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Research Article

Ammonia excretion dynamics of the silverside (*Basilichthys microlepidotus*) during the juvenile-to-adult grow-out phase, under different body sizes and stocking densities in a recirculating aquaculture system (RAS)

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ABSTRACT. The Chilean silverside (*Basilichthys microlepidotus*) is an endemic freshwater species with emerging potential for aquaculture and conservation-oriented production. However, key bioengineering parameters required for system design and biomass management remain poorly defined. This study evaluated how body size and stocking density affect total ammonia nitrogen (TAN) excretion rates in a recirculating aquaculture system (RAS) under controlled conditions. Two stocking densities (1.1 and 1.8 kg m^{-3}) and two size ranges (7.8-77.8 g and 80-297 g), as well as their interaction, were tested using a factorial design with triplicate tanks per treatment. Mean TAN excretion rates ranged from 24.5 to 30.6 mg TAN kg^{-1} fish h^{-1}. A clear diurnal excretion pattern was observed, with peak TAN release occurring 2-4 h after feeding, followed by a gradual decline. Neither body size nor stocking density significantly affected TAN excretion within the evaluated ranges, indicating that these results provide bioengineering parameters for RAS design and scaling.

Key words: *Basilichthys microlepidotus*; excretion; ammonia; bioengineering; recirculating aquaculture system (RAS)

INTRODUCTION

Aquaculture is one of the most recently developed activities and holds significant potential for global growth. For this reason, it is playing a growing role in global food security: fisheries and aquaculture production reached a record 235 million tons in 2024, with aquaculture surpassing 100 million tons of aquatic animal production for the first time and becoming the principal driver of growth in aquatic food supply (FAO 2026). The FAO actively promotes culturing native species to enhance regional food security through aquaculture (FAO 2022, 2024).

In Chile, one of the most representative freshwater fish genera is *Basilichthys*, belonging to the subfamily Atherinopsidae. Among its most notable endemic species is *Basilichthys microlepidotus* (Jenyns, 1841), which is distributed from the Huasco River (28°30'S) to the Aconcagua River (32°20'S) (Dyer 2000). A phylogenetic study suggests that *B. australis* may be the same species as *B. microlepidotus*, with a distribution extending south to Chiloé Island (42°18'S) (Véliz et al. 2012). This species faces multiple threats, including invasive species, anthropogenic pressure from overexploitation, and habitat fragmentation and degradation (Vega-Retter et al. 2020, Cortés-Miranda et al. 2022). It is currently classified as "Vulnerable" to extinction (Colin 2023). As a conservation measure, the government established a 15-year harvesting ban, effective from October 6, 2011, to October 5, 2026

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(Ministerio de Economía, Fomento y Turismo 2011). Therefore, it is necessary to develop programs and tools to propagate and conserve this species by implementing hatcheries that can play a key role in reducing its vulnerability, at least temporarily. At the same time, broader ecological issues are addressed (Webb et al. 2023, Treanor et al. 2024). In this context, this species' culture could support restocking-oriented aquaculture to recover depleted natural populations (Rojas et al. 2016).

Regarding aquaculture-oriented research on *B. microlepidotus*, only a few studies have been conducted. Among them, studies describing sexual differentiation and evaluating gonadal development to induce spawning in captivity stand out (Huaquin et al. 1984), as does the characterization of reproductive cycle duration (Comte & Vila 1992). Reproduction of adult specimens in captivity has been achieved, with natural spawning occurring after approximately 18 months of culture under temperature conditions of ~15-22°C and dissolved oxygen levels of ~7.0-12.0 mg L^{-1}, once reproductive maturity was reached (~23-24 cm total length) (Rojas et al. 2016). However, the nutritional requirements, optimal rearing conditions, bioengineering parameters, and system design requirements for its culture remain unknown.

The two most important bioengineering parameters in aquaculture are considered to be the oxygen consumption rate and the ammonia excretion rate, which are critical aspects for water quality management in aquaculture systems, as they serve as direct indicators of fish metabolism (Timmons et al. 2002, Chabot et al. 2016, Svendsen et al. 2016). Moreover, information on nitrogenous waste excretion rates is essential for estimating maximum biomass or stocking density, designing recirculating aquaculture systems (RAS), setting water flow rates, and sizing biofilters (Leung et al. 1999a, Tng et al. 2008). Ammonia, the main byproduct of protein metabolism, is primarily excreted through the gills and is highly toxic in its un-ionized form (NH_3), which can negatively affect growth, immune function, antioxidant capacity, and survival (Randall & Tsui 2002, Xu et al. 2021, Sun et al. 2024). The proportion of un-ionized ammonia increases with rising temperature, pH, and salinity (Bower & Bidwell 1978, Colt & Armstrong 1981). In RAS, ammonia accumulation can be mitigated through nitrification and denitrification processes carried out by specific bacterial communities (Timmons & Ebeling 2010). These bacteria convert ammonia to nitrite and then to nitrate, which is ultimately removed from the system as gaseous nitrogen (Guo et al. 2025).

