progressively more particulate with increasing density (Fig. 6).

The percentage of organic matter present in the uneaten detritus for both experiments and in the initial detritus is shown in Fig. 7. Organic matter in the uneaten dry detritus was significantly higher than in the uneaten wet detritus or the initial one, while content in the wet detritus did not present differences with the initial conditions (Table 2).

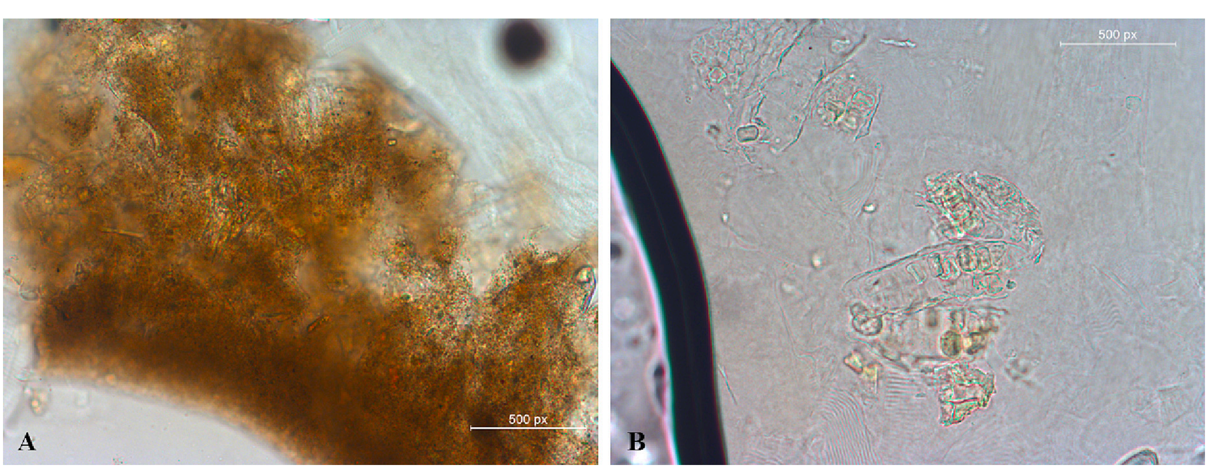

Fig. 3. Examples of the gut content (detritus in A, vegetal tissue in B) of *G. insensibilis* used in the experiment (A) or sampled from a marsh pond (B) observed under a microscope (x400).

Table 2
Results of the two-way ANOVAs (survival, daily food consumption, percentage of organic matter in the uneaten detritus and concentration of ammonium, nitrate and nitrite) and the two-way ANCOVA (total consumption). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. L = low; M = medium; H = high; C = control.

