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Ammonia tolerance of Japanese anchovy *Engraulis japonicus*: Implications for cost reduction in a skipjack pole-and-line fishery

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Abstract

The Japanese anchovy *Engraulis japonicus* is used as live bait in skipjack pole-and-line fisheries. Japanese anchovies are transported to fishing grounds by fishing boats on voyages that can take from 4 to 50 days. Because ammonia excreted by Japanese anchovies might affect their survival during transportation, fishers empirically attempt to maintain them in healthy condition by exchanging water aboard ship holding tanks at a rate of 160–500 % volume h^{-1}. To develop protocols for more efficient and cost-effective transportation of Japanese anchovies, we evaluated their ammonia tolerance and effects of stocking density and variable water exchange on their survival. We estimated median lethal concentrations (with 95 % confidence intervals) of un-ionized ammonia nitrogen (UIAN), at which 50 % of Japanese anchovies had died within 24 h and 48 h, as 0.770 (0.751–0.790) mg l^{-1} and 0.706 (0.661–0.750) mg l^{-1} at 15 °C and 0.634 (0.466–0.802) mg l^{-1} and 0.450 (0.379–0.521) mg l^{-1} at 25 °C, respectively. While the UIAN concentration increased to lethal levels and severe fish mortality (dependent on stocking density) occurred at 25 °C with no water exchange, the UIAN concentration could be maintained within non-lethal levels at 25 °C and with a one-third water exchange every 12 h. Our results suggest that present-day empirically deduced water exchange rates applied aboard commercial vessels in holding tanks of Japanese anchovies could be reduced, implying a possible cost reduction for the skipjack pole-and-line fisheries.

Key words: ammonia toxicity; median lethal concentration; fish culture; fish transportation

Introduction

In Japan's pelagic and inshore skipjack pole-and-line fishery (SPLF), the Japanese anchovy *Engraulis japonicus* is transported live to fishing grounds by fishing boats equipped with holding tanks for use as a live bait (Yamashita et al. 2011). For pelagic SPLF boats operating in tropical seas, transportation of live Japanese anchovies by fishing boats can take up to 50 days, while for vessels working inshore this transportation can take as little as 4 days.

In the pelagic SPLF, to maintain healthy Japanese anchovies aboard vessels, fishers empirically reduce the high temperature (~30 °C) of ambient seawater to 15 °C using a cooling system and exchange water in tanks by a flow-to-waste system at a rate ≥ 160 % volume h^{-1} (Kimura et al. 2012). This cooling of water is costly; with depressed fish and soaring fuel prices, reducing costs is increasingly important to ensure the continued viability of the pelagic SPLF.

For inshore boats the water in tanks containing

Japanese anchovies is not cooled, and water quality is maintained by a flow-to-waste system at 500 % of its volume h^{-1} (Kurosaka et al. 2012). However, during summer when seawater temperatures can exceed 29 °C, mass death of Japanese anchovies often occurs in holding tanks.

In the SPLF, therefore, for efficient and cost-effective transportation of Japanese anchovies to fishing grounds, knowledge of their upper temperature limits and lower water exchange rates is essential to ensure their viability in transit.

The upper thermal tolerance of Japanese anchovies was reported by Oda et al. (2018) after exposing fish to 15, 20, 25, 30, and 35 °C for 120 h. Anchovy survival was high (> 98 %) at temperatures ranging 15–25 °C, but all fish died at temperatures ≥ 30 °C. Accordingly, holding tank water temperature during transfer of Japanese anchovies to pelagic and tropical fishing grounds could be increased from the current 15 °C to 25 °C, but it would be necessary to control temperatures below lethal limits during summer for the inshore fishery.

Fish generally excrete ammonia as a principal waste product, and they are very sensitive to ammonia toxicity (Handy and Poxton 1993; Wang and Walsh 2000; Ip et al. 2001). Therefore, high ammonia concentrations might negatively affect survival of Japanese anchovies during transportation, if fish are being fed, and in instances where water exchange was limited. An understanding of ammonia tolerance limits of Japanese anchovies would enable appropriate water exchange rates when transporting them aboard SPLF boats to be determined. Despite this, no study of which we are aware has reported the ammonia tolerance of Japanese anchovy.