The ammonia excretion rate is influenced by both exogenous and endogenous factors, such as diet, oxygen and ammonia concentration, temperature, pH, stocking density, circadian rhythm, environmental conditions, species, body mass, trophic groups, and life stage (Peck et al. 2003, Bolner & Baldisserotto 2007, Green & Hardy 2008, Lam et al. 2008, Tng et al. 2008, Ip & Chew 2010, García et al. 2012, Nerici et al. 2012, Souza-Bastos et al. 2014, Bucking 2017, Xu et al. 2020, Godoy-Olmos et al. 2022, Ott et al. 2025).

Stocking density is another critical factor in aquaculture systems because it directly affects water quality, feed efficiency, growth performance, and overall fish welfare (Roy et al. 2024, Metwaly et al. 2025). High-protein diets and increased stocking densities increase the system's ammonia load, requiring continuous monitoring to prevent toxicity (Hargreaves & Tucker 2004, Ott et al. 2024). Given the limited information on ammonia excretory metabolism in *B. microlepidotus*, this study aimed to evaluate ammonia excretion rates under two stocking densities, two body size classes, and their interaction. The results provide valuable insights for designing more efficient culture systems and may help develop water quality guidelines for natural systems, considering the influence of native species.

MATERIALS AND METHODS

Ethical considerations

For species capture and transport, the Subsecretaría de Pesca y Acuicultura granted a research fishing permit E-2023-716, with oversight from the Servicio Nacional de Pesca y Acuicultura. In addition, all animal procedures conducted in this study complied with the guidelines for the care and use of laboratory animals. The Ethics and Biotechnology Committee of the Universidad Católica del Norte (UCN), Coquimbo, Chile, reviewed and approved the study.

Experimental animals

Fish were obtained by net capture in the Elqui River and transported to the Central Marine Aquaculture Laboratory of the Faculty of Marine Sciences, UCN, Coquimbo, Chile (29°57'S-71°21'W). The animals were subsequently transferred from cultivation ponds to circular cement tanks (12,000 L) with constant water flow (5 L s^{-1}) and continuous aeration to acclimate them to the experimental conditions. The fish were acclimated for one month to the culture conditions, after which 151 fish were selected and distributed into four experimental groups defined by the combination

of two stocking densities and two size ranges: low density-small size (D1S1), low density-large size (D1S2), high density-small size (D2S1), and high density-large size (D2S2). Stocking densities were 1.1 kg m^{-3} for the low-density condition (D1) and 1.8 kg m^{-3} for the high-density condition (D2), while size ranges were 7.8-77.8 g for S1 and 80-297 g for S2. Each combination was performed in triplicate, with fish randomly distributed among the culture tanks. The number of fish and total biomass varied among replicate tanks according to individual body size and the target stocking density: D1S1 tanks held 18-19 fish (18.3 ± 0.6) with a total biomass of 425.8-504.4 g (466.5 ± 39.4 g); D1S2 tanks held 3-6 fish (4.7 ± 1.5) with a total biomass of 536.8-591.8 g (566.1 ± 27.7 g); D2S1 tanks held 21-23 fish (22.3 ± 1.2) with a total biomass of 845.5-866.0 g (858.0 ± 10.9 g); and D2S2 tanks held 5 fish in all three replicates with a total biomass of 719.0-781.1 g (758.8 ± 34.5 g). The relatively wide body-weight ranges used to define each size class (7.8-77.8 g for S1 and 80-297 g for S2) reflect the natural size-frequency distribution of wild-caught *B. microlepidotus* available from the Elqui River, since no hatchery-reared, size-graded cohorts were available for this vulnerable, harvest-banned species. A comparably broad size-class approach for wild-caught Atheriniformes has been used previously by our research group (Méndez et al. 2026a), and the two-way ANOVA design applied here explicitly tests whether this within-class variability translates into a detectable size effect on total ammonia nitrogen (TAN) excretion.

Experimental design

To determine the ammonia excretion rate, a RAS with a total volume of 7 m^3 was used. The system consisted of 12 cylindrical-conical tanks (each 500 L in capacity) with dimensions of 1 m in diameter and 1 m water column height. The tanks were connected to a 1,000 L settling tank and a sand filter (10.8 m^3 h^{-1}; Emaux, model P500). The system also included a 200 L submerged moving-bed biofilter filled with Kaldnes media (specific surface area: 600 m^2 m^{-3}) and a UV sterilization unit (20 W; BioLight, model WUV20-1). All components were connected by a centrifugal pump (1 HP; ESPA, model SILEN 100) with a maximum flow rate of 14 m^3 h^{-1}. Each rearing tank was equipped with a centrally positioned Aero-Tube™ air diffuser hose and a hydraulic aeration pipe connected to a 2.5 HP blower (Sweetwater®). A natural photoperiod and rearing conditions similar to those used in culture systems were maintained. The municipal-supplied freshwater was aerated continuously for 24 h to ensure dechlorination. The absence of residual chlorine was verified using a free chlorine checker HI701 (Hanna Instruments). Additionally, the tanks were cleaned daily to remove uneaten feed and fecal residues.