Source of variation	df	SS	MS	*F* value	*P* value
Two-way ANOVAs					
Survival					
Diet (Di)	1	0.0012	0.0012	0.1605	0.6914
Density (Den)	2	0.0514	0.0257	3.3098	0.0519
Di x Den	2	0.0127	0.0063	0.8071	0.4556
Residual	32	0.2484	0.0078		
Levene Test	5			0.8626	0.5174
Transformation			arcsine		
Daily food consumption					
Diet (Di)	1	120.27	120.27	5.500	0.0261*
Density (Den)	2	2523.70	1261.85	57.703	0.000***
Di x Den	2	30.65	15.33	0.6857	0.0512
Residual	29	634.17	21.87		
Levene Test	5			2.1852	0.0855
Transformation	none				
Tukey Test	L > M; L > H; M = H				
Percentage of organic matter in uneaten detritus					
Diet (Di)	2	0.3107	0.1554	10.0431	0.000***
Density (Den)	3	0.0831	0.0277	1.7913	0.1600
Di x Den	3	0.0462	0.0154	0.9954	0.4027
Residual	53	0.81992	0.0155		
Levene Test	8			1.6351	0.1386
Transformation	arcsine				
Tukey Test	Dry>Initial; Dry>Wet; Initial = Wet;				
Ammonium concentration					
Diet (Di)	1	0.69	0.689	0.7027	0.4073
Density (Den)	3	370.43	123.476	126.0132	0.000***
Di x Den	3	9.66	3.220	3.2864	0.0313*
Residual	37	36.26	0.980		
Levene Test	7			1.0487	0.4151
Transformation	none				
Tukey Test	(Di x Den) Dry: H > C; L = C; M > C; H > L; H > M; M = L				
	Wet: H > C; L = C; M > C; H > L; H > M; M > L				
Nitrate concentration					
Diet (Di)	1	0.0459	0.0459	20.8017	0.000***
Density (Den)	3	0.0643	0.0214	9.6999	0.000***
Di x Den	3	0.0206	0.0069	3.1023	0.0376*
Residual	39	0.0862	0.0022		
Levene Test	7			1.1334	0.3627
Transformation	none				
Tukey Test		(Di x Den)	Dry: H = M = L = C		
			Wet: H = M = C; L > H; L > M; L > C		
Nitrite concentration					
Diet (Di)	1	0.0334	0.0334	32.765	0.000***
Density (Den)	3	0.0533	0.0178	17.421	0.000***
Di x Den	3	0.0557	0.0186	18.196	0.000***
Residual	39	0.0398	0.0010		
Levene Test	7			1.8835	0.0988
Tukey Test		(Di x Den)	Dry: H = M = L = C		
			Wet: H = M = C; L > H; L > M; L > C		
Two-way ANCOVA					
Total consumption					
Biomass (covariable)	1	122.9	122.9	14.262	0.000***
Diet (Di)	1	166.0	166.0	19.259	0.000***
Density (Den)	3	956.9	319.0	37.016	0.000***
Biomass x Di	1	1.9	1.9	0.220	0.6425
Biomass x Den	2	29.9	14.9	1.734	0.1957
Di x Den	3	115.0	38.3	4.450	0.0115*
Biomass x Di x Den	2	15.7	7.9	0.914	0.4131
Residual	27	232.7	8.6		
Levene Test	7			0.7089	0.6646
Tukey Test	Dry > Wet				
	Dry and Wet: H > C; M > C; L > C; H = M = L				

3.3. *Inorganic nitrogen concentrations and LC_{50}–96 h for G. insensibilis*

Results for chemical analyses on water samples from dry diet and wet diet experiments are presented in Table 3. Concerning N-NH_4^+ concentrations, the two-way ANOVA analyse did not show significant differences between diets (Table 2). However, significantly higher concentrations of N-NH_4^+ were observed when animals were reared at high densities. Values in the medium density treatment were also significantly higher than those in the control for the dry diet experiment, and higher than those in the low density treatment and the control for the wet diet experiment (Table 2).

Significant relationships were found between N-NH_4^+ concentrations and DFC in all treatments of both experiments, demonstrating the toxic effect of ammonium on intake (Fig. 8; Table 4).

A similar pattern (significant differences and interactions) was observed for N-NO_3^- and N-NO_2^- concentrations (Table 3): for the dry diet experiment, no differences were detected between densities while for the wet diet experiment low density treatment presented significantly higher concentrations than medium, high and control treatments (Table 2).

Regarding the lethal effect of nitrogen compounds, the LC_{50}–96 h results for *G. insensibilis* are presented in Table 5. For this experiment, significant relationships were found between the three compounds concentrations and the mortality rates observed (Table 5). The highest toxicity level was found for N-NH_4^+.

4. Discussion

This is the first study exploring the waste removal rate in *G. insensibilis*. Indeed, to our knowledge, there are no previous studies in amphipod crustaceans which quantify the amount of detritus removed from the environment by these organisms. It is also the first time that inorganic nitrogen lethal concentrations are calculated for this amphipod.

For all treatments tested and experiments, survival was always higher than 80%. This result confirms the adequacy of detritus as a diet in terms of survival and highlight the potential of *G. insensibilis* for mass culture, having no effect being promoted by density on survival. When compared with data from previous studies on *G. insensibilis* or other amphipods (Guerra-García et al., 2016; Hyne et al., 2005; Jiménez-Prada et al., 2020), survival appears in concordance with these authors, as they worked at very low densities (between 3 and 60 individuals L^{-1}) but performed longer experiments. Likewise, since high occupation percentages of the digestive tracts were observed, fish waste also appears as a suitable diet for *G. insensibilis* concerning intake and confirms that this species is a vegetal-and-animal detritivore (Constantini and Rossi, 1995).