To improve transportation methods of Japanese anchovies and SPLF cost efficiency, we conducted experiments that 1) evaluated the ammonia tolerance ability of Japanese anchovy; and 2) examined the effects of stocking density and water exchange rate, and 3) water exchange frequency on survival of Japanese anchovies.

Materials and Methods

Experimental fish

Japanese anchovies were captured with purse seine fishing or fixed shore net fishing along the west coast of Nagasaki Prefecture, Japan, for use in the SPLF in November 2009 (for experiment 1) and in February 2010 (for experiments 2 and 3). Fish were then transported live by truck to the Shibushi Field Station laboratory, FRA, Kagoshima Prefecture, Japan, and stocked in an 80-kl concrete tank (70 kl seawater volume) with flow-through water (exchange rate 12.5 % volume h^{-1}, temperature 14–22 °C, salinity 32 PSU), and cultured with formulated feed pellet (Iwashitairyou; Marubeni Nisshin Feed Co., Ltd., Tokyo, Japan) daily at a ratio of 0.6 % of fish body weight (employed in the SPLF). Feeding ceased the day before and during each experiment.

Experiment 1: evaluating anchovy ammonia tolerance

To determine the ammonia tolerance of Japanese anchovies under the current temperature regime of the pelagic SPLF (15 °C) and upper safe temperature (25 °C) for culturing Japanese anchovies (Oda et al. 2018), we exposed test fish to seawater with total ammonia nitrogen (TAN) at concentrations of 40, 80, 120 and 160 mg l^{-1} at 15 °C, and 10, 20, 40 and 80 mg l^{-1} at 25 °C. We prepared the sump tanks of 2 kl volume with seawater containing concentrations of TAN, adjusted using ammonium chloride (NH_4Cl; Nacalai Tesque Inc., Kyoto, Japan). A control sump tank in which no ammonium chloride was added was also prepared. We used two 200-l black polyethylene tanks (actual water volume 175 l) for each treatment. Seawater was supplied to experimental tanks from sump tanks using small pumps at an exchange rate of 20.8 % volume h^{-1}. Tanks were aerated using airstones.

Ammonia tolerance experiments were conducted on December 14, 2009 at 25 °C and from February 3, 2010 at 15 °C, for 48 h. Tests were run under conditions of constant light (700–1900 lx) used in the pelagic SPLF. Average anchovy body size was 95.4 ± 4.9 mm total length (TL) and 5.2 ± 1.0 g body weight (BW) (mean ± standard deviation, N = 30) for experiments at 25 °C, and 104.6 ± 8.1 mm TL and 7.3 ± 1.6 g BW for experiments at 15 °C (a body size comparable to that of bait fish used in the SPLF). Water temperature was controlled by heater. The stoking density of fish in each test tank was ~13 kg fish kl^{-1} used in the pelagic SPLF (~400 individuals at 25 °C and ~320 individuals at 15 °C). To acclimate for 3 days prior to commencing experiments, we stocked fish into test tanks and roughly counted their number in a short time (to minimize stress on test animals). Japanese anchovies were observed every 3 h during experiments; dead fish were counted and removed. On completion of experiments, the final numbers of surviving fish were counted; the initial numbers of stocked fish in test tanks were determined as 384–417 at 25 °C, and 297–342 at 15 °C.

To determine TAN concentrations, 50 ml samples of seawater were collected from each tank every 3 h and stored at −80 °C. Temperature was measured using a stem thermometer, dissolved oxygen (DO) and pH was determined using DO (HQ-30d; Hach Company, Loveland, CO) and pH (PB-11; Sartorius Japan, Tokyo, Japan) meters, respectively. On completion of the experiment, frozen seawater samples were thawed, centrifuged at 5000 rpm for 10 min, and filtered with a syringe filter (Minisart-plus; Sartorius Japan) with 0.2 µm pore size attached to a 5 ml syringe to remove extraneous material. TAN concentrations were measured using a salicylate method by spectrophotometer, according to the manufacturer's instructions (DR2010; Hach Company). TAN is present in seawater as unionized ammonia (NH_3) and ionized ammonium (NH_4^+); environmental pH, temperature, and salinity affect the equilibrium of NH_3 and NH_4^+, and ammonia toxicity is particularly related to NH_3 concentration (Handy and Poxton 1993; Wang and Walsh 2000; Ip et al. 2001). The concentration of highly toxic un-ionized ammonia nitrogen (UIAN) was calculated from the TAN concentration, pH and temperature at 32 PSU salinity according to Kido et al. (1991) based on reports of Whitfield (1974) and Bower and Bidwell (1978) by: X (%) = 100/(1 + antilog (9.35 + 0.0324 (298 – T) – pH)), where X is the percentage of UIAN to TAN, and T the temperature (°K). Oxygen saturation (%) in test water was also calculated based on the saturated DO concentrations (100%) in seawater (32 PSU) at designated temperatures following Truesdale et al.