The experimental design included three replicate rearing tanks per treatment (n = 3 tanks per treatment), for a total of 12 experimental tanks (4 treatments × 3 replicates). This design allowed evaluation of the main effects of stocking density and body size, as well as their interaction, on ammonia excretion rates. The three 8 h batch blocks described below (comprising one continuous 24-h sampling cycle per tank) were repeated in triplicate per tank, separated by 8 h of normal recirculation between replicate cycles, to obtain stable estimates of TAN excretion.

Determination of ammonia excretion

Measurements were conducted in three 8-h blocks (08:00-16:00, 16:00-00:00, and 00:00-08:00 h), in triplicate under closed-flow conditions in each rearing tank, to determine the diurnal cycle of ammonia excretion rate under culture conditions across the four treatments. The ammonia excretion rate was determined using a mass balance approach under closed-flow (batch) conditions (Merino et al. 2007). During batch operation, recirculation was halted, and a 300 W Sera™ heater was placed in each rearing tank to maintain a constant temperature, while aeration was kept active. Water samples (10 mL) were collected in triplicate from each tank at 0 h and subsequently every 2 h while the system operated under batch conditions. At the end of each 8-h experimental period, the system was returned to normal recirculating operation. Normal recirculation and biofilter connection were re-established for 8 h between consecutive replicate cycles, allowing water-quality parameters to return to baseline before the next cycle began.

TAN concentration was quantified colorimetrically using the salicylate method (Hach Method #8155 low-range: 0.01-0.50 mg TAN L^{-1}) employing ammonium salicylate and ammonium cyanurate reagents, following the procedure described by Hach for the Hach DR3900 spectrophotometer. Instrument verification was performed using certified reference standards (Hach AccuVac® ampules). All samples were analyzed in triplicate; samples with a coefficient of variation (CV) exceeding 5% were re-analyzed. Un-ionized ammonia (NH_3, mg N L^{-1}) was calculated from TAN, using the corresponding pH and temperature values, according to the formula proposed by Emerson et al. (1975). Dissolved oxygen, temperature, and pH were recorded *in situ* at each TAN sampling point (i.e. every 2 h) using the same multiparameter probe, so that the

pH and temperature values used to calculate un-ionized ammonia corresponded to the same sampling time as each TAN measurement. The TAN excretion rate was estimated by a mass balance analysis under conditions similar to those of normal culture. The tanks were considered as discontinuous systems open to the atmosphere. To calculate the specific hourly TAN excretion rate under culture conditions, the following equation was used:

$$\text{TAN excretion rate (mg TAN kg}^{-1}\text{ fish h}^{-1}) = \left(\frac{Ct1-Ct0}{t1-t0}V\right)/B \quad (1)$$

where: Ct1 is the TAN concentration at time t1; Ct0 is the concentration at time t0; V is the tank volume in liters (L); and B is the total fish biomass.

During the experimental period, dissolved oxygen, temperature, and pH were measured using a Hach HQ40d multiparameter probe. Nitrate levels were recorded weekly using Method 8039 (cadmium reduction method, 0 to 30.0 mg L^{-1} NO_3-N), and nitrite was measured using Method 8507 (diazotization method, 0 to 0.300 mg L^{-1} NO_3-N) (Hach Company 2003). Total alkalinity was determined by titration using the Bromophenol Blue method with the HI3811 alkalinity test kit (Hanna Instruments). Total suspended solids (TSS) were measured following Method 2540D of the American Public Health Association (APHA 1995).

Feeding regime

Fish were fed a commercial diet (Biomar ORB Intro) containing 50.13-54.13% crude protein, 18.5-20% lipids, a maximum of 10% moisture, and up to 12% ash. These ranges reflect the manufacturer's guaranteed analysis for this commercial diet; the same feed was used across all treatments and size classes throughout the trial. Fish were fed once daily, as a single meal, between 08:00 and 09:00 h. The feeding rate was set at 1% of body weight. Because the TAN-excretion trial itself consisted of three replicate 24 h sampling cycles per tank separated by 8 h recirculation periods (see Determination of ammonia excretion), biomass and stocking density were effectively constant over this short measurement window, and no biologically meaningful weight gain would be expected; the biweekly ration adjustment therefore reflects standard husbandry practice during the longer rearing/ acclimation period that preceded the trial, rather than an adjustment applied during the trial itself.

Statistical analysis

To compare the results, a two-way analysis of variance (ANOVA) was performed. Temporal variations in TAN excretion were analyzed using a repeated-measures ANOVA, with time (h) as the within-subject factor and tank as the experimental unit. When sphericity was violated, Greenhouse-Geisser corrections were applied. *Post-hoc* pairwise comparisons were performed using Tukey's HSD test. Before the analysis, the assumptions of normality (Shapiro-Wilk test) and homogeneity of variances (Levene's test) were verified. A significance level (α) = 0.05 was used for all statistical tests (Sokal & Rohlf 1969). All data are presented as mean ± standard deviation (SD). Statistical analyses were conducted using R software, version 3.5.3 (R Core Team 2020).