In contrast, total consumption was similar in all treatments independently of the number of gammarids and no advantages were observed for removal rates when density was increased. Thus, DFC was clearly higher at low densities in both experiments. This is likely related with higher N-NH_4^+ concentrations (i.e. 9.08 and 7.77 mg L^{-1}) found in the high density treatments, being significantly correlated to their lower feeding rates. This is a normal pattern in aquaculture that has been already observed in other crustaceans (Naqvi et al., 2007) or fishes (Hargreaves and Tucker, 2004; Meade, 1985) and the use of an open culture system with water renewal or a recirculating system (RAS) equipped with a biofilter would make possible to reduce the high ammonium concentrations present in the water (van Rijn, 2013). Gammarids feeding and foraging behaviour also explains the lower DFC rates at higher densities (Cozzoli et al., 2022; Felten et al., 2008; Maranhão et al., 2001). A large number of specimens swimming and exploring their surrounding space could contribute to breakdown the

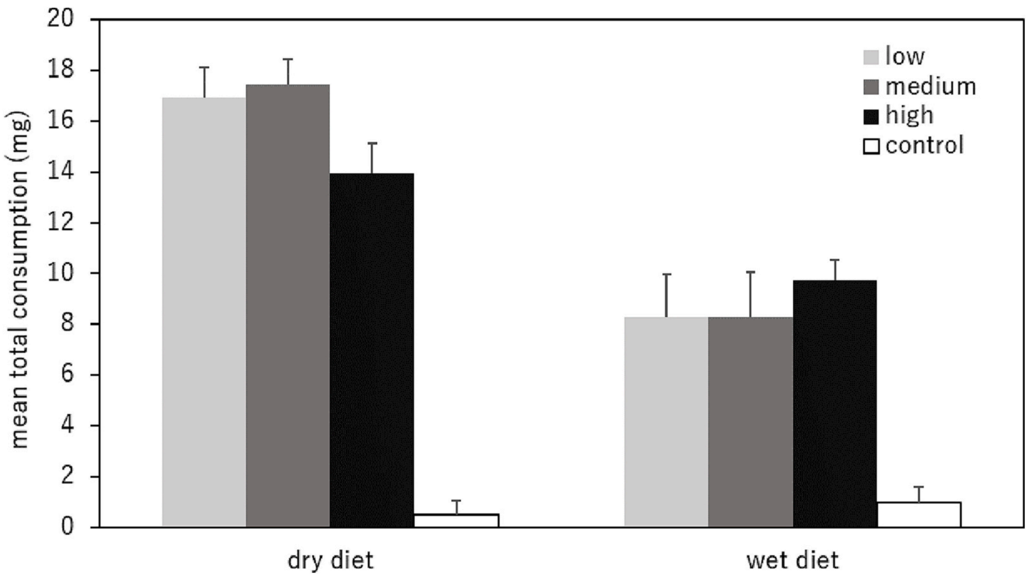

Fig. 4. Mean total consumption (mg of detritus in DW) of *G. insensibilis* reared at low (100 ind L^{-1}), medium (500 ind L^{-1}) or high (1000 ind L^{-1}) densities and feed dry (left) or wet (right) detritus-based diet. Data are mean ± SE (n = 6).

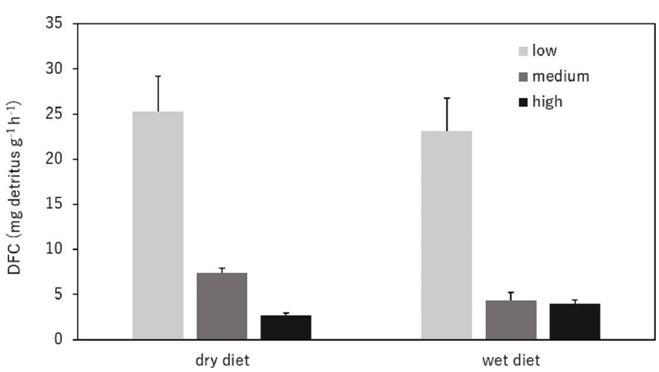

Fig. 5. Daily food consumption (mg g^{-1} h^{-1}) of *G. insensibilis* reared at low (100 ind L^{-1}), medium (500 ind L^{-1}) or high (1000 ind L^{-1}) densities and feed dry (left) or wet (right) detritus-based diet. Data are mean ± SE (n = 6).

