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RESEARCH

Pharmacokinetics, pharmacodynamics and withdrawal time of doxycycline in white leg shrimp (*Litopenaeus vannamei*) after oral administration

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Abstract

This research aimed to investigate the pharmacokinetics (PK), withdrawal time (WT) and hepatopancreas histological impact of doxycycline (DOX) in white leg shrimp (*Litopenaeus vannamei*). To determine PK parameters in hemolymph, hepatopancreas, and muscle, a single oral dose of 20 mg DOX/kg body weight was administered, following by interval sampling during 24 h. DOX concentrations were quantified by high-performance liquid chromatography coupled with tandem mass spectrometry, and PK parameter estimation was done using a one-compartmental model. The maximum concentrations in shrimp hemolymph, hepatopancreas and muscle were 1.09 µg/mL, 2.37 µg/g and 0.46 µg/g at 1.6 h, 0.23 h and 2 h, respectively. The pharmacokinetics/pharmacodynamics (PK/PD) properties were integrated based on the concentration-time curves of DOX in the hepatopancreas over the minimum inhibitory concentration (MIC) of DOX against *V. parahaemolyticus*. It was indicated that the time during which the DOX concentration in the hepatopancreas was above the MIC (T>MIC) was approximately 4 h, corresponding theoretically to an 8-hour dosing interval. However, this PK/PD interpretation is presented as supportive information and should be interpreted with caution. A depletion study was conducted by administering DOX-medicated feed once and twice daily for three consecutive days at the same dose. DOX residues in shrimp muscle dropped below the limit of detection after 14 days of stopping medication. Based on a maximum residue limit (MRL) of 50 µg/kg, the estimated withdrawal time at 26.5 °C ranged from 8 to 10 days, depending on the dosing regimen (once or twice a day). Regarding histological analysis, the effect of DOX on hepatopancreas was significant during the twice a day dosing treatment, but recovery of hepatopancreatic cells was observed within seven days after medication.

Keywords Doxycycline · Hepatopancreas · Pharmacokinetics · Pharmacodynamics · White leg shrimp · Withdrawal time

Introduction

Aquaculture industry plays a critical role in global food security, with white leg shrimp (*Litopenaeus vannamei*) being one of the most widely cultivated species in Vietnam with an annual production of 1.26 million tons in 2024 (VASEP 2025). Nevertheless, the intensive and super-intensive shrimp farming has led to increased vulnerability to bacterial diseases, which can significantly impact production yields and economic stability (Nguyen et al. 2020). Regarding bacterial disease, *Vibrio* spp., particularly *V. parahaemolyticus*, is a prevalent pathogen responsible for acute hepatopancreatic necrosis disease (AHPND), which

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has been firstly reported in Vietnam since 2010, with massive mortality of farmed shrimp in the Mekong Delta (Tran et al. 2013; Nghia et al. 2015). To control *Vibrio* pathogen and other bacteria in aquaculture, effective disease management strategies used by farmers often involve the use of antibiotics, including doxycycline (DOX) (Phu et al. 2016; Chi et al. 2017; Dao et al. 2022).

DOX (α-6-deoxy-5-hydroxytetracycline) is a second-generation, broad-spectrum tetracycline antibiotic with bacteriostatic activity, its antimicrobial activity targets bacterial ribosomes, where it inhibits protein synthesis by preventing the addition of amino acids to polypeptide chains within this cellular organelle (Etebu et al. 2016). DOX is highly effective against Gram-positive and Gram-negative bacteria (Chopra and Roberts 2001). This antibiotic is permitted for use in aquaculture in several countries under national regulatory frameworks. In Vietnam, DOX is included in the list of veterinary drugs allowed for circulation and use (Ministry of Agriculture and Rural Development, 2024). However, improper use of antibiotics such as high doses and non-compliance with antibiotic withdrawal periods has resulted in the presence of antibiotic residues found in exported shrimp products. Between 2020 and 2024, the Rapid Alert System for Food and Feed (RASFF) from the European Union reported that exceeding residue limits of antibiotics including DOX, oxytetracycline, tetracycline, sulfamethoxazole, furazolidone and ciprofloxacin were detected in frozen shrimp products imported from many countries including Vietnam (RASFF 2025). Moreover, residual levels of antibiotics that exceed the maximum residue limit (MRL) pose potential risks to human health (Quesada et al. 2013; Guidi et al. 2018), and contribute to the issue of antimicrobial resistance (Chopra and Roberts 2001). Nevertheless, to our knowledge, no data are available about the pharmacokinetics (PK) and tissue residue depletion of DOX in white leg shrimp.

Research on the PK of DOX in crustaceans remains limited. To date, only one PK study of DOX has been reported in a freshwater crustacean, the crayfish (*Procambarus clarkii*) (Xu et al. 2022), in which oral administration at 20 mg/kg resulted in a maximal concentration (C_{max}) of 17.58 µg/g and a time to reach maximal concentration (T_{max}) of 6 h in the hepatopancreas. While, no comprehensive PK data are available for penaeid shrimp. In contrast, PK studies on tetracycline-class antibiotics, particularly oxytetracycline, have been conducted in several shrimp species, including white shrimp (*Litopenaeus setiferus*) (Reed et al. 2004), black tiger shrimp (*Penaeus monodon*) (Uno et al. 2006), and Pacific white shrimp (*Litopenaeus vannamei*) following single and multiple oral administrations (Uno et al. 2010; Ma et al. 2019). These studies reported measurable oxytetracycline concentrations in shrimp hemolymph, with peak hemolymph concentrations (C_{max}) generally ranging from approximately 3 to 28 µg/mL, and T_{max} values occurring within approximately 4 to 10 h, depending on shrimp species, dosage, and experimental conditions. In addition, tissue-specific distribution kinetics of oxytetracycline in shrimp, such as accumulation in the exoskeleton, have also been reported (Faroongsarng et al. 2013). Whereas, PK characteristics of DOX have mainly been investigated in fish species such as rainbow trout (Altan at al. 2024), striped catfish (Vinh et al. 2024), channel catfish (Xu et al. 2020; Ai et al. 2011), African catfish (Ibrahim et al. 2019), tilapia (Yang et al. 2014). In addition, PK data for DOX in terrestrial animals have been widely reported and were summarized by Yang et al. (2014). Despite the availability of these oxytetracycline PK studies, the distribution, tissue exposure, and elimination kinetics of DOX in penaeid shrimp remain largely unexplored. Therefore, information on shrimp-specific PK/PD relationships for doxycycline is still lacking. Establishing appropriate PK/PD indices for antibacterial drugs in aquaculture is essential for optimizing dosing strategies against various bacterial infections, which helps ensuring effective treatment while reducing the risk of antimicrobial resistance in aquatic species (Mothadaka et al. 2023). Additionally, integrating PK parameters such as C_{max} and $AUC_{0-24\,h}$ along with a suitable pharmacodynamic (PD) parameter, such as the minimum inhibitory concentration (MIC) to figure out the appropriate therapeutic dose and treatment interval when using antibiotics, ensures their effective application in controlling bacterial infections in aquatic species (Mothadaka et al. 2023).

The impact of antibiotics on shrimp hepatopancreas histology is another key aspect that needs to be evaluated. The hepatopancreas is a multifunctional organ responsible for digestion, metabolism, and immune responses, it plays a central role in shrimp health and overall growth (Manan et al. 2015). Adverse histological changes in this organ can occur with exposure to improperly used antibiotics or chemicals (Maftuch et al. 2017; Bray et al. 2006). A study on the hepatopancreas histology of white leg shrimp revealed that cefotaxime administration had no negative impact on hepatopancreatic structure or health (Huynh et al. 2024). Despite this, there has been no research specifically examining the adverse effects of DOX on the hepatopancreas of white leg shrimp.

In general, due to the common use of DOX in white leg shrimp aquaculture, its PK/PD properties and effect on shrimp hepatopancreas as well as muscle residue depletion should be investigated to rationalize DOX use in aquaculture and ensure food safety for consumers.

Materials and methods

Chemicals

A commercial product "Doxy 10%", labeled to contain 10% (w/w) of DOX, purchased from a veterinary medicine supplier in Vietnam (Vemedim Co Ltd). Prior to the experiment, the absence of DOX residues in hemolymph and tissues (hepatopancreas and muscle) of white leg shrimp was confirmed. In addition, the actual concentration of DOX in the commercial product was quantitatively verified by LC–MS/MS using an analytical-grade doxycycline standard for calibration. C18 Bondesil powder with a particle size of 40 µm was supplied by Agilent (Santa Clara, CA, USA). High-purity chemicals above 98%, including the DOX standard obtained from Dr. Ehrenstorfer® (Augsburg, Germany) and Doxycycline D3 (internal standard, IS) obtained from TLC Pharmaceutical Standards Ltd. (Ontario, Canada). In addition, methanol, acetonitrile, distilled water and other chemicals were sourced from Merck (Darmstadt, Germany).

Vibrio parahaemolyticus strains

The *V. parahaemolyticus* strain associated with AHPND on white leg shrimp was isolated and obtained from the culture collection of the Faculty of Aquatic Pathology, College of Aquaculture and Fisheries, Can Tho University. Sample collection included 42 bacterial strains isolated from shrimp disease cases recorded between 2022 and 2024 across seven provinces including Ca Mau, Kien Giang, Soc Trang, Can Tho, Dong Thap, Tra Vinh, and Tien Giang of the Mekong Delta, Vietnam. The bacterial strains were reactivated prior to the experiment following the procedure described in "Antibiotic susceptibility testing" section.