RESULTS

Water quality

The average values of the water quality parameters are presented in Table 1. Overall, the physicochemical parameters in the culture tanks were similar across treatments, indicating no interference with the ammonia excretion data. Water conditions such as temperature, dissolved oxygen, and pH remained consistent across treatments during the experimental period, with no significant differences ($P > 0.05$). The average concentrations of nitrogenous compounds in the recirculating aquaculture system were as follows: TAN (NH_3-N), 0.03 ± 0.01 mg L^{-1}; nitrite (NO_2-N), 0.06 ± 0.01 mg L^{-1}; and nitrate (NO_3-N), 18.17 ± 0.06 mg L^{-1}. Alkalinity averaged 202 ± 1.73 mg CaCO3 L^{-1}, and TSS were 12.82 ± 4.56 mg L^{-1}.

Ammonia excretion rate

TAN excretion data were highly consistent across replicate measurement cycles within each tank, supporting the reliability of the treatment means. During the experimental period, no mortality was recorded in any treatment group. The daily ammonia excretion rate (TAN, mg TAN kg $fish^{-1}$ h^{-1}) under routine conditions was 30.6 ± 41.1, 29.2 ± 33.6, 24.5 ± 29.1, and 24.6 ± 27.0 for D1S1, D1S2, D2S1, and D2S2, respectively. No significant differences were observed in ammonia excretion rates among treatments ($P > 0.05$) (Fig. 1). The relatively high variability observed in hourly TAN excretion values reflects pronounced diurnal fluctuations associated with postprandial metabolism rather than inconsistencies among experimental replicates.

Table 1. Water quality parameters of silverside *Basilichthys microlepidotus* in a recirculating aquaculture system. D1S1: low density-small size, D1S2: low density-large size, D2S1: high density-small size, and D2S2: high density-large size.

Parameters	D1S1	D1S2	D2S1	D2S2
Temperature (°C)	20.07 ± 0.11	20.39 ± 0.33	20.07 ± 0.06	20.06 ± 0.11
Dissolved oxygen (mg L^{-1})	9.14 ± 0.07	9.17 ± 0.14	9.08 ± 0.08	9.16 ± 0.09
Oxygen saturation (%)	98.23 ± 0.81	98.68 ± 1.67	97.46 ± 1.15	98.44 ± 1.04
pH	8.38 ± 0.05	8.38 ± 0.05	8.37 ± 0.05	8.37 ± 0.06

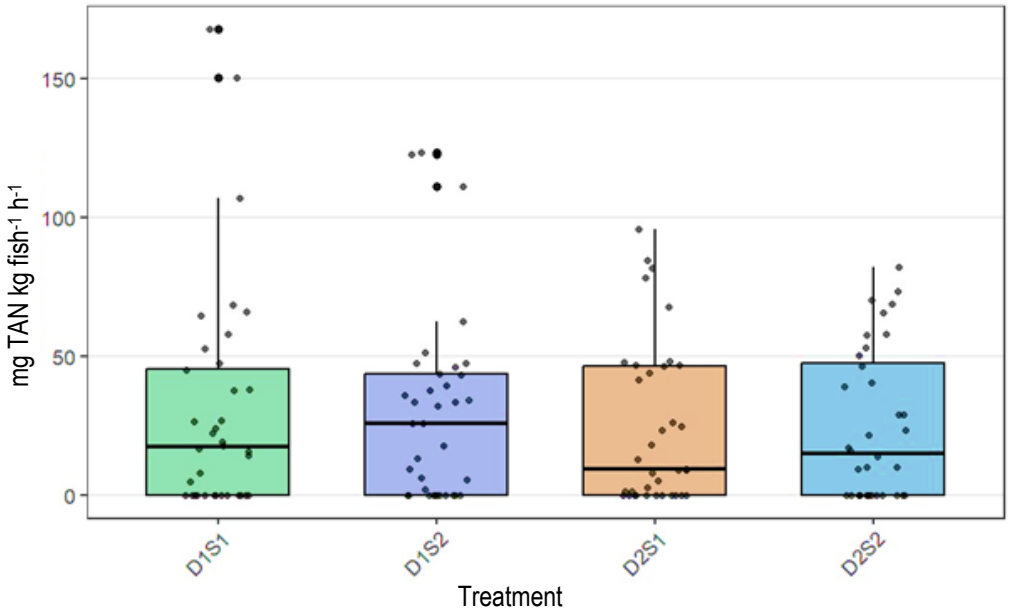


Figure 1. Total ammonia excretion rate (mg TAN kg $fish^{-1}$ h^{-1}) in silverside *Basilichthys microlepidotus* subjected to different treatments. D1S1: low density-small size, D1S2: low density-large size, D2S1: high density-small size, and D2S2: high density-large size.