detritus into finer particles that became less available for feeding, showing a food-item-size dependence of the intake. In another hand, ecological patterns of space use by amphipods indicate that a lower availability of space per individual causes a decrease in the dispersal ability and in locomotion costs, which would reduce the need for ingestion. However, all these observations are highly related to the small laboratory scale used in our experiments. Further research is needed in order to improve a *G. insensibilis* mass culture in open systems at high densities that could be valorised on IMTA frameworks.

Although daily food consumption was higher when gammarids were fed on a dry diet, the uneaten dry detritus presented a higher percentage of organic matter than the uneaten wet one, indicating that gammarids consumed a higher amount of organic matter when they were fed on a wet diet, which seems a more nourishing option. As observed by Zamora and Jeffs (2011), a conditioned diet (dry diet) could decrease the availability of OM in available food, increasing particle selection and ingestion rate and reducing nutrient absorption. Moreover, as no differences in the percentage of OM were found between the given detritus and the uneaten wet detritus, it seems that *G. insensibilis* consumes as much organic matter as inorganic matter, reflecting compensatory feeding, usually observed in deposit feeders when high quality food is scarce (Lopez and Levinton, 1987). This behaviour could also be explained as an effect of the level of hunger due to the starvation period (Alexander et al., 2015), showing a non-food-selective feeding after starvation. In general, this result did not show a clear advantage of the dry diet over the wet one. Working with wet mass data usually shows limits related to the ecological variability in the water content of organic matter (Madsen, 1993), but using DW is time consuming and impractical for larger experiments or industrial applications. In our work, a wet diet is proposed in order to perform more realistic experiments, where WW data can be transformed into DW for a better comparisons between experiments, as already advocated by Bickel and Perrett (2016) and Rice et al. (2012).

Mean removal rates reached 169.78 mg DW g^{-1}WW day^{-1} and 154.28 mg DW g^{-1}WW day^{-1} when gammarids were fed on dry or wet detritus respectively, at a low density (data units have been transformed for easier comparison with some studies). These rates are promising as they are comparable to those of other extractive species already employed on IMTA, even if there are few data available on this topic (summary of reviewed works on Table 6). Orr et al. (2014) showed that the sea urchin *Strongylocentrotus droebachiensis* fed on fish wastes can remove between 7.35 and 9.19 mg DW g^{-1}WW day^{-1} when reared at 1.6 Kg m^{-2}. Several studies have been performed on sea cucumbers: following Grosso et al. (2021), *Holothuria tubulosa* fed on sea urchin wastes removed between 1.32 and 2.86 mg DW g^{-1}WW day^{-1} (it is important to note that this species can be reared at a density around 0.3 Kg m^{-2}; Tolon et al., 2017). The brown sea cucumber *Australostichopus mollis* fed mussel wastes (Slater et al., 2009) or abalone wastes (Maxwell et al., 2009) removed 330 mg DW g^{-1}WW day^{-1} or 31–59 mg DW g^{-1}WW day^{-1}, respectively reared at 0.55 Kg m^{-2} and 0.25–0.5 Kg m^{-2} (for this last study, experiment was realized with isolated individuals). Concerning polychaetes, Honda and Kikuchi (2002) demonstrated that *Perinereis nuntia vallata* fed fish wastes can remove between 12 and 63 mg DW g^{-1}WW day^{-1} when reared at approximately 0.004 Kg m^{-2}.