(1955) as: K (mg l^{-1}) = 14.161 – 0.3943T + 0.0007714T^2 – 0.0000646T^3 – 32(0.0841 – 0.00256T + 0.0000374T^2), where K is the saturated DO concentration, and T the temperature (°C).

Ammonia tolerance of Japanese anchovy was evaluated by median lethal concentration (MLC) as the UIAN concentration at which 50% of test fish died after 24 h or 48 h from the onset of experimentation. MLC values were estimated by applying a generalized linear model (GLM) with a binomial distribution using the *glm* function (quasibinomial family, logit link) in R statistical software version 4.0.2 (R Core Team 2020). In the GLM, the number of live or dead fish after 24 or 48 h was the two-vector response variable; the mean value of the UIAN concentration until 24 or 48 h was the explanatory variable. MLC values with 95% confidence intervals were estimated based on GLM results using the *invest* function implemented in the investr package (Greenwell and Schubert Kabban 2014) in R.

Experiment 2: effects of stocking density and water exchange rate on anchovy survival

Our three treatments were: 1) high stocking density (~49 kg fish kl^{-1}) with water exchange at 500% volume h^{-1} (control), 2) high stocking density (~49 kg fish kl^{-1}) without water exchange, and 3) low stocking density (~24.5 kg fish kl^{-1}) without water exchange. Treatment 1 represented standard inshore SPLF conditions.

We used two 200-l black polyethylene tanks (actual water volume 100 l) in each treatment. Fish of 112.7 ± 6.5 mm TL and 7.8 ± 2.0 g BW were stocked 2 days before initiating an experiment for acclimation to tank environments. Pure oxygen was provided to tank water by airstone, as is sometimes performed on inshore SPLF boats during summer. The experiment commenced on April 29, 2010 for 96 h at 25 °C under constant illumination. Dead fish were counted and removed every 6 h; the initial number of fish stocked in low-density tanks was 333–335 individuals, and in high-density tanks, 649–655 individuals. TAN concentration was measured, and UIAN concentration was estimated every 6 h as in experiment 1.

Experiment 3: effect of water exchange method on anchovy survival

Of two treatments, the first, the control, had tank water continuously exchanged at a rate of 500% volume h^{-1} (as operated on inshore SPLF boats). Treatment 2 had one third of the tank water exchanged with the same volume of fresh seawater every 12 h. Fish of 112.9 ± 4.0 mm TL and 8.3 ± 1.6 g BW were stocked in tanks at ~49 kg fish kl^{-1}; the experiment was conducted from May 8, 2010. The initial number of stocked fish in replicate tanks in the two treatments ranged 511–535; methodology is otherwise as in experiment 2.

Results

Experiment 1: evaluating anchovy ammonia tolerance

Water tank environmental parameters are presented in supplementary Tables S1 and S2. TAN concentrations were higher than designated concentrations in some tanks, but differentiation of TAN concentrations could be maintained between treatments.

At 15 °C most Japanese anchovies survived in the control and 40 mg l^{-1} treatments, with mean survival rates after 48 h 99.8 % and 99.4 %, respectively (Fig. 1A). In the 80, 120 and 160 ppm treatments, fish mortality increased sharply as TAN concentration increased, with mean survival rates after 48 h being 82.6 % in the 80 mg l^{-1} treatment, 12.9 % in the 120 mg l^{-1} treatment, and 0 % after 33 or 36 h in the 160 mg l^{-1} treatment. DO and oxygen saturation tended to decrease with increased TAN concentration, particularly early in experimentation (Table S1). Oxygen saturation varied from ~38–76 %.

The 24 h and 48 h MLC values (+ 95 % confidence intervals) of UIAN were estimated as 0.770 (0.751–0.790) mg l^{-1} and 0.706 (0.661–0.750) mg l^{-1}, respectively, based on GLM analysis (Fig. 2A; Table 1).