A diurnal pattern of TAN excretion was observed across all groups, characterized by two main peaks: the first occurring 2 to 4 h (10:00-14:00 h) after feeding, and a second peak at approximately 02:00 h. However, significant differences were detected only in the excretion rates recorded at 08:00 h (Fig. 2). Excretion values fluctuated between 0 and 167.8 mg TAN kg $fish^{-1}$ h^{-1} throughout the day.

DISCUSSION

All water physicochemical parameters were maintained within the ranges recommended for this species (Rojas et al. 2016, Rojas & Espinoza 2018). Dissolved oxygen concentration and saturation directly influence fish physiology and metabolic rates; therefore, maintaining oxygen saturation near 100% is recommended (Thorarensen et al. 2017). For most fish species, dissolved oxygen levels above 5 mg L^{-1} are suggested to ensure adequate feed intake and growth (Zhou et al. 2024). Oxygen is also essential for nitrifying bacteria involved in nitrification (Timmons & Ebeling 2010). High pH values (>8.8), combined with elevated alkalinity, can disrupt critical metabolic processes, inducing respiratory and metabolic alkalosis in freshwater fish (Yao 2024). High pH and alkalinity levels may inhibit ammonia excretion (Parra & Baldisserotto 2007, Zhao et al. 2024). In this study, pH values ranged from 7.0 to 9.0 and remained comparable between daytime and nighttime sampling points, which favors nitrifying bacteria (Chen et al. 2006). An alkalinity level between 100 and 150 mg CaCO3 L^{-1} is recommended because it promotes nitrification and prevents large pH fluctuations within the system (Van Wyk & Scarpa 1999, Ebeling et al. 2006). Because pH

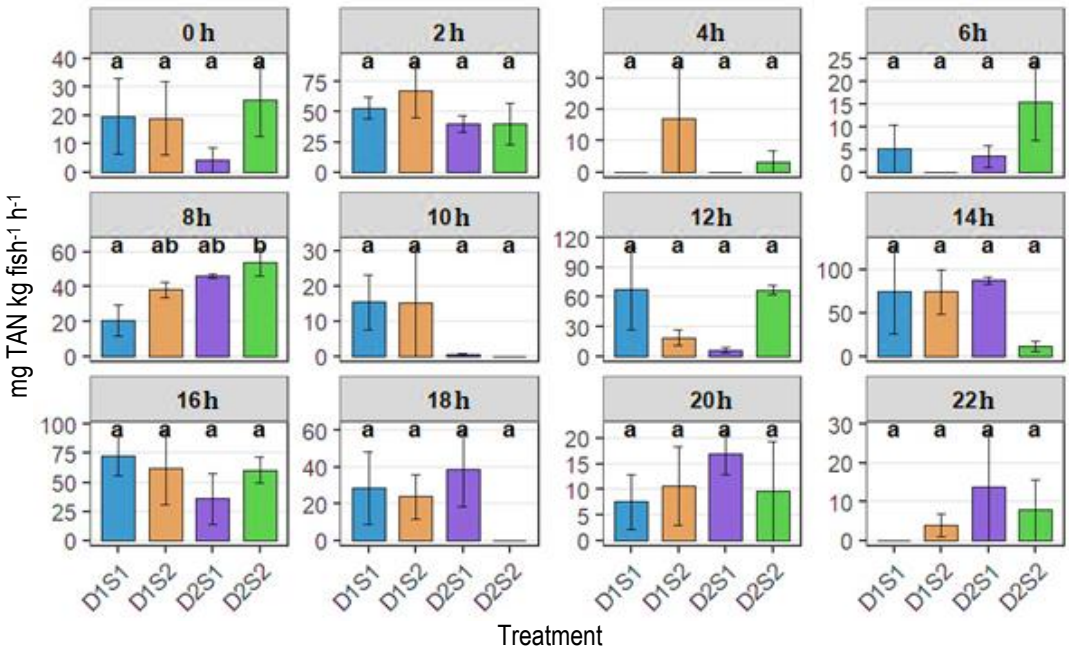


Figure 2. Daily profile of total ammonia nitrogen (TAN) excretion (mg TAN kg fish^{-1} h^{-1}) in silverside *Basilichthys microlepidotus* reared in a recirculating aquaculture system (RAS). Bars represent mean values ± standard deviation; panel labels indicate hours (h) after the start of the trial. Different letters indicate significant differences ($P < 0.05$). D1S1: low density-small size, D1S2: low density-large size, D2S1: high density-small size, and D2S2: high density-large size.

and temperature remained stable and within a narrow range throughout the trial (Table 1), the postprandial and nocturnal TAN/NH_3 peaks described below are unlikely to be primarily driven by short-term pH shifts; instead, they more plausibly reflect the metabolic dynamics of protein catabolism discussed in the following sections.