It seems difficult to evaluate and compare different extractive species performance on IMTA as, according with Nederlof et al. (2022), their responses to aquaculture waste vary between studies (species, experimental methodology or waste nature and composition can contribute to these variations). It is interesting to relate these responses to the different species rearing densities in order to facilitate comparison for future researches and for the IMTA industry development (Table 6). Thus, results in the present study are in the range of the works cited above and are comparable or even higher to those for sea cucumber or polychaetes. In an other hand, there is very few information about how

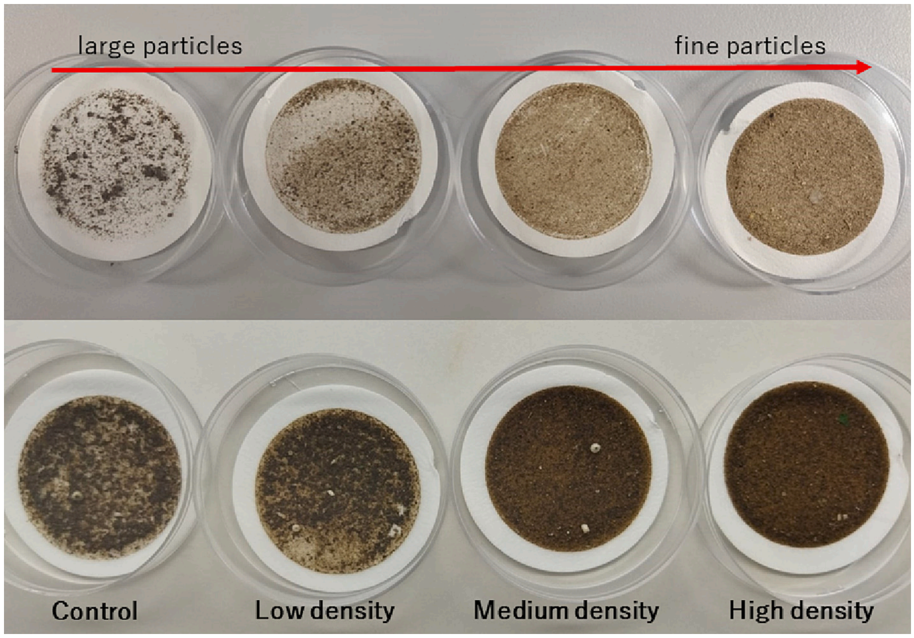

Fig. 6. Example of uneaten detritus after 48 h (freeze-dried samples above and non-freeze-dried samples below) in the four treatments: control (without animals), low density (100 ind L^{-1}), medium density (500 ind L^{-1}) and high density (1000 ind L^{-1}). Detritus particles are progressively finer as the density of animals increases.

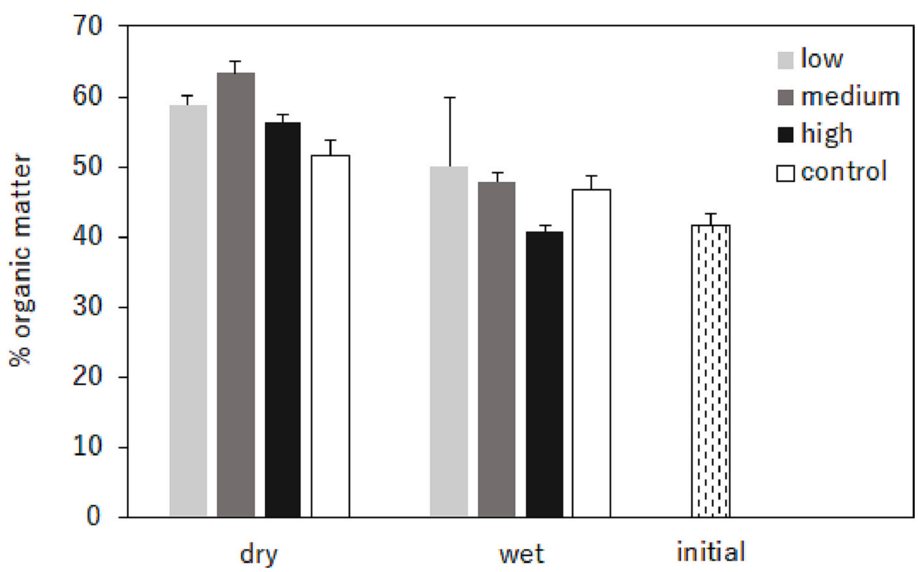

Fig. 7. Percentage of organic matter present in the uneaten detritus after the dry diet experiment and the wet diet experiment compared with the percentage of organic matter present in the initial detritus (not given to the animals). Low = 100 ind L^{-1}, Medium = 500 ind L^{-1}, High = 1000 ind L^{-1}. Data are mean ± SE (n = 6).