Antibiotic susceptibility testing

V. parahaemolyticus was cultured in nutrient broth supplemented with NB+ (1.5% NaCl) and incubated at 28 °C for 18 h. The bacteria were then transferred to tryptic soy agar plates supplemented with TSA+ (1.5% NaCl) and incubated for an additional 18 h. The colony color and shape were recorded, and Gram staining was performed to confirm the purity of the bacterial culture. To increase biomass, pure colonies were cultured in NB + at 28 °C for 24 h. Afterward, the bacterial concentration was adjusted to approximately 10^8 CFU/mL using McFarland´s 0.5 Barium Sulfate Standard Solution (Sotomayor et al. 2019).

The antibiotic resistance of *V. parahaemolyticus* was assessed using the agar disk diffusion method, based on the protocols of Balouiri et al. (2016) and Jiang et al. (2020). Commercial DOX disks (30 µg; Oxoid, UK) was applied in this test. One hundred microliters of the bacterial suspension were evenly spread on the surface of a TSA+ plate. After 15-min, the DOX disk was carefully placed on the agar surface with sterile fine-pointed forceps to ensure proper contact. The plates were then incubated at 28 °C for 24 h. The inhibition zone diameters (including the disk diameter) were visually measured and the size of the clear zone was used to categorize the isolates as susceptible, intermediate, or resistant (CLSI M100, 2018).

Minimum inhibitory concentration

V. parahaemolyticus was activated following the method previously described. The minimum inhibitory concentration (MIC) of DOX was determined using the broth macro dilution method with modifications as described by Ali et al. (2019). A 128 mg DOX standard solution was prepared by dissolving with 10 mL of sterile distilled water to reach a final concentration of 1280 µg/100 µL. This solution was then subjected to a series of 12 successive dilutions.

For the test, 1 mL of the bacterial solution was diluted 200-fold with TSB+ medium. Fourteen sterilized test tubes, each with screw caps, were labeled and filled with 4.9 mL of the diluted bacterial solution. Then, to each test tube, 100 µL of the prepared DOX standard solution was added to achieve final concentrations of 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, and 0.125 µg/mL. In addition, a positive control (TSB+ solution with bacteria) and a negative control tube (TSB+ solution without bacteria) were prepared as well. All test tubes were incubated at 28 °C for 24 h. The MIC was determined as the lowest concentration of DOX where no visible bacterial growth was observed. MIC values are interpreted using the interpretive criteria for *Vibrio* spp. from Clinical and Laboratory Standards Institute (CLSI M45, 2015). The percentile function from Excel software (Microsoft Office 2019) was utilized to calculate the MIC50 and MIC90 from the MIC dataset.

White leg shrimp

Healthy white leg shrimp (15.4±2.3 g/shrimp) were supplied by the Faculty of Marine Science and Technology, College of Aquaculture and Fisheries, Can Tho University. The shrimp were confirmed to be negative for AHPND, which is primarily caused by *Vibrio* spp., particularly *Vibrio parahaemolyticus* and *Enterocytozoon hepatopenaei* (EHP) based on molecular diagnostic methods (PCR). The shrimp were reared from the post-larval stage without the use of antibiotics for 45 days before conducting the experiment. Shrimp were stocked into 2 m³ tanks at a density of 100 shrimp/m³ and acclimated for 1-week. During the acclimatisation period, shrimp were fed a commercial pellet feed

containing 40% crude protein (Proconco, Vietnam) four times a day at 6:00 am, 10:00 am, 2:00 pm, and 6:00 pm, with the feed amount corresponding to 3% of body weight of shrimp. Any leftover feed was removed by siphoning after 30 min of feeding (lower than 5% of the total amount). The water temperature was recorded at 26.5 ± 0.9 °C. To maintain proper oxygen levels above 5 mg/L, the experimental tanks were fully equipped with an aeration system. Additionally, water quality parameters such as alkalinity (adjusted to 140–160 mg HCO_3^-/L), salinity (adjusted to 10‰), temperature, pH, and nitrite (NO_2^-), were regularly monitored and controlled to ensure optimal conditions for shrimp health and normal development throughout the entire experiment. This salinity level falls within the typical range (10–15‰) commonly used in white leg shrimp farming systems in Vietnam.

Pharmacokinetics of doxycycline after oral administration

White leg shrimp were stocked and acclimated in 6 tanks, with each tank representing one replicate (2 kg shrimp per tank). A single dose of 20 mg DOX/kg body weight was administered to the shrimp, and this dosage was chosen based on the standard therapeutic dose recommended by the manufacturers of commercial products, and in previous studies where the same dose was applied to treat bacterial infections in fish and shrimp via oral administration (Ibrahim et al. 2019; Xu et al. 2019a, b, 2021; Vinh et al. 2024). The DOX commercial product used in this study contained 10% active ingredient. Shrimp were fed at 3% body weight per day, corresponding to 60 g feed per tank per day, divided into four feedings. For the pharmacokinetic experiment, medicated feed was administered only during the first feeding (6:00 am), while non-medicated feed was provided at subsequent feeding times, i.e. at 10:00 am, 2:00 pm, and 6:00 pm. Based on a shrimp biomass of 2 kg per tank, a total of 40 mg active DOX per tank was required to achieve the target dose. Since only 15 g feed was administered during the medicated feeding, the medicated feed was prepared to contain 2667 mg active DOX/kg feed, corresponding to 26.67 g commercial product/kg feed. The medicated feed was prepared by dissolving the calculated amount of DOX product in tap water, thoroughly mixing it with pelleted feed. After that, the medicated feed was coated with 2% squid oil, and then the feed was left for 15 min to allow the drug and oil to be fully absorbed into the pellets before being administered to the shrimp. The concentration of DOX in medicated feed was 1502 mg/kg feed, analyzed by LC-MS/MS.

Following administration, hemolymph, muscle, and hepatopancreas of shrimp were collected at 0.5, 1, 2, 4, 8, 12, and 24 h post-medication. In this experiment, the tank was considered the experimental unit. At each time point, three shrimp from each tank were collected. For hemolymph collection, approximately 0.5 mL hemolymph per shrimp was collected from the pericardial cavity using a 1 mL syringe pre-filled with an anticoagulant solution (EDTA 0.01 M, NaCl 0.338 M, glucose 0.115 M, trisodium citrate dihydrate 0.03 M), corresponding to a 1:1 (v/v) ratio of anticoagulant to hemolymph. Hemolymph from three shrimp from the same tank was pooled to form one composite sample per tank at each sampling time, transferred to 1.5 mL centrifuge tubes, and centrifuged at 9500 *g* for 5 min. After centrifugation, the supernatant was collected as cell-free hemolymph (Lorenzon et al. 1999). Simultaneously, the hepatopancreas and muscle tissues from those shrimps were collected and pooled into marked plastic bags to obtain one pooled tissue sample for each tank, forming one replicate. Thus, each sampling time included six independent pooled samples corresponding to six replicate tanks ($n = 6$). All hemolymph, muscle, and hepatopancreas samples were then frozen and stored at -80 °C for subsequent analysis.

Doxycycline muscle tissue depletion and hepatopancreas histological examination

The experiment was carried out with two groups, each group consisting of three replicate tanks with the density of 100 shrimp/m³. In group 1, shrimp were administered DOX-medicated feed at a dose of 20 mg DOX/kg body weight per day, delivered as a single feeding at 6:00 am for three consecutive days (medicated feed was prepared as described earlier). In group 2, the medicated feed was provided to the shrimp twice daily, at 6:00 am and 6:00 pm, for three consecutive days. In this group, the dose of 20 mg DOX/kg body weight was administered at each feeding, resulting in a total daily dose of 40 mg DOX/kg body weight. After the three-day period of DOX-medicated feed administration, non-DOX medicated feed was given for the next 21 days, four times daily at 6:00 am, 10:00 am, 2:00 pm, and 6:00 pm. Shrimp were sampled on days 1 and 3, at 1 h after feeding DOX medicated feed. Further sampling was carried out on days 1, 3, 7, 14, and 21 after the cessation of medicated feed. At each sampling time, 10 shrimp were randomly collected from each tank, and shrimp muscles were pooled, minced, and stored at -80 °C for later analysis.

Additionally, to assess the impact of DOX on the shrimp's hepatopancreas, histological examination was also performed in this experiment. Samples were collected at three time points: one day before medication, one hour after the last dose of medication, and seven days after the cessation of medication. At each sampling time, three individual shrimp per tank were collected (three replicate tanks per

treatment group), resulting in a total of nine shrimp ($n=9$) per treatment group, which were separately processed for histological analysis.