Ammonia levels remained within safe ranges for the organisms, preventing potential effects on excretion. The highest recorded values during the experiment were 0.61 mg TAN L^{-1} and 0.04 mg NH_3 L^{-1}. Freshwater fish are less sensitive to ammonia than marine fish, and the mean acute toxicity values reported are 2.79 mg NH_3 L^{-1} for freshwater species and 1.86 mg NH_3 L^{-1} for marine species (Randall & Tsui 2002); in a chronic toxicity test with tilapia (*Oreochromis niloticus*), un-ionized ammonia is recommended to remain below 0.89 mg L^{-1} (Sánchez et al. 2023).

No previous data have been reported on ammonia excretion for this species, and no bioengineering metabolic parameters exist for any other Chilean Atherinopsidae; this study therefore represents the first quantitative TAN excretion record for the family under RAS conditions. The mass-balance protocol used here follows established methodology (Merino et al. 2007, Nerici et al. 2012), and the same approach has since been validated by our research group for other RAS-reared taxa, including the pejerrey *Odontesthes bonariensis* (Méndez et al. 2026a) and the freshwater prawn *Macrobrachium caementarius* (Méndez et al. 2026b).

The average TAN excretion rates observed in *B. microlepidotus* (24.5-30.6 mg TAN kg^{-1} fish h^{-1}) were markedly higher than those reported for its congener, the Argentine silverside *O. bonariensis*, under comparable RAS conditions (4.6-10.8 mg TAN kg^{-1} fish h^{-1}; Méndez et al. 2026a), the only other Atheriniformes for which comparable bioengineering data exist, and were also higher than most other freshwater teleosts examined for comparison. In juveniles of *Leporinus macrocephalus* (2.92-17.98 g), values ranged from 2.24 to 38.02 mg TAN kg^{-1} fish h^{-1} (Maltez et al. 2016); in *Tinca tinca* juveniles (15-19 g), between 1.95 and 8.80 mg TAN kg^{-1} fish h^{-1} (Zakęś et al. 2006); in *Perca fluviatilis* L. (44.8 to 336.2 g), 5.2 to 13.8 mg TAN kg^{-1} fish h^{-1} (Stejskal et al. 2009), and in *Piaractus brachypomus* (46.1-520.0 g), from 79.2 to 177.3 mg TAN kg^{-1} fish h^{-1} (Ruales & Vásquez-Torres 2014). Among marine species reared under aquaculture conditions, *Paralichthys californicus* exhibited excretion

rates ranging from 3.4 to 4.3 mg TAN kg^{-1} fish h^{-1} (Merino et al. 2007), while *Seriolella violacea* showed values between 10.7 and 16.4 mg TAN kg^{-1} fish h^{-1} in RAS systems (Nerici et al. 2012). Variations among studies can be attributed to multiple factors, including experimental methods, feed type and composition, feeding rate, stocking density, photoperiod, salinity, dissolved oxygen, fish size, trophic group, and pH, all of which significantly influence fish growth and bioenergetics (Pilati & Vanni 2007, Bucking 2017, Godoy-Olmos et al. 2022, Ott et al. 2025).

Regarding diurnal ammonia excretion, teleost fish generally increase excretion after feeding, driven by digestive, absorptive, and assimilatory processes, as well as hepatic catabolism of amino acids not used for protein synthesis. These amino acids are converted via transamination, followed by glutamate deamination (Ballantyne 2001, Tng et al. 2008). Our results are consistent with previous studies reporting a significant increase in ammonia excretion rates after feeding, peaking between 2 and 11 h postprandially, followed by a progressive decline until the next feeding event (Dosdat et al. 1996, Phillips et al. 1998, Leung et al. 1999a, Zakęś et al. 2006, Fang et al. 2017, Guo et al. 2025, Ott et al. 2025). This postprandial increase is particularly important as it provides insight into managing TAN loads under RAS conditions.

During the diurnal ammonia excretion cycle, notable differences were observed around 08:00 h, which may be attributed to several factors, including an increase in metabolic rate related to the circadian rhythm, leading to higher amino acid catabolism (Tng et al. 2008). Laboratory activities at this time may also induce disturbances and stress, increasing swimming activity and consequently ammonia production (Whiles et al. 2009). Another potential mechanism involves cellular protein degradation; however, this process depends on the activation of the ubiquitin-proteasome and autophagy-lysosome pathways, which are highly regulated and complex (Bucking 2017).

Stocking density is a critical factor in aquaculture, as it directly affects survival, growth, water quality, and production efficiency in fish and shrimp farming (Azhar et al. 2022, Méndez et al. 2024, Metwaly et al. 2025). However, in intensive systems, increased density is limited by the accumulation of toxic metabolic wastes, which deteriorate water quality and compromise the health of cultured organisms (Adineh et al. 2019). Studies examining how stocking density affects ammonia excretion are scarce. In hybrid striped bass (*Morone saxatilis* × *M. chrysops*), ammonia excretion increased with biomass under high-density conditions (0.7, 1.4, and 2.8 kg m^{-3}) (Liu et al. 2009).