At 25 °C high fish mortality occurred in one control tank when aeration was (accidentally) not provided; results for this tank have been excluded from analysis. The survival rate of control and 10 mg l^{-1} treatment fish reached about

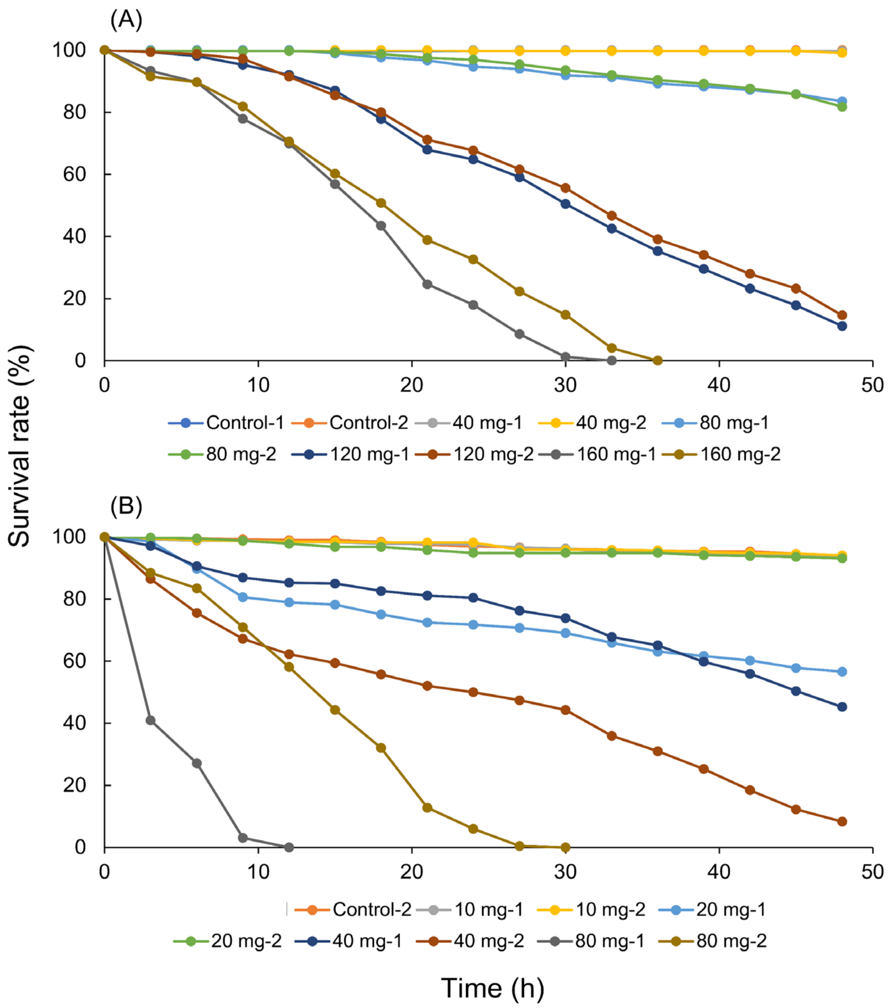

Fig. 1. Survival rate of Japanese anchovies exposed to seawater with different total ammonia nitrogen concentrations at 15 °C (A) and 25 °C (B), experiment 1 (with two replicate tanks per treatment). At 25 °C, one control tank was excluded because high fish mortality occurred when aeration was (accidentally) not provided. Ammonia concentration controlled at 10–160 mg l^{-1} by adding NH_4Cl to water (the control tank received no NH_4Cl).