Quantification of doxycycline in shrimp hemolymph and tissues (hepatopancreas and muscle)

DOX quantification was performed using a Waters ACQUITY Ultra-High-Performance Liquid Chromatography system (Waters, Milford, MA, USA) coupled to a Xevo™ TQ-S triple quadrupole mass spectrometer combined with electrospray ionization (ESI) operated in the positive ion mode (Waters). The system was equipped with an Acquity UPLC CSH C18 column (2.1 × 50 mm, 1.7 µm) (Waters, Milford, MA, USA). Mass spectrometry parameters, including capillary voltage (2.8 kV), ion source temperature (150 °C), cone gas flow (50 L/h), desolvation gas flow (800 L/h), and desolvation temperature (400 °C), were optimized. Argon was used as the collision gas at a pressure of 4×10^{-3} mbar. The following ion transitions (precursor > product ion, *m/z*) were monitored for quantification and confirmation:

- Doxycycline (DOX):
  - Quantification: *m/z* 445.08 > 428.1 (collision energy: 18 eV)
  - Confirmation: *m/z* 445.08 > 154.06 (collision energy: 28 eV)
- Doxycycline D3 (Internal Standard, IS):
  - Quantification: *m/z* 448.2 > 431.1 (collision energy: 18 eV)

Liquid chromatographic separation was performed using mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile) at a flow rate of 0.25 mL/min. The initial mobile phase composition (90% A and 10% B) was maintained for 0.6 min. A linear gradient was then applied to reach 10% A and 90% B at 2.5 min. This composition was held for 0.5 min, after which the system was returned to the initial conditions (90% A and 10% B) over 0.5 min and maintained for an additional 1 min for column equilibration.

Hemolymph sample preparation

DOX extraction from white leg shrimp hemolymph was performed using a modified method of Vinh et al. (2024). In brief, 0.5 mL of hemolymph was spiked with 10 µL of 1 µg/mL Doxycycline D3 as an internal standard and vortexed after addition of 3 mL of acetonitrile. After centrifugation at 9500 *g* for 10 min, the supernatant was collected and evaporated to dryness under a gentle nitrogen stream at 40 ± 1 °C using a nitrogen evaporator. The residue was reconstituted in 1 mL of mobile phase in a ratio of 95% A: 5% B. The mobile phase included two solvents: solvent A (50% acetonitrile + 50% methanol containing 0.1% formic acid) and solvent B (0.1% formic acid in water). Before LC–MS/MS injection, the reconstituted sample was filtered through a 0.22 µm nylon membrane filter (Advantec MFS, CA, USA) and an aliquot of 10 µL was injected into the LC–MS/MS system.

Tissue and feed sample preparation

DOX extraction from feed and shrimp tissues (hepatopancreas and muscle) was performed after mincing using an Ultra Turrax homogenizer (T-25, IKA, China). Samples (3.00 ± 0.1 g) were transferred into a 50 mL tube, fortified with 60 µL of IS (Doxycycline D3, 1000 ng/mL), and left at room temperature for 15 min. The extraction process involved adding 1 mL of 0.1 M phosphate buffer at pH of 8.0, vortexing for 1 min, and then 20 mL of acetonitrile was introduced, followed by vortexing for 30 s and sonication for 15 min in an Elma Ultrasonic bath (Elma Hans Schmidbauer, Singen, Germany). To collect the supernatant, the tubes were then centrifuged at 3501 *g* for 5 min. Acetonitrile was adjusted to a final volume of 24 mL and 8 mL of this was transferred into a 15 mL glass test tube containing 40 mg of Bondesil C18. After centrifugation again at 3501 g for 4 min, the supernatant was collected in a 10 mL glass test tube and evaporated at 40 ± 1 °C in a water bath under nitrogen flow. The dried residues were reconstituted in 1 mL of mobile phase before injection into the LC–MS/MS system. If DOX concentrations exceeded the calibration range, samples were diluted with a blank sample extract containing IS at the same concentration as the samples before reinjection.

Method validation

The analytical methods were validated in accordance with European Commission Decision 2002/657/EC (EC 2002) and met the required acceptance criteria to ensure accurate and reliable DOX quantification in shrimp muscle tissue. Key validation parameters are presented in Luy (2017). The limits of detection (LOD) and limits of quantification (LOQ) of the analytical methods were 2.5 and 5 µg/kg or µg/L in tissue and hemolymph, respectively. The standard calibration curve, prepared using blank samples, exhibited a linear range of 5–100 µg/kg. The extraction recovery ranged from 94% to 100%, ensuring reliable quantification. The method demonstrated strong linearity with coefficients

of determination (R^2) above 0.99 and high specificity with interference levels below 2.5%. Precision was also confirmed through repeatability (relative standard deviation of 3.05%–4.59%) and within-laboratory reproducibility (relative standard deviation of 2.06%–4.47%). The apparent recovery varied between 97% and 100%. The decision limit (CC_α) and detection capability (CC_β) were calculated to be 53.8 µg/kg and 57.6 µg/kg, respectively. To minimize potential matrix effects, quantification was performed using matrix-matched calibration curves prepared from blank shrimp samples, e.g. shrimp muscle, hepatopancreas and hemolymph.

Pharmacokinetics estimation

The concentration of DOX in hemolymph, hepatopancreas, and muscle was modelled using a naïve pooled population approach based on a one-compartmental model with first-order absorption and elimination. This approach was applied because the destructive sampling design and limited number of samples obtained from individual shrimp prevented repeated measurements from the same individuals, making standard non-compartmental analysis (NCA) unreliable and mixed-effects modeling unsuitable. However, the naïve pooled method does not allow quantification of inter-individual variability, and the resulting PK estimates should therefore be interpreted as descriptive or approximate typical PK rather than a true population model.

For hemolymph, the following PK parameters were calculated: absorption rate constant (ka), absorption half-life ($T_{1/2}a$), maximal hemolymph concentration (C_{max}), time to reach maximal hemolymph concentration (T_{max}), area under the hemolymph concentration–time curve from time 0 to infinity (AUC_{0-inf}), elimination rate constant (k_{el}), elimination half-life ($T_{1/2el}$), apparent total body clearance after oral administration (CL/F) and apparent volume of distribution after oral administration (V_d/F).

For the hepatopancreas and muscle tissues, the PK parameters calculated included maximal tissue concentration (C_{max}), time to reach maximal tissue concentration (T_{max}), and area under the tissue concentration–time curve from time 0 to infinity (AUC_{0-inf}), which was determined using the linear-up log-down trapezoidal method. All data were processed using Phoenix 8 (Certara, Princeton, NJ, USA).

Withdrawal times estimation

The withdrawal time for DOX was estimated following the European Medicines Agency guidelines (EMA 2022), utilizing the statistical software WT 1.4 developed by Hekman (2004). For withdrawal time estimation, residue depletion data used for WT calculation were obtained at 1 h, 1, 3, and 7 days after the final dose. DOX concentrations in muscle were measured at multiple time points after treatment and analyzed using linear regression based on time. The used unit was degree-days, which were determined by multiplying the average daily water temperature (°C) by the total number of days the temperature was recorded. The withdrawal period was defined as the time when the upper one-sided 95% tolerance limit for DOX residue concentration dropped below the MRL, with 95% confidence level. For ease of interpretation, withdrawal times were also expressed in calendar days based on the mean rearing temperature.

Hepatopancreas histological analysis

The histology of shrimp hepatopancreas was examined using the methods as described in Huynh et al. (2024). Hepatopancreas histological sections were examined using a microscope (Novex, Arnhem, the Netherlands). The counts of B and F cells were performed according to Nima et al. (2022) with slight modifications. Both cell types were counted in the same microscopic fields, with different fields randomly selected, and a total of 40 tubules were counted per individual shrimp sample (Huynh et al. 2024).

To assess the effects of dosing and/or sampling on the hepatopancreas, statistical analyses on histopathology results were carried out using one-way and two-way ANOVA with SPSS software (version 20.0). When significant differences were detected, Duncan's multiple range test was employed as a post hoc analysis. A significance level of $p < 0.05$ was used to evaluate statistical differences.

Results

Minimum inhibitory concentration (MIC) of doxycycline and its susceptibility to *Vibrio parahaemolyticus*

The MIC values of DOX against 42 *V. parahaemolyticus* isolates–collected from diseased shrimp in seven provinces of the Mekong Delta–ranged between 2 and over 256 µg/mL (Table 1). For 15/42 isolates MIC values of ≤4 µg/mL were noted, a smaller portion (only 8 isolates) clustered at 8 µg/mL, and the most common group showed MIC values≥16 µg/mL as observed in 19 isolates. Generally, regarding strains showed to be inhibited in a specific DOX concentration (29 strains) the calculation showed that 50% and 90% of tested strains had the MIC smaller or equal to 4 µg/mL and 19.2 µg/mL (corresponding to MIC50 and MIC90), respectively.