High stocking density, acting as a form of chronic stress, has been shown to alter various physiological and biochemical blood parameters in fish (Jia et al. 2022, Köprücü et al. 2024). This effect likely relates to intensified social interactions at higher densities, which increase the energy expenditure required to maintain physiological balance and cope with stress (Diao et al. 2023, 2024). In this context, physiological stress from crowding and competition for food and space may also reduce growth (Maucieri et al. 2019). In our study, stocking density did not significantly affect TAN excretion within the range tested (1.1-1.8 kg m^{-3}). Therefore, stocking density appears to be species-specific and life-stage dependent (Li et al. 2021, Mugwanya et al. 2022).

Water temperature and fish body size are considered key factors influencing growth and bioenergetics in aquatic organisms (Sun & Chen 2014, Vanni & McIntyre 2016). Body mass strongly affects metabolic processes, following a universal biological principle: metabolic rate per unit body weight generally decreases as body size increases (Gillooly et al. 2001). This pattern aligns with Kleiber's law, which states that larger animals exhibit lower mass-specific metabolic rates than smaller ones (Leiva & Schramski 2022). This relationship has been confirmed in fish, where ammonia excretion rates decrease with increasing body weight, indicating that smaller individuals excrete more ammonium per gram of body mass than larger ones (Dosdat et al. 1996, Jatteau 1997, Zakęś et al. 2007, Stejskal et al. 2009, Shrestha et al. 2023). The fact that larger fish excrete less ammonia per unit of body mass suggests a lower metabolic rate (Leung et al. 1999b, Tao et al. 2025). Smaller juvenile fish likely experience higher oxidative catabolism to meet their energy requirements compared with adults, which may reflect increased rates of protein catabolism during active growth (Bucking 2017). However, in our study, no significant differences related to body size were observed, consistent with previous findings in *Phoxinus phoxinus*, *Tinca tinca* and *Paralichthys californicus*, where body weight did not significantly affect nitrogen excretion (Cui & Wootton 1988, Merino et al. 2007, Nowosad et al. 2013). Thus, it can be proposed that the efficiency of dietary energy utilization slightly improves with increasing body size within certain ranges. This trend may occur in a wide variety of fish species (Sun & Chen 2014). Another possible explanation is that dietary energy can be allocated to somatic growth or reproductive maturation, rather than used solely for energy production (Xie et al. 1997, Yigit et al. 2003). Excess dietary protein may also increase ammonia excretion, as catabolizing surplus amino acids

incurs additional energy costs (Green & Hardy 2008). Consequently, the lower the proportion of dietary protein used for energy, the greater the nitrogen retention in tissues, indicating more efficient utilization of dietary protein for growth (Guo et al. 2012, Oliveira-Cunha et al. 2022). In this study, a commercial trout feed was used, containing 50.13-54.13% crude protein, rather than a diet specifically formulated for *B. microlepidotus*. Therefore, trials are needed to determine this species' optimal protein requirements at different developmental stages to improve production efficiency. Nutrient load reduction and feed efficiency improvement could be achieved through refined nutritional strategies and associated technologies, including the formulation of diets with optimal protein-to-energy ratios, balanced essential amino acids, highly digestible ingredients, functional additives, and life stage-specific formulations tailored to environmental conditions (Neto & Ostrensky 2013).

The absence of significant body-size and stocking-density effects on TAN excretion in *B. microlepidotus* has a direct practical implication for RAS design: within the ranges evaluated here (7.8-297 g; 1.1-1.8 kg m^{-3}), ammonia loading can be estimated from total cultured biomass alone, without separate correction factors for size class or crowding level, simplifyng the system sizing relative to species in which body size or density more strongly modulate mass-specific excretion, such as the congeneric *O. bonariensis* (Méndez et al. 2026a), and is broadly consistent with reports for other freshwater teleosts in which nitrogen excretion scales primarily with feed intake rather than with body size once fish are beyond early larval stages (Cui & Wootton 1988, Nowosad et al. 2013), even though mass-specific ammonia output is generally expected to decrease with body size and increase with temperature across teleosts in general (Zhang et al. 2004).

Sizing the biological filtration component of a RAS requires matching the stock's ammonia production rate to the biofilter media's areal nitrification capacity under the system's operating conditions. Surface-specific TAN removal rates reported for submerged and moving-bed biofilters (MBBR) in freshwater RAS typically range from 0.2 to 0.6 g TAN m^{-2} d^{-1}, depending on media type, hydraulic loading, biofilm maturity, and, critically, water temperature (Chen et al. 2006, Rusten et al. 2006). Using Kaldnes™ K1 media, Rusten et al. (2006) reported a maximum TAN removal rate of 0.30 g TAN m^{-2} d^{-1} at a loading of 0.45 g TAN m^{-2} d^{-1} and 9°C, while Suhr & Pedersen (2010) measured a surface-specific removal rate of 0.27 ± 0.02 g TAN m^{-2} d^{-1} in MBBR treating cold (8°C) effluent from a large commercial outdoor rainbow trout RAS. Because nitrification kinetics are strongly temperature-dependent - Kinyage & Pedersen (2016) reported a six-fold increase in surface-specific TAN removal (0.04 to 0.25 g TAN m^{-2} d^{-1}) across a 6-30°C range in freshwater MBBR - the warmer temperatures used in the present study (~20°C) would be expected to support higher areal nitrification rates than the conservative, cold-water values reported above, provided the biofilter has reached biofilm maturity and organic loading is controlled (Chen et al. 2006, Ebeling et al. 2006).