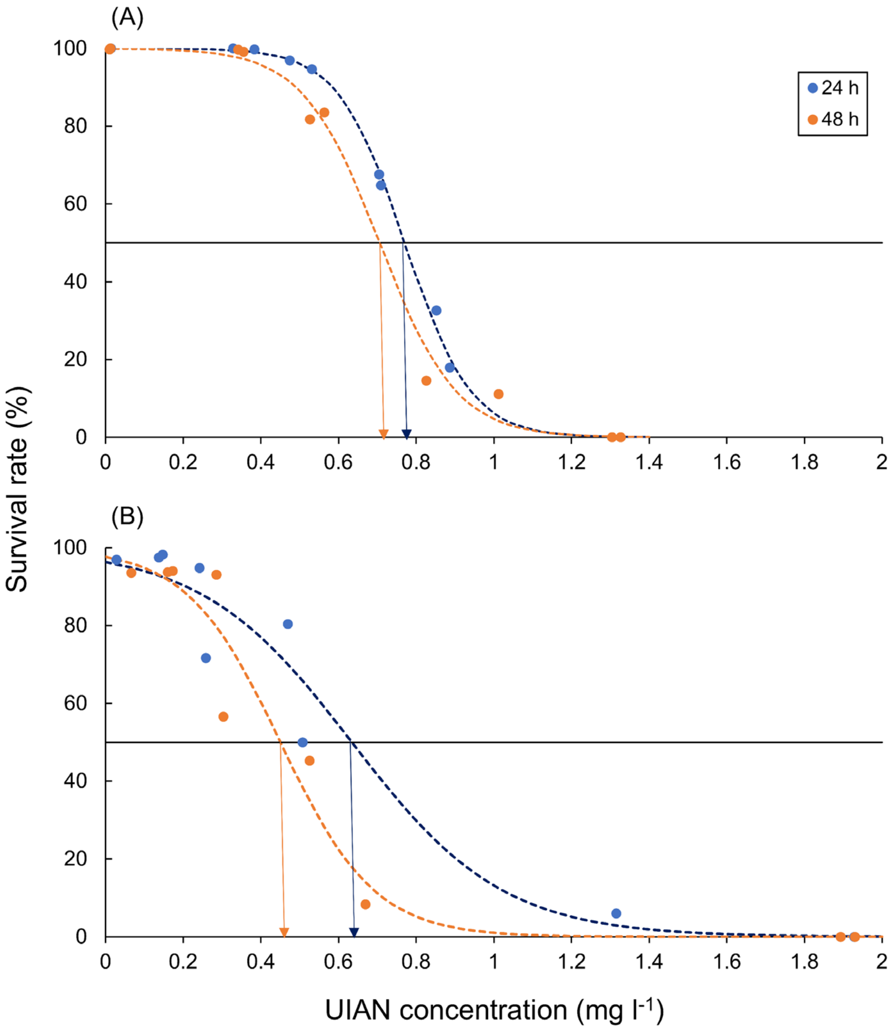

Fig. 2. Survival response of Japanese anchovies to different concentrations of un-ionized ammonia nitrogen (UIAN) after 24 h or 48 h at 15 °C (A) and 25 °C (B), experiment 1. Curves illustrated using coefficient estimates from the generalized linear model to evaluate relationships between UIAN and anchovy survival (Table 1). Arrows indicate the median lethal concentration at which 50 % of test anchovies died.

Table 1. Coefficient estimates with standard errors (SE) in the generalized linear model with a binomial distribution for evaluating relationships between un-ionized ammonia nitrogen concentration (Doses) and survival of Japanese anchovies, experiment 1.

Experimental temperature (°C)	Time	Coefficient	Estimate	SE	t value	p value
15	24 h	Intercept	9.07	0.73	12.35	< 0.0001
		Doses	−11.78	0.97	−12.16	< 0.0001
	48 h	Intercept	7.18	0.78	9.20	< 0.0001
		Doses	−10.17	1.14	−8.95	< 0.0001
25	24 h	Intercept	3.27	0.57	5.72	0.000717
		Doses	−5.15	1.27	−4.06	0.004803
	48 h	Intercept	3.73	0.64	5.83	0.000641
		Doses	−8.29	1.55	−5.34	0.001079

94 % (Fig. 1B). In the 20 mg l^{-1} and 40 mg l^{-1} treatments fish mortality varied between replicate tanks, the number of dead fish increased with time, and the mean survival rate after 48 h was 74.8 % and 26.8 %, respectively. In the 80 mg l^{-1} treatment, the decrease in survival rate was pronounced, with 100% mortality after 12 or 30 h. DO and oxygen saturation tended to fluctuate as it did at 15 °C (Table S2). Oxygen saturation varied from ~51 %–112 %.

The UIAN 24 h and 48 h MLC values (+ 95 % confidence intervals) were estimated as 0.634 (0.466–0.802) mg l^{-1} and 0.450 (0.379–0.521) mg l^{-1}, respectively, based on GLM analysis (Fig. 2B; Table 1).