Regarding the disk diffusion assay, the inhibition zone diameters for the strains from various regions of the Mekong

Table 1 Minimum inhibitory concentration and inhibition zone diameters of DOX against *V. parahaemolyticus* isolates associated with shrimp disease

Isolate no.	MIC (µg/mL)	MIC interpretation	Inhibition zone (mm)	Zone interpretation
1	4	S	21	S
2	16	R	13	I
3	32	R	15	S
4	16	R	16	S
5	2	S	18	S
6	4	S	19	S
7	4	S	16	S
8	2	S	18	S
9	2	S	16	S
10	8	I	17	S
11	8	I	19	S
12	16	R	20	S
13	32	R	13	I
14	>256	R	0	R
15	>256	R	0	R
16	>256	R	6	R
17	>256	R	6	R
18	2	S	20	S
19	>256	R	8	R
20	8	I	25	S
21	8	I	20	S
22	8	I	19	S
23	2	S	17	S
24	2	S	18	S
25	2	S	17	S
26	8	I	14	S
27	>256	R	12	I
28	>256	R	10	R
29	2	S	18	S
30	64	R	14	S
31	8	I	19	S
32	>256	R	11	I
33	8	I	19	S
34	2	S	19	S
35	2	S	20	S
36	2	S	20	S
37	>256	R	6	R
38	>256	R	6	R
39	4	S	18	S
40	>256	R	17	S
41	>256	R	0	R
42	>256	R	7	R

Isolate number from 1 to 17 in Ca Mau regions, 18–19 in Kien Giang regions, 20–30 in Soc Trang regions, 31–33 in Can Tho regions, 34 in Dong Thap region, 35–39 in Tra Vinh regions, 40–42 in Tien Giang regions

S – susceptible, I – intermediate, R – resistant. MIC interpretations were assigned according to CLSI M45 (2015) interpretive criteria for Vibrio spp. Zone diameter interpretations were applied according to CLSI M100 (2018)

Delta were as follows: Ca Mau (isolates 1–17, ranging from 0 to 21 mm), Kien Giang (isolates 18–19, ranging from 8 to 20 mm), Soc Trang (isolates 20–30, ranging from 10 to 25 mm), Can Tho (isolates 31–33, ranging from 11 to 19 mm), Dong Thap (isolate 34, 19 mm), Tra Vinh (isolates 35–39, ranging from 6 to 20 mm), and Tien Giang (isolates 40–42, ranging from 0 to 17 mm) (Table 1).

Pharmacokinetics of doxycycline after oral administration

Figure 1 illustrates the concentration-time curves of DOX in hemolymph, hepatopancreas, and muscle of white leg shrimp following an oral administration of a single dose at 20 mg DOX/kg body weight. The concentration of DOX over time was highest in shrimp hepatopancreas, followed by shrimp hemolymph, and then shrimp muscle.

The PK parameters of DOX in shrimp hemolymph (Table 2) include the absorption rate constant (k_a) of 1.68 h^{-1}, absorption half-life ($T_{1/2a}$) of 0.41 h, peak hemolymph concentration (C_{max}) of 1.09 µg/mL, and the time to reach maximum concentration (T_{max}) at 1.6 h and area under the concentration-time curve (AUC_{0-inf}) of 9.45 µg.h/mL (Table 2). The apparent total body clearance (CL/F) was 2.12 L/kg/h, apparent volume of distribution (V_d/F) of 14.61 L/kg, elimination rate constant (k_{el}) of 0.15 h^{-1}, and elimination half-life ($T_{1/2el}$) of 4.78 h (Table 2).

When examining the PK of DOX in shrimp tissues, i.e. hepatopancreas and muscle, as shown in Table 3, it is clear that the C_{max} in the hepatopancreas (2.37 µg/g) obtained after 0.23 h (T_{max}) were markedly five times higher compared to that in the muscle which was only 0.46 µg/g after 2 h of dosing. In terms of the AUC_{0-inf} parameter, the value of hepatopancreas (31.72 µg.h/g) was significantly higher than that in muscle (5.55 µg.h/g), i.e. 5.7 times.

Residue depletion and withdrawal time estimation of doxycycline

The DOX concentrations in the shrimp muscle when feeding once and twice a day for 3 consecutive days are presented in Table 4. On the first day of treatment, the DOX levels in shrimp muscle were 226 µg/kg for the once-a-day regimen and 559 µg/kg for the twice-a-day regimen. After that, these concentrations reached the highest concentration at day 3 of medication for both treatments, peaking at 323 µg/kg for the once-a-day treatment and 599 µg/kg for the twice-a-day treatment. After stopping medication, DOX concentrations in shrimp muscle gradually decreased, the values declined to 7.27 µg/kg in once-a-day feeding and 10.33 µg/kg for twice-a-day feeding at day 7 post medication. The

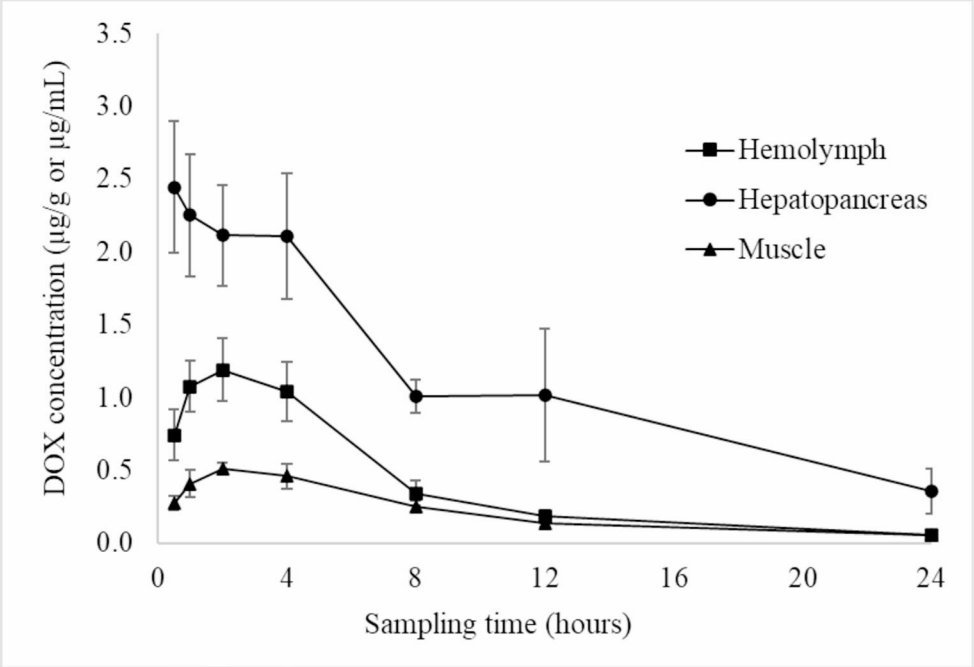

Fig. 1 DOX concentrations in hemolymph, hepatopancreas and muscle of white leg shrimp after oral administration of a single dose at 20 mg/kg body weight, the data are presented as mean ± standard deviation ($n = 6$)

Table 2 PK parameters of DOX in white leg shrimp hemolymph after a single oral administration at a dose of 20 mg DOX/kg body weight

PK parameter	Unit	Hemolymph estimate
k_a	h^{-1}	1.68
$T_{1/2a}$	h	0.41
C_{max}	µg/mL	1.09
T_{max}	h	1.60
CL/F	L/kg/h	2.12
V_d/F	L/kg	14.61
AUC_{0-inf}	µg.h/mL	9.45
k_{el}	h^{-1}	0.15
$T_{1/2el}$	h	4.78

Values represent population PK parameter estimates derived from six independent tank-based concentration–time profiles ($n = 6$). Absorption rate constant (k_a), absorption half-life ($T_{1/2a}$), maximal hemolymph concentration (C_{max}), time to maximal hemolymph concentration (T_{max}), area under the hemolymph concentration−time curve from time 0 to infinity (AUC_{0-inf}), elimination rate constant (k_{el}), elimination half-life ($T_{1/2el}$), apparent total body clearance after oral administration (CL/F) and apparent volume of distribution after oral administration (V_d/F)

Table 3 Main PK parameters of DOX in hepatopancreas and muscle tissues of white leg shrimp after a single oral administration at a dose of 20 mg DOX/kg body weight

PK parameter	Unit	Hepatopancreas estimate	Muscle estimate
C_{max}	µg/g	2.37	0.46
T_{max}	h	0.23	2.00
AUC_{0-inf}	µg.h/g	31.72	5.55

Values represent population PK parameter estimates derived from six independent tank-based concentration–time profiles ($n = 6$). Maximal tissue concentration (C_{max}), time to maximal tissue concentration (T_{max}) and area under the tissue concentration−time curve from time 0 to inf (AUC_{0-inf})

Table 4 The concentration of DOX in white leg shrimp muscle during and after medication at a dose of 20 mg DOX/kg body weight when feeding shrimp with DOX-medicated feed once daily and twice daily for 3 consecutive days

Period	Sampling time (days)	DOX concentrations (µg/kg)	
		Once daily treatment	Twice daily treatment
Medication	1	226 ± 62.2	559 ± 16.8
	3	323 ± 52.0	599 ± 35.6
After medication	1	54.33 ± 22.50	57.00 ± 13.08
	3	20.67 ± 7.64	28.33 ± 7.09
	7	7.27 ± 2.41	10.33 ± 4.04
	14	< LOD	< LOD
	21	< LOD	< LOD

Values are expressed as mean ± standard deviation, $n = 3$. LOD=limit of detection of 2.5 µg/kg

DOX levels were below the LOD (2.5 µg/kg) after 14 days onwards in both feeding regimes.