Applying these bioengineering principles to the present dataset, a production module holding 100 kg of *B. microlepidotus* biomass would generate a daily TAN load of 58.8-73.4 g TAN d^{-1} (based on the 24.5-30.6 mg TAN kg^{-1} h^{-1} routine range measured here). Sizing a MBBR at a conservative, cold-water areal removal rate of 0.3 g TAN m^{-2} d^{-1} (Rusten et al. 2006, Suhr & Pedersen 2010) would require 196-245 m^2 of protected media surface area, equivalent to 0.33-0.41 m^3 of Kaldnes™ K1-type media (600 m^2 m^{-3}). If, on the other hand, one assumes the higher rate of 0.6 g TAN m^{-2} d^{-1} -reported for submerged filters in freshwater (Chen et al. 2006), and more representative of the ~20°C conditions used in this study, the required area would be approximately 98-122 m^2 (0.16-0.20 m^3 of media). Because peak postprandial TAN excretion reached 167.8 mg TAN kg^{-1} h^{-1} (more than five times the routine rate), and because nitrification performance can be transiently compromised by temperature fluctuations, incomplete biofilm maturation, or elevated organic loading (Zhu & Chen 2001, Kinyage & Pedersen 2016), a design safety factor of 1.5-2.0x over the routine-rate estimate is recommended, consistent with general RAS design guidance (Timmons & Ebeling 2010).

From an applied perspective, these bioengineering parameters are relevant to two related production contexts for *B. microlepidotus*. First, for restocking-oriented hatchery programs -a priority given the species' vulnerable conservation status and the ongoing harvesting ban (Ministerio de Economía, Fomento y Turismo 2011)- biofilter sizing based on the excretion rates reported here would allow hatchery managers to maintain safe water-quality conditions for broodstock and juveniles without over- or under-building biofiltration capacity, reducing both capital cost and the risk of ammonia-related stress during early life stages. Second, for small- and medium-scale semi-intensive diversification ventures, the finding that TAN excretion is stable across the evaluated size and density ranges means producers can scale stocking density upward,

within the range tested (up to 1.8 kg m^{-3}), without a disproportionate increase in mass-specific ammonia loading, simplifying operational planning. These implications should be interpreted within the boundaries of the conditions tested here (single diet, single temperature, sizes up to 297 g, densities up to 1.8 kg m^{-3}); extrapolation to larger-scale commercial densities, other life stages, or other temperatures should be validated experimentally, ideally alongside oxygen-consumption measurements to close the bioenergetic picture for this species. However, experimental methods, feed type and composition, feeding rate, stocking density, photoperiod, salinity, dissolved oxygen, and pH can also influence ammonia excretion in fish. Future studies should quantify nitrogen losses through fecal output and the fraction excreted as urea, since fish may release small amounts of ammonia through these pathways (Sayer & Davenport 1987, Shrestha et al. 2023). Additionally, estimating the proportion of ingested nitrogen converted into biomass would help improve nitrogen-use efficiency and reduce the environmental impact of aquaculture operations.

In conclusion, this study provides the first quantitative data on TAN excretion in the Chilean silverside *B. microlepidotus* under RAS conditions. Neither body size (7.8-297 g) nor stocking density (1.1-1.8 kg m^{-3}) significantly affected TAN excretion under the water-quality conditions maintained during the trial, and a clear postprandial diel pattern was evident, with peaks 2-4 h after feeding and near 02:00 h. These findings indicate that, within the evaluated ranges, ammonia loading in this species can be estimated from total biomass alone, providing baseline bioengineering parameters for biofilter sizing and RAS design.

Nevertheless, factors such as feed type and composition, feeding rate, photoperiod, salinity, and dissolved oxygen can also influence ammonia excretion in fish. They should be considered when extrapolating these parameters to other production settings. Future studies should quantify nitrogen losses through fecal output and the fraction excreted as urea (Sayer & Davenport 1987, Shrestha et al. 2023), and estimate the proportion of ingested nitrogen converted into biomass, which would help refine nitrogen-use efficiency for this species. Complementary studies on oxygen consumption across ontogenetic stages, together with species-specific dietary protein requirements, would further strengthen the bioengineering parameters reported here for RAS design and biofilter sizing.

Credit author contribution

C.A. Mendez: conceptualization, data curation, formal analysis, investigation, methodology, writing - original draft and writing, review & editing; C. Galleguillos: data curation, formal analysis and review & editing; C. Harris-Toro & M.L. Nava: data curation and review & editing; M.C. Morales: conceptualization, project administration, funding acquisition and review & editing. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

Ethical statement

The data presented in this study are original and have not been previously published in any journal.

Data availability

Data will be made available upon request.

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