Experiment 2: effects of stocking density and water exchange rate on anchovy survival

No significant decrease in survival rates of Japanese anchovies was observed in the control treatment at high stocking density with a water exchange; mean survival rate after 96 h was 87.5 % (Fig. 3A). Mean survival rate in the high- and low-density treatments without a water exchange decreased sharply after 30 h and 54 h, with 100 % mortality by 54 h and 96 h, respectively.

The UIAN concentration in the control treatment remained at about 0.01 mg l^{-1} but

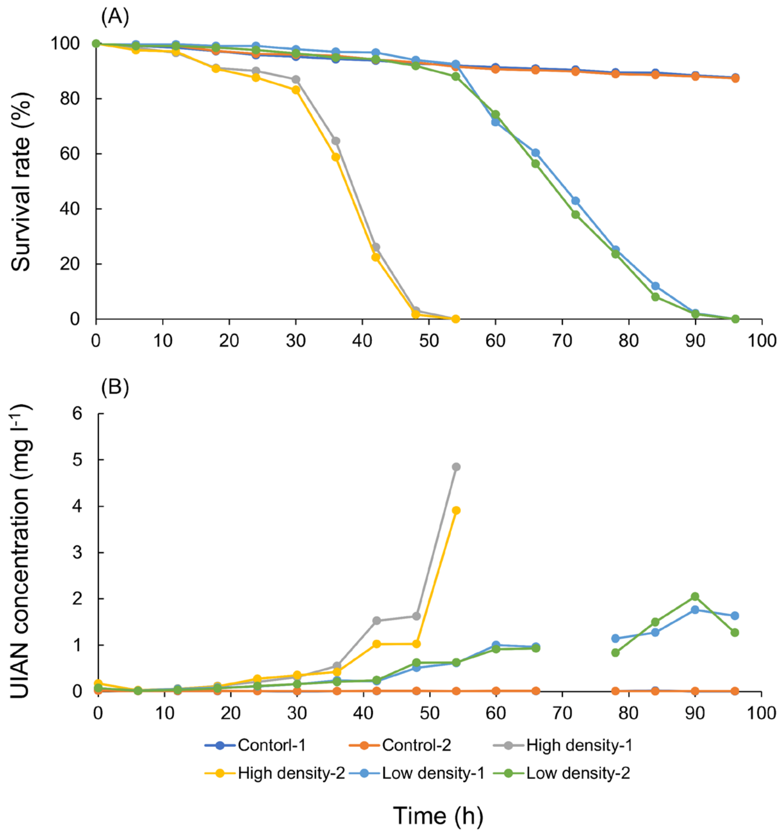

Fig. 3. Survival rate of Japanese anchovies (A) and un-ionized ammonia nitrogen (UIAN) concentration (B), experiment 2 at 25 °C for three treatments: 1) high stocking density with water exchange (control), 2) high stocking density without water exchange, and 3) low stocking density without water exchange. Two tanks were used for each treatment. Ammonia concentration was not examined after 72 h.

increased in high- and low-density treatments, exceeding 0.4 mg l^{-1} after 36 h and 48 h in the high- and low-density treatments, respectively (Fig. 3B; Table S3), at which point survival rate declined sharply (Fig. 3A); a substantial increase in the UIAN concentration was observed in the high-density treatment (Fig. 3B). DO was supersaturated during experimentation (Table S3).

Experiment 3: effect of water exchange method on anchovy survival

Survival of Japanese anchovies with an intermittent (test) and continuous (control) water exchange gradually differed from 12 h; the mean survival rate after 96 h was 71.5% in the control treatment and 50.8% in the test treatment, with survival rate variable in the test treatment (tank 1