The WT of DOX in white leg shrimp muscle was estimated in this experiment after feeding shrimp with DOX medicated feed once-a-day and twice-a-day for three consecutive days at 20 mg/kg body weight and an ambient temperature of 26.5 °C. Based on an MRL of 50 µg/kg set by Japan (JFCRF 2017), the results for the WT of DOX in shrimp muscle were 186 degree-days (corresponding to 8 days at 26.5 °C) for feeding once-a-day treatment (Fig. 2A), and 241 degree-days (equating to 10 days) for twice-a-day treatment (Fig. 2B). WT of DOX in shrimp muscle were shorter when applying the MRL of 100 µg/kg as set by the European Union (EMA 2015), namely 140 degree-days (equating to 6 days) and 190 degree-days (approximately

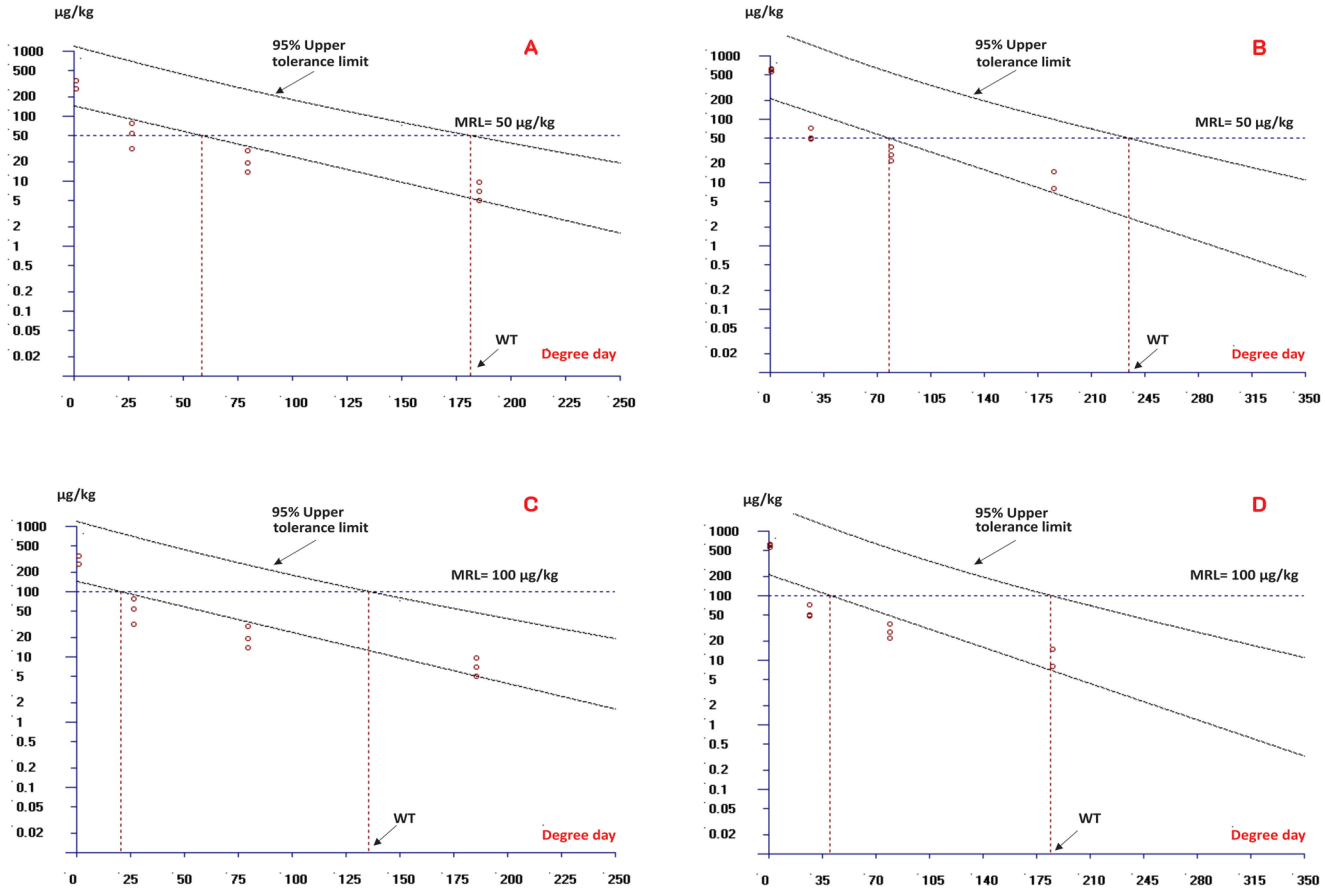

Fig. 2 Plot of DOX residual levels in white leg shrimp muscle recorded at 1 h, 1, 3, and 7 days, corresponding to 1.1, 26.5, 79.5, and 185.5 degree-days, respectively, following the cessation of treatment as a function of degree-days. The WTs were calculated as the time when the one-sided 95% upper tolerance limit was below the MRL (50 µg/kg and 100 µg/kg) when feeding shrimp with DOX-medicated feed once daily (**A**, **C**) and twice daily (**B**, **D**) during 3 consecutive days at a dose of 20 mg/kg body weight, at an average ambient temperature of 26.5 °C

8 days) in once-a-day (Fig. 2C) and twice-a-day regimens (Fig. 2D), respectively.

Hepatopancreas histology of white leg shrimp

Before antibiotic application, the histological examination of the hepatopancreas revealed no abnormalities, with the tissue structure displaying the normal presence of both B and F cells (Fig. 3A). Following doxycycline administration, histological changes in the hepatopancreas were observed in both once daily and twice daily dosing regimens. On day 3 of medication, shrimp receiving doxycycline once daily showed mild structural alterations in the hepatopancreatic tubules, including changes in the distribution and morphology of epithelial cells (Fig. 3B). Whereas, more pronounced alterations were observed in shrimp receiving doxycycline twice daily on day 3, with apoptosis-like morphological changes were observed in the hepatopancreatic tubule epithelial cells, characterized by cell shrinkage and nuclear condensation (Fig. 3C). By day 7 after stopping medication, the recovery of the hepatopancreatic structure was evident in both treatment groups, with improved tubule organization and cellular appearance in the once-daily (Fig. 3D) and twice-daily (Fig. 3E) regimens, approaching the normal histological architecture observed before treatment.

Following the administration of medicated feed once daily and twice daily for 3 consecutive days at a dose of 20 mg/kg body weight, the results concerning the count of B and F cells in the hepatopancreas are presented in Figs. 4 and 5. Upon statistical evaluation using two-way ANOVA, no significant difference in the number of B cells ($p > 0.05$) in the shrimp hepatopancreas was found between the two doses applied (Fig. 4). However, a significant variation in B cell counts was observed over experimental duration ($p < 0.05$) i.e., by day 3 of medication, the number of B cells in the shrimp hepatopancreas was significantly reduced compared to prior to medication ($p < 0.05$). By day 7, following the cessation of the treatment, the B cell count had rebounded and returned to levels comparable to those observed before treatment with no significant difference