productive a waste-fed sea cucumber, polychaete or sea urchin culture is and the existing data also present a high variability between studies. For instance, the study of Grosso et al. (2021) on *H. tubulosa* showed a somatic growth rate of 0.13–0.28%, while Slater et al. (2009) observed a SGR in *A. mollis* of 30%. The population of the polychaete *Hediste diversicolor* fed on fish wastes increased by 90% in a 5 months IMTA trial (Marques et al., 2017). Regarding sea urchins, several studies have focused on their use on IMTA systems and its culture seems warranted at least at a semi-industrial scale (Shpigel et al., 2018). However, to our knowledge, there is no growth performance data on sea urchins fed aquaculture wastes. Further research is also needed in order to determine how productive a waste-fed culture of gammarids can be, as they have several potential applications on the aquaculture industry. Gammarids are already used as a partial replacement (10–20%) of fish meal (Harlıoğlu and Farhadi, 2018) and have a great nutritional profile as live food (Baeza-Rojano et al., 2013b). These studies pointed that today, gammarids products (live, dried flakes, or powdered) are derived from wild caught specimens and can fetch a high price. Therefore, in addition to the environmental benefits derived from the bioremediation of wastes, *G. insensibilis* aquaculture will also likely have great economic benefits.

Concerning LC_{50}, *G. insensibilis* appears to be more sensitive to ammonia (33.23 mg L^{-1}) than other marine amphipods (Kohn et al., 1994), but it still tolerates higher levels that those usually found in aquaculture facilities (Parra and Yúfera, 1999) and those tolerated by other high-value cultured crustacean species as the white shrimp

Table 3
Nitrogen compounds concentration in the seawater after 48 h experimentation. Low density = 100 ind L^{-1}, medium density = 500 ind L^{-1}, high density = 1000 ind L^{-1} (data are mean ± SE; n = 6).

		N-NH_4^+ (mg L^{-1})	N-NO_3^- (mg L^{-1})	N-NO_2^- (mg L^{-1})
Clean seawater		0.001	0.303	0.004
Dry diet experiment	low density	2.70 ± 0.16	0.22 ± 0.03	0.06 ± 0.01
	medium density	4.20 ± 0.67	0.21 ± 0.02	0.09 ± 0.004
	high density	9.08 ± 0.72	0.14 ± 0.02	0.05 ± 0.003
	control	0.84 ± 0.09	0.22 ± 0.03	0.08 ± 0.03
Wet diet experiment	low density	1.89 ± 0.13	0.22 ± 0.02	0.23 ± 0.02
	medium density	5.31 ± 0.36	0.11 ± 0.01	0.09 ± 0.01
	high density	7.77 ± 0.43	0.09 ± 0.02	0.06 ± 0.01
	control	0.95 ± 0.18	0.12 ± 0.01	0.11 ± 0.01

Litopenaeus vanameii (1.2 mg L^{-1}; Valencia-Castañeda et al., 2018). Indeed, this study highlighted that freshwater and brackish water species are more sensitive to nitrogen compounds, showing a salinity effect on the nitrogen LC_{50}. The same trend has also been observed in previous studies addressing nitrite LC_{50}, with a more toxic response being recorded at low salinity (Alonso and Camargo, 2006; Barbieri et al., 2016), which is in concordance with the present study, performed at high salinity (NO_2-LC_{50} 27.65 mg L^{-1}). Finally, *G. insensibilis* showed a higher tolerance to nitrate concentration (1308 mg L^{-1}), as nitrate is in general less toxic compared with ammonium and nitrite (Romano and Zeng, 2013). However, nitrate is the final step of the nitrification process and it can be accumulated in aquaculture systems, where it can reach levels of 500 mg L^{-1} (Camargo et al., 2005). With these tolerances,

Table 4
Equations describing the relationships between ammonium concentration (mg L^{-1}) in the seawater and DFC (mg g^{-1} h^{-1}) of *G. insensibilis* reared at three different densities and fed dry detritus-based diet or wet dry detritus-based diet.