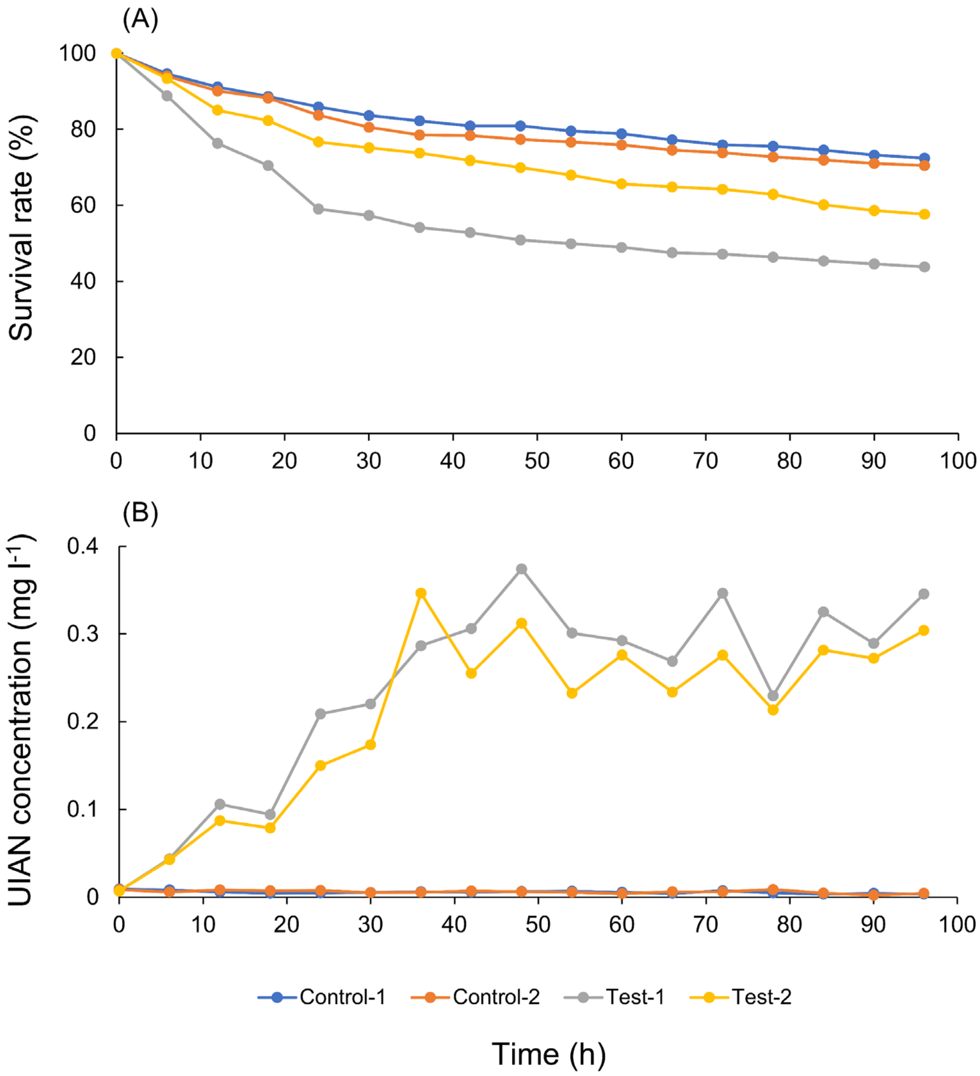

Fig. 4. Survival rate of Japanese anchovies (A) and un-ionized ammonia nitrogen (UIAN) concentration (B), experiment 3 at 25 °C for two treatments: 1) tank water continuously exchanged (control), and 2) one third of the tank water drained and replenished with the same volume of fresh seawater every 12 h (test). Two tanks were used for each group.

43.8 %, tank 2 57.7 %) (Fig. 4A). The UIAN concentration in the control group was less than 0.01 mg l^{-1} during experimentation, but in the test group reached ~0.3 mg l^{-1} by 36 h, then fluctuated between ~0.21 and 0.35 mg l^{-1} (Fig. 4B; Table S4). DO was supersaturated during experimentation (Table S4).

Discussion

In experiment 1, we determined the acute toxicity of UIAN to Japanese anchovies of 5-7 g BW, and estimated 24 h and 48 h MLC values (+ 95 % confidence intervals) at 15 °C to be 0.770 (0.751–0.790) mg l^{-1} and 0.706 (0.661–0.750) mg l^{-1}, and at 25 °C, 0.634 (0.466–0.802) mg l^{-1} and 0.450 (0.379–0.521) mg l^{-1}, respectively. Ammonia toxicity has been reported for several marine fish species, e.g., 96 h MLC values of UIAN between 1.7 and 2.6 mg l^{-1} for seabass *Dicentrarchus labrax*, seabream *Sparus aurata* and turbot *Scophthalmus maximus* juveniles of 6–163 g BW under optimal environmental conditions (17–18 °C, 34 PSU, and > 75 % oxygen saturation) (Person-Le Ruyet et al. 1995). Person-Le Ruyet et al. (1995) also calculated 6 h, 12 h, 24 h, and 48 h MLC values of UIAN for these fish juveniles and documented that the MLC values did not change significantly from 24 to 96-h exposure and were not related to fish size. Thus, ammonia tolerance ability appeared to be lower in Japanese anchovies than in juveniles of seabass, seabream and turbot.