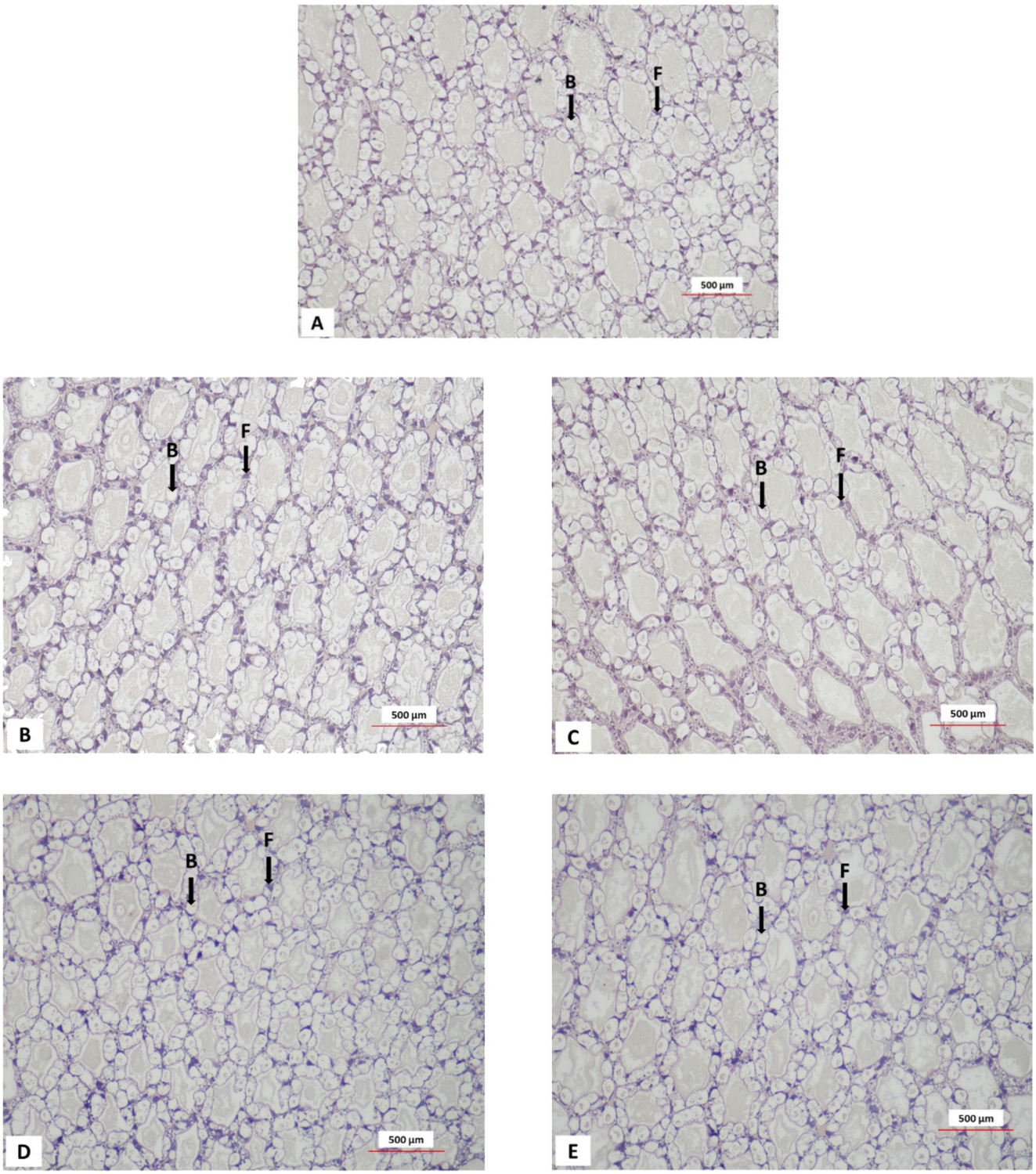

Fig. 3 Representative histological sections of hepatopancreas of white leg shrimp before treatment (**A**) and after administering DOX-medicated feed once daily (**B**) and twice daily (**C**) on day 3 of medication, and at 7 days post-treatment for once daily (**D**) and twice daily (**E**) groups (100× magnification). B means blister cells, F means fibrillar cells

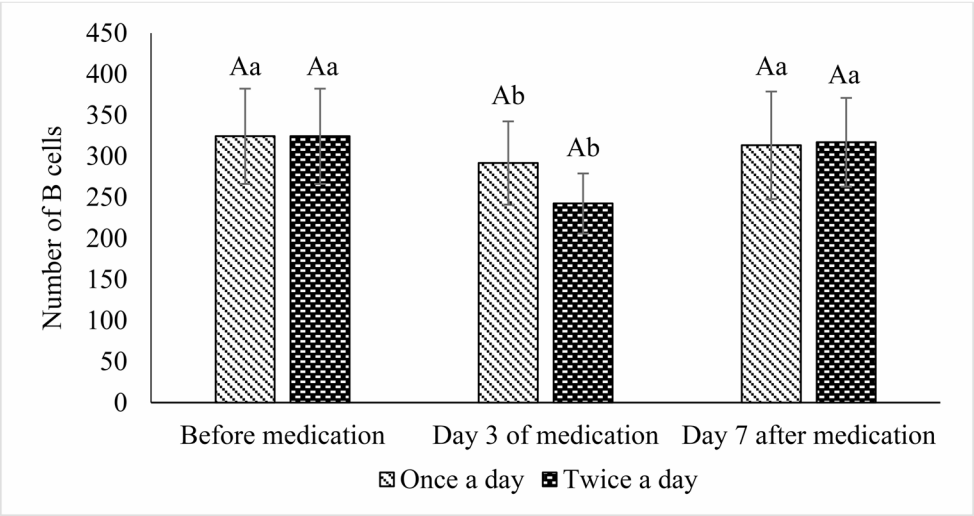

Fig. 4 The number of B cells of shrimp hepatopancreas (mean ± SD, $n = 9$) before medication, on day 3 of medication and on day 7 post-medication for once daily (left column) and twice daily (right column) feeding regimens. Value with identical capital letters indicate no significant different between the applied doses ($p > 0.05$). Value with matching lowercase letters denote no significant different between sampling time ($p > 0.05$)

noted when compared to before medication ($p > 0.05$). Furthermore, no interaction between the dosage regimen and experimental duration was detected with regard to B cell counts ($p > 0.05$).

Regarding the F cell counts in the shrimp hepatopancreas (Fig. 5), as two-way ANOVA revealed a significant interaction between dosing regimen and experimental duration ($p < 0.05$), interpreted the two factors were accessed by one-way ANOVA. The result showed that on day 3 of medication, the F cell number of shrimp in the twice daily treatment was significantly lower than all other groups ($p < 0.05$). However, after 7 days stopping medication, the number of F cells of twice daily treatment were increased and recovered and no significant difference was found when comparing with the counts of F cells of shrimp sampled before medication ($p > 0.05$).

Discussion

Antimicrobial activity of doxycycline against *Vibrio parahaemolyticus* strains

Based on Clinical and Laboratory Standards Institute classification (CLSI 2018), fifteen isolates were susceptible to DOX with a sensitivity rate of 35.7% (MIC ≤ 4 µg/mL), while 19% (8/42) of strains were intermediately resistant (MIC = 8 µg/mL). Additionally, 19 isolates showed resistance to DOX at a rate of approximately 45% (MIC ≥ 16 µg/mL). In the disk diffusion assay, according to CLSI guidelines (2018), the classification of the antimicrobial susceptibility of bacterial strains includes three categories based on the diameters of the inhibition zones i.e., ≥ 14 mm indicates susceptibility, 11–13 mm denotes intermediate susceptibility,

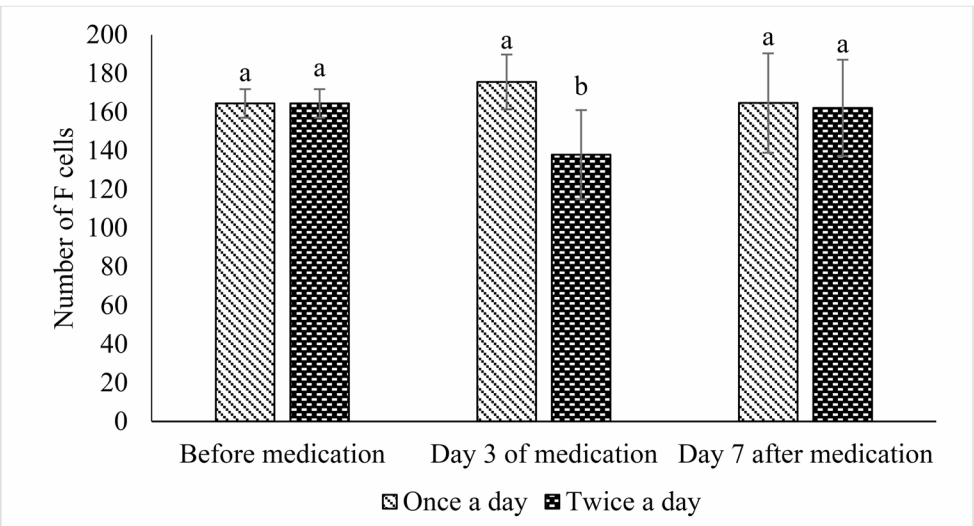

Fig. 5 The number of F cells of shrimp hepatopancreas (mean ± SD, $n = 9$) before medication, on day 3 of medication and on day 7 post-medication for once daily (left column) and twice daily (right column) feeding regimens. Values with different lowercase letters indicate significant differences among groups based on simple-effects comparisons due to a significant interaction between dosing regimen and sampling time (two-way ANOVA, $p < 0.05$)

and ≤ 10 mm indicates resistance. Based on these criteria, *V. parahaemolyticus* strains examined in this study were classified as susceptible, intermediately resistant and resistant to DOX with the proportions of 66.7% (28/42), 9.5% (4/42) and 23.8% (10/42), respectively. Several differences were noted between susceptibility classification based on MIC and disk diffusion. According to Balouiri et al. (2016), MIC determination provides a quantitative measure of antibacterial activity under standardized broth conditions, whereas disk diffusion results are influenced by antibiotic diffusion properties, agar composition, and bacterial growth characteristics. Furthermore, a standard disk diffusion breakpoint for *Vibrio* spp. has not been formally established by CLSI, and the inhibition zone diameter criterion applied in this study was taken from published literature. Moreover, MIC values in current study were considered for calculating the PK/PD index and interpreting dosage, while disk diffusion was used as a supportive susceptibility screening method. These findings underscore the varying levels of antibiotic susceptibility among *V. parahaemolyticus* strains collected from different geographical locations. These results were aligned with previous finding from *V. parahaemolyticus* isolated from shrimp, i.e., Letchumanan et al. (2015) indicating that *V. parahaemolyticus* isolates from shrimp in Malaysia exhibited high susceptibility to tetracycline (82%), meanwhile, the investigation on *V. parahaemolyticus* showed 36.6% of resistance (Saifedden et al. 2016).