	Equation	R^2	*F*	*P*
Dry diet experiment	y = −2.3988× + 25.039	0.4205	10.8858	< 0.01
Wet diet experiment	y = −1.2678× + 12.977	0.266	4.7123	< 0.05

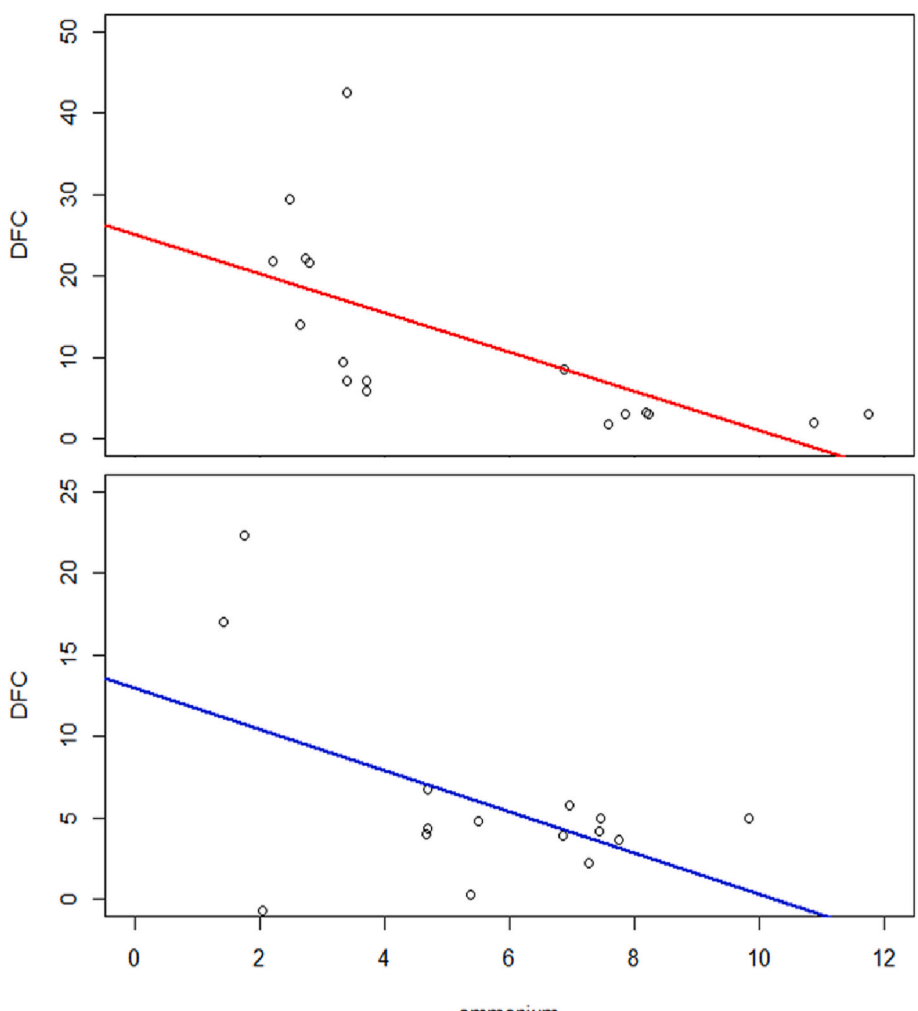

Fig. 8. Linear regressions describing the relationships between ammonium concentration (mg L^{-1}) in the seawater and daily food consumption (mg g^{-1} h^{-1}) of *G. insensibilis* reared at three different densities and fed dry detritus-based diet (red line) or wet detritus-based diet (blue line) (n = 6). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 5
Mean ± SE mortality (%) observed and LC_{50} (mg L^{-1}) for *G. insensibilis* exposed to three different concentrations of ammonium, nitrate and nitrite after 96 h ($n = 5$). Linear regressions describing relationships between nitrogen compounds concentrations and mortality are presented. Concentrations are expressed as mg L^{-1} of the chemical compound used for solutions and as mg L^{-1} of nitrogen from ammonium, nitrate or nitrite.