In experiment 1, DO and oxygen saturation tended to decrease with increasing ammonia concentration, possibly because of increased oxygen consumption by fish affected by elevated ammonia concentration (Lemarié et al. 2004). Because the toxicity of ammonia to fish increases with decreasing oxygen (Wajsbrot et al. 1991), it should be noted that our MLC estimates are based on the survival of Japanese anchovies that may have been synergistically affected by increased ammonia concentration and decreased oxygen (minimum saturation 38 % at 15 °C and 51 % at 25 °C). Further study is required to determine the ammonia toxicity level for Japanese anchovies under non-oxygen limited conditions with pure oxygen supply as employed in experiments 2 and 3.

In the pelagic SPLF, to maintain healthy Japanese anchovies, fishers empirically reduce holding tank water to 15°C and have a high water-exchange rate (≥ 160 % volume h^{-1}) (Kimura et al. 2012). The UIAN concentration of water containing Japanese anchovies aboard pelagic SPLF boats was ≤ 0.011 mg l^{-1} (Kimura et al. unpublished data), which is much lower than our estimated 24 h and 48 h MLC values for UIAN for Japanese anchovies at 15 °C. The mean UIAN concentration of 0.34–0.36 mg l^{-1} at 15 °C in the TAN 40 mg l^{-1} treatment with oxygen saturation of ~70 % (Table S1) had a survival rate exceeding 99 % over 48 h (Fig. 1). Consequently, aboard pelagic vessels, the water in tanks holding Japanese anchovies might be being replaced more often than is necessary given anchovy ammonia tolerance.

In experiment 2, mortality of Japanese anchovies was density dependent at 25 °C when held without a water exchange. Mortality was highly associated with increased UIAN concentration in tank water because severe fish mortality occurred at UIAN concentrations exceeding 0.4 mg l^{-1} (Fig. 3), equivalent to 48 h MLC of Japanese anchovies at 25 °C. Thus, UIAN concentration manifested lethal effects on Japanese anchovies at similar levels in different oxygen saturation conditions in experiments 1 (limited) and 2 (saturated). In experiments 2 and 3 at 25 °C, the UIAN concentration in control treatments (standard protocols aboard inshore vessels: water exchange rate 500 % volume h^{-1}) was very low ($\leq$ 0.01 mg l^{-1}) and survival rate of test fish was relatively high (88 % in experiment 2, and 72 % in experiment 3) at 96 h (Figs. 3 and 4). In experiment 3, in the test group, the UIAN concentration did not reach MLC, and was maintained at around 0.3 mg l^{-1} by means of intermittent renewal of holding tank water (Fig. 4). This water exchange frequency maintained the mean survival rate of test fish at 51 %, which is 71 % the survival rate (72 %) of the control treatment. The upper UIAN concentration to keep Japanese anchovies healthy may be ~0.1 mg l^{-1}, as recorded during the experiment until 12 h before the anchovy survival rate tended to separate in control and test treatments (Fig. 4; Table S4).

We reveal that exchanging water of or below 25 °C to reduce the UIAN below chronic toxicity levels is necessary for efficient and cost-effective transfer of Japanese anchovies from port to fishing grounds in the SPLF. For pelagic SPLF, the fuels costs would be reduced by decreasing water exchange rates to control the UIAN concentration below the upper safe level (~0.1 mg l^{-1}) under the upper safe temperature condition (25 °C) in transit. For inshore SPLF which require relatively short voyages to transport Japanese anchovies to fishing grounds, to reduce costs of cooling system installation, seawater ice could be used to cool anchovy water to or below 25 °C before departing port; then, a second stock tank could be prepared with seawater ice to maintain temperature and UIAN concentration below the upper safe levels (25 °C and ~0.1 mg l^{-1}, respectively) by means of intermittent renewal of holding tank water in transit.

Further study is required to determine appropriate and practical water exchange rates and methodologies for commercial application, in addition to a trial aboard a commercial vessel to verify if costs can be reduced by the methodology that we advocate herein for the pelagic and inshore SPLF.

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