The sensitivity rate of *V. parahaemolyticus* to DOX in present study (66.7%) was consistent with the report of Thi et al. (2025), the disk diffusion results of 32 strains of *V. parahaemolyticus* isolated from farmed shrimp in Ben Tre and Soc Trang provinces in the Mekong Delta, Vietnam exhibited that these strains were sensitive to DOX at a proportion of 68%. However, another study showed that *V. parahaemolyticus* isolated from AHPND shrimp cultured in the Bac Lieu province of Mekong Delta was highly sensitive to DOX, with a rate of 94% (Luan et al. 2023). Besides, the results of the inhibition zone diameter in this investigation also aligns with a previous study on *V. parahaemolyticus* isolated from shrimp diseases, the diameter was 16.05 ± 1.3 mm and showed sensitivity to DOX (Yudiati et al. 2021). Results from the antibiotic sensitivity test and MIC determination in current research were however higher when compared with the study of Dang et al. (2022) evaluating the susceptibility of 58 *V. parahaemolyticus* bacteria isolated from diseased shrimp in Mekong Delta, Vietnam towards DOX, with MIC values ranging from 2 to 4 µg/mL, and a resistance rate of 3.5% for isolates based on disk diffusion test result. According to Nikaido (2009) and Prescott et al. (2000), plasmids are considered a major factor contributing to antibiotic resistance in bacteria, as they carry genes encoding resistance to various classes of antibiotics, including tetracyclines, β-lactams, macrolides, trimethoprim/sulfamethoxazole, aminoglycosides, and phenicols. The drug resistance becomes more serious when plasmids are transferred between bacteria of the same or different species through conjugation. This horizontal gene transfer enables the spread of resistance traits, which partly explains the variation in susceptibility rates among isolates of the same bacterial species observed across different geographical regions (Thi et al. 2014).

Overall, the findings from the current investigation demonstrated that the susceptibility of *V. parahaemolyticus* strains toward DOX varied depending on regions. Therefore, it is crucial that the antimicrobial effectiveness of DOX continues to be monitored regularly, particularly with its increasing usage and its extensive application in treating shrimp diseases, to ensure sustained effectiveness and minimize the risk of resistance development over time.

Pharmacokinetics of doxycycline in white leg shrimp

PK studies provide essential information on the absorption, distribution, metabolism, and excretion of drugs in aquatic species (Rodgers and Rowland 2006; Jambhekar and Bree 2009). These parameters are crucial to optimize treatment regimens as well as minimize residual risk. In the current research, the maximum DOX concentration in the hepatopancreas of shrimp was approximately twice as high as that in hemolymph and about five times higher than that in muscle. This trend aligns with findings of Xu et al. (2022), who investigated the PK of DOX in crayfish (*Procambarus clarkii*) administered orally at a dose of 20 mg/kg, where DOX accumulation in the crayfish hepatopancreas was observed to be the highest, nevertheless, the maximal concentrations in hepatopancreas (17.58 µg/g), muscle (3.13 µg/g) and hemolymph (3.07 µg/mL) found in crayfish were higher than those in our study. A similar distribution pattern of tetracycline-class antibiotics has been reported in shrimp species. For example, PK studies of oxytetracycline in Pacific white shrimp (*Litopenaeus vannamei*) demonstrated that drug concentrations were highest in the hepatopancreas, followed by hemolymph and muscle after oral administration (Ma et al. 2019). In that study, peak oxytetracycline concentrations reached 27.77 µg/mL in hemolymph and 149.52 µg/g in the hepatopancreas following a single oral dose, which were markedly higher than the concentrations observed for DOX in the present study. Similarly, oxytetracycline PK in black tiger shrimp (*Penaeus monodon*) showed a C_{max} of 21.1 µg/mL following oral administration (Uno et al. 2006). In Pacific white shrimp (*Litopenaeus vannamei*), hemolymph C_{max} values of 3.37 and 17.4 µg/mL with T_{max} values of 7 and 10 h were reported at doses of

10 and 50 mg/kg bw, respectively (Uno et al. 2010). These values are much higher than the peak hemolymph concentration observed in the present study (1.09 µg/mL). Such differences may be attributed to variations in antibiotic compound, dosage, and experimental conditions, as well as differences in PK properties between DOX and oxytetracycline in shrimp.

The hepatopancreas in crustaceans plays a vital role not only for metabolism and excretion but also for the substance absorption (Verri et al. 2001; Faroongsarng et al. 2007). In this study, the T_{max} values in white leg shrimp hepatopancreas (0.23 h) and muscle (2 h) were significantly shorter (6 h in hepatopancreas and 4 h in muscle) compared to crayfish, but the C_{max} and AUC_{0-inf} values of shrimp hepatopancreas, muscle and hemolymph were largely lower than those in crayfish with the same dose of application as in our study (Xu et al. 2022). The persistent high DOX concentration in those crayfish organs resulted in a notably longer elimination half-life ($T_{1/2el}$), with a value of 69.5 h for hemolymph compared with white leg shrimp (4.78 h) in our study.

When comparing the PK of DOX following a single dose of 20 mg/kg body weight after oral administration with other aquatic animals, particularly in the plasma of several fish species, the observed C_{max} value in this study (1.09 µg/mL) was lower than the findings in rainbow trout, striped catfish, African catfish and tilapia which exhibited values of 1.12, 5.06. 2.29 and 2.27 µg/mL, respectively (Vinh et al. 2024; Altan et al. 2024; Ibrahim et al. 2019; Yang et al. 2014). In addition, the T_{max} value in white leg shrimp was shorter than that reported for these fish species. Such interspecies differences in PK parameters are consistent with recognized physiological and anatomical variation among taxa, which can influence drug absorption, distribution, metabolism and elimination (Toutain et al. 2010). In particular, oral drug absorption is highly dependent on gastrointestinal tract structure and function. Shrimp lack a true vertebrate stomach and have a relatively short digestive tract, which likely contributes to more rapid but less extensive systemic uptake compared to fish (Vinarov et al. 2021). In addition, this apparent discrepancy due to differences in circulatory systems between species, in the case of an animal with an open circulatory system, all of the organs and internal structures are constantly bathed in hemolymph (Fang et al. 2018). Concerning the value of AUC_{0-inf}, the DOX concentration distribution in shrimp hemolymph (9.45 µg.h/mL) was significantly lower than that observed in rainbow trout and tilapia which applied at the same dose (20 mg/kg bw), which had values of 242.25 and 113.45 µg.h/mL, respectively, reported by Altan et al. (2024) and Yang et al. (2014), suggesting lower systemic exposure. These differences may reflect combine effects of reduced oral bioavailability and more rapid elimination mechanisms in shrimp. The apparent volume of distribution (V_d/F) for DOX in shrimp was 14.61 L/kg, indicating efficient DOX distribution from hemolymph, and the value was higher than that reported in rainbow trout (4.74 L/kg) and striped catfish (6.55 L/kg) albeit possible differences in oral bioavailability need to be accounted for (Altan et al. 2024; Vinh et al. 2024). It is noteworthy that research on PK of DOX in African catfish (Ibrahim et al. 2019) and tilapia (Yang et al. 2014) presented a biphasic hemolymph concentration profile, characterized by two C_{max} peaks after oral administration at a single dose of 20 mg/kg body weight, indicating enterohepatic recirculation, however, this phenomenon was neither observed in white leg shrimp in our study nor in crayfish (Xu et al. 2022), and may be attributed to fundamental anatomical and physiological differences between crustaceans and vertebrates. Following absorption and distribution, DOX is eliminated from the shrimp's body. In the current study, DOX was eliminated relatively rapidly from the hemolymph of white leg shrimp, with an elimination half-life ($T_{1/2el}$) of 4.78 h. The elimination was faster when compared to fish, where $T_{1/2el}$ values were reported as 18.5 h, 39.78 h, 5.81 h, and 77.2 h in striped catfish, rainbow trout, African catfish, and tilapia, respectively (Vinh et al. 2024; Altan et al. 2024; Ibrahim et al. 2019; Yang et al. 2014). Additionally, the apparent total body clearance (CL/F) in this study was 2.12 L/kg/h, which was higher than the 0.8 L/kg/h reported in striped catfish by Vinh et al. (2024). The $T_{1/2el}$ value was markedly shorter than those reported in fish species, which is in agreement with the higher apparent total body clearance observed in shrimp. This indicates a more rapid elimination of DOX in white leg shrimp compared to striped catfish because clearance reflects the elimination of drug from the body. Furthermore, PK parameters can differ significantly between or within species, influenced by factors such as species specificity, health status, geographical location, age, size, salinity, water temperature, drug administration routes, and other experimental conditions (Björklund and Bylund 1991; Zhang and Li 2007). Generally, these physiological differences provide a mechanistic basis for the observed PK parameter variations between shrimp and fish species.

Toutain et al. (2010) reported that PK parameters may vary due to several factors like geographical region, genetic diversity resulting from artificial selection and breeding activities even within the same aquatic species. Additionally, drug metabolism in ectothermic animals is considerably affected by environmental factors, particularly water temperature (Vinh et al. 2024). Luo et al. (2019) also suggested that different routes of drug administration may influence dosage regimens. For example, Xu et al. (2022) presented that the time to maximal concentration of DOX in crayfish hepatopancreas differed based on the administration route, occurring at 6 h, 8 h and 12 h via oral administration, intramuscular injection

and intrasinus injection, respectively. These findings highlight the importance of administration routes in influencing PK parameters in aquatic species. In shrimp aquaculture in Vietnam, the most common method applied by shrimp farmers is feeding shrimp orally.

Regarding PK/PD indices, according to Mothadaka et al. (2023), the critical breakpoints for PK/PD surrogate markers vary across different antimicrobial groups, the minimum required values should be C_{max}/MIC > 8–10, $AUC_{0–24\ h}$/MIC > 125, and T > MIC > 50% which depend on whether concentration-dependent or time-dependent antibiotics are considered to achieve optimal therapeutic outcomes. It is worth noting that the PK/PD guidelines regarding therapeutic performance for various antibacterial drugs are quite different. DOX is considered as both a concentration-dependent and time-dependent antibiotic, in the case of concentration-dependent, the ratio of C_{max}/MIC or $AUC_{0–24\ h}$/MIC is used for dosage calculation (Toutain et al. 2002). Mothadaka et al. 2023; Altan et al. 2024). However, in the present study, the DOX concentrations achieved in shrimp tissues resulted in relatively low C_{max} and AUC values, which limited the applicability of AUC/MIC-based PK/PD modeling for robust dose calculation. In addition, Cunha (2003) and Xu et al. (2020) reported that DOX is also classified as a time-dependent antibiotic, meaning that T > MIC a suitable parameter for determining appropriate dosing intervals. However, in this study, T > MIC was included to illustrate the relationship between tissue concentrations and the MIC, without implying its use as a primary PK/PD index for dose optimization, which is more commonly based on AUC/MIC for tetracyclines. In the current study, taking the MIC of DOX towards *V. parahaemolyticus* strains of 2 µg/mL (Table 1), the time during which the DOX concentration in the hepatopancreas was above the MIC (T > MIC) was 4 h. This MIC value corresponded to the lowest observed MIC among the tested isolates and was selected because DOX concentrations achieved in shrimp tissues were relatively low; therefore, PK/PD indices in the present study could only be meaningfully evaluated for susceptible strains within the range of attainable tissue concentrations. For time-dependent antimicrobials such as DOX, antibacterial efficacy is generally associated with T > MIC covering approximately 40–50% of the dosing interval (AliAbadi and Lees 2000). Based on this theoretical criterion, a T > MIC of approximately 4 h would correspond to a dosing interval of approximately 8 h; however, according to the calculation, DOX showed efficiency in only 10% of the collected strain, and both MIC50 and MIC90 were higher than the maximum concentration of DOX in the investigated organs. These findings suggest that the administered dose may be insufficient to achieve adequate therapeutic exposure against many field isolates. Therefore, the dosage should be increased to prolong the interval time but the dose must be lower than the safety threshold which needs further investigation. Nevertheless, this interpretation should be approached with caution, as T > MIC is not considered the principal PK/PD index for DOX, and other indices such as AUC/MIC may provide a more robust basis for dose optimization. In addition, in this study, the use of hepatopancreas concentrations for PK/PD interpretation was considered biologically relevant, as this organ is the main target of AHPND and the primary site of pathological damage caused by *V. parahaemolyticus* in shrimp. The application of DOX in practice should be based on the MIC database in the region and dose regimes should be optimized.

Depletion and withdrawal time of doxycycline in white leg shrimp

The elimination of DOX residues in the muscle of shrimp was relative quick, with concentrations dropping below the LOD (2.5 µg/kg) from 14 days onwards after stopping administration of medicated feed, whether given once or twice daily. In comparison to previous studies, the DOX residual concentrations in white leg shrimp muscle were significantly lower than those reported for fish species. For instance, after stopping DOX-medicated feed for 3 consecutive days at the same dosage used in this study (20 mg DOX/kg body weight), residual levels in grass carp muscle were found to be 49.9 µg/kg on day 42 (Xu et al. 2019b) and 20.9 µg/kg in yellow catfish muscle on day 28 (Xu et al. 2021).

According to Avunje et al. (2021), the WT refers to the period needed for drug residues in edible tissues to decrease to a safe level for consumer consumption. Applying a MRL of 50 µg/kg, the corresponding WT for this study was estimated at approximately 8 days and 10 days at 26.5 °C for feeding shrimp with DOX at the given dose in once and twice-a-day treatments, respectively. The results demonstrated that the WTs in this study were shorter than those of other species at this MRL. For example, in yellow catfish, the WT was 27 days in muscle after 3 daily oral administrations at 20 mg DOX/kg body weight at 24 °C (Xu et al. 2021). Similarly, Xu et al. (2019b) observed that the WT of DOX in grass carp was longer (50 days) at 24 °C after oral administrations for 3 consecutive days at a dose of 20 mg DOX/kg body weight. In contrast, Vinh et al. (2024) reported a short WT of 7 days at 29.4 °C when stripped catfish were fed DOX-medicated feed for 5 consecutive days at the same dosage regimen. The varying values of WTs among species may be due to different species, size, dosage regimen and temperature. Regarding the temperature, Corum et al. (2023) found that at an MRL of 50 µg/kg, the WTs of DOX in rainbow trout muscle were 43 days and 35 days following oral administration at 20 mg/kg for 5 consecutive days at 10 and 17 °C, respectively. The result indicated that

higher temperatures facilitate faster elimination of antibiotic residues, resulting in a shortened WT. Additionally, the depletion and WT of DOX in white leg shrimp in this study were longer than those of other antibiotics commonly used in shrimp farming, such as cefotaxime (Huynh et al. 2024), oxytetracycline (Wang et al. 2004; Avunje et al. 2021), sulphamethoxazole (Wang et al. 2004).

Impact of doxycycline on hepatopancreas histology of white leg shrimp

Hepatopancreas of shrimp plays a pivotal role in digestion and metabolism and is one of the indicators which help to predict the health status of shrimp (Caceci et al. 1988; Manan et al. 2015). Due to its vital functions, any cellular damage or structural alteration in this organ can have detrimental effects, including reduced growth and immunological activity, and even increased mortality. Caceci et al. (1988) identified that there are four types of epithelial cells in hepatopancreases, each with specific functions, which include embryonic cells (E cells), responsible for mitotic division; fibrillar cells (F cells), responsible for extracellular digestion; resorptive cells (R cells), which store glycogen and reserve lipid droplets, and blister cells (B cells), which contain digestive enzymes. For the purpose of the current study, a relatively detailed histological examination of the hepatopancreas was conducted, focusing specifically on B and F cells. The selection of these cell types was based on the previous research, Nima et al. (2022) found that the proportion of R cells in the hepatopancreas remains relatively unchanged in both normal and growth-retarded shrimp, whereas the number of B cells are significantly reduced in shrimp with slowed growth compared with normal shrimp. Another reason was the observations from shrimp farmers, who noted that shrimp growth tends to slow when medicated feed containing antibiotics is used. This growth retardation is often linked to compromised digestive functions in the hepatopancreas. Both B and F cells are integral to digestion - F cells synthesize and store digestive enzymes in large vesicles, which is later transformed into B cells (Hu and Leung 2007). These enzymes are then released into the hepatopancreatic lumen via a holocrine secretion process facilitated by B-cells (Nunes et al. 2014).

In the present study, the hepatopancreatic cell numbers fully recovered after seven days of stopping DOX-medicated feed at a dose of 20 mg/kg body weight, administered either once or twice daily for 3 consecutive days. This reversible and transient histological alteration is consistent with the pharmacokinetic characteristics observed in the present study, namely the relatively low tissue exposure and rapid elimination of DOX from the shrimp body. The low C_{max} and short elimination half-life in hemolymph and hepatopancreas suggest limited systemic accumulation of DOX, which may explain the absence of severe or persistent histopathological damage. This outcome is consistent with findings from previous research on cefotaxime in white leg shrimp, which similarly showed no histopathological effects on hepatopancreatic cells at a dose of 25 mg/kg body weight (Huynh et al. 2024). In contrast, studies on other antibiotics or other veterinary medicines, such as enrofloxacin, oxytetracycline, and maduramicin, have reported significant adverse effects on shrimp health, particularly on sensitive organs like the hepatopancreas (Avunje et al. 2021; Gao et al. 2021; Maftuch et al. 2017, Bray et al. 2006). The hepatopancreas is known as a primary site of xenobiotic metabolism, where the drug and its residual compounds are catabolized to be eliminated from the shrimp body (Rewitz et al. 2006). Therefore, the mild and reversible histological changes observed in this study likely reflect the role of the hepatopancreas in drug processing rather than toxic injury, further supporting the PK findings of low tissue exposure and rapid drug clearance at the applied dose. Several research reports have demonstrated that higher doses of drugs correlate with more severe histological damage to the hepatopancreas. For instance, Gao et al. (2021) reported that exposure to 0.7 mg/L of maduramicin in crayfish (*Procambarus clarkii*) reduced the number of hepatic epithelial cells, while a higher concentration of 7.0 mg/L induced more severe pathological changes in crayfish hepatopancreas. The finding highlights the risks associated with excessive antibiotic use and serve as a warning to shrimp farmers. Overuse or prolonged exposure to high doses of antibiotics or chemicals can result in serious health issues on shrimp.

Conclusion

The findings of this study have provided valuable insights into the PK of DOX as well as its optimal use in shrimp to control pathogens while ensuring consumer safety. The results indicate that after a single dose of 20 mg/kg body weight, DOX is quite rapidly absorbed and eliminated in hemolymph, hepatopancreas, and muscle of white leg shrimp. Regarding food safety, after oral administration, DOX requires 8 days and 10 days to deplete in shrimp muscle at 26.5 °C for once and twice daily regimens, respectively. In addition, DOX oral medication caused slight effect on hepatopancreas but the recovery was found after 7 days of cessation of medication. For managing *V. parahaemolyticus* infections, however, the dose should be increased to prolong the interval time in future studies while taking possible adverse effects on the hepatopancreas into account. Optimizing dosage is essential not only for effective disease control but also for minimizing antibiotic resistance risks.

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Authors' contributions All authors contributed to the