$[NH_4Cl]$ (mg L^{-1})	$[N\text{-}NH_4^+]$ (mg L^{-1})	mortality (%) mean ± SE	LC_{50}–96 h (mg L^{-1} $N\text{-}NH_4^+$)	equation	R^2	*F*	*P* value
90	23.78	30 ± 7.07					
120	31.7	44 ± 8.47	**33.23**	y = 0.5667× −22	0.44	10.237	0.0069
150	39.63	64 ± 5.48					
$[NaNO_3]$ (mg L^{-1})	$[N\text{-}NO_3^-]$ (mg L^{-1})		**LC_{50}–96 h (mg L^{-1} $N\text{-}NO_3^-$)**				
7000	1156	44 ± 6.19					
9000	1456	56 ± 4.65	**1308**	y = 0.0055× + 5.8333	0.34	6.656	0.0229
11,000	1816	66 ± 6.19					
$[NaNO_2]$ (mg L^{-1})	$[N\text{-}NO_2]$ (mg L^{-1})		**LC_{50}–96 h (mg L^{-1} $N\text{-}NO_2^-$)**				
150	30.44	42 ± 9.69					
300	60.87	52 ± 3.74	**39.79**	y = 0.0984× + 25.514	0.43	9.823	0.0079
500	101.46	76 ± 9.27					

Table 6
Summary table with relevant examples of studies on IMTA extractive species fed on wastes removal rate (listed from highest to lowest removal rates related to rearing density order).

Extractive species	Group	Removal rate (mg DW g WW^{-1} day^{-1})	Waste nature	Rearing density	Survival	Growth rate	Value added	References
Australostichopus mollis	Echinodermata: Holothuriida	330	Green-lipped mussel (*Perna canaliculus*)	0.55 Kg m^{-2}	87–100%	30%	Premium market food	Slater et al., 2009
Australostichopus mollis	Echinodermata: Holothuriida	31–59	Black-foot abalone (*Haliotis iris*)	0.25–0.5 Kg m^{-2}	no data	no data	Premium market food	Maxwell et al., 2009
Strongylocentrotus droebachiensis	Echinodermata: Echinacea	[1]7.35–9.19	Sablefish (*Anoplopoma fimbria*)	[1]1.6 Kg m^{-2}	no data	no data	[2]Premium market food	[1]Orr et al., 2014; [2]Castilla-Gavilán, 2018
Gammarus insensibilis	Crustacea: Amphipoda	154.98–169.78	Meagre (*Argyrosomus regius*) Senegalese sole (*Solea senegalensis*)	0.016 Kg m^{-2}	83.3–96.3%	no data	[1,2]Live feed, [3]fish meal substitute	Present work; [1]Baeza-Rojano et al., 2010; [2]Baeza-Rojano et al., 2013b; [3]Harlıoğlu and Farhadi, 2018
Holothuria tubulosa	Echinodermata: Holothuriida	[1]1.32–2.86	Purple sea urchin (*Paracentrotus lividus*)	[2]0.3 Kg m^{-2}	[1]100%	0.13–0.28%	[3]Premium market food	[1]Grosso et al., 2021; [2]Tolon et al., 2017; [3]Robinson and Lovatelli, 2015
Perinereis nuntia vallata	Annelida: Polychaeta	12–63	Japanese flounder (*Paralichthys olivaceus*)	0.004 Kg m^{-2}	50–70%	7.1–18.9%	Sport fishing bait	Honda and Kikuchi, 2002

waste-fed gammarids can be cultured in sub-lethal conditions without detriment to survival, but special attention should be paid to safe conditions avoiding a decrease in the intake. As discussed above, lower intakes were observed in the present study when the water presented high ammonium concentrations. More research could be done in order to investigate safe inorganic nitrogen concentrations for *G. insensibilis*.

5. Conclusions

It is likely that *G. insensibilis* has effectively a great potential as a detritivorous link for IMTA systems, being able to remove fish wastes and uneaten feeds. Further research is required to determine the effect of a fish-waste diet on *G. insensibilis* growth and reproduction in the long term. Authors recommend the use of non-conditioned wastes and open culture systems in order to perform high density cultures without exceeding safe ammonium concentration levels (no detriment to intake).

CRediT authorship contribution statement

M. Castilla-Gavilán: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization, Supervision. **J.M. Guerra-García:** Conceptualization, Visualization, Supervision, Project administration. **J.M. Moreno-Oliva:** Conceptualization, Methodology, Investigation. **I. Hachero-Cruzado:** Conceptualization, Visualization, Supervision, Project administration, Funding acquisition.

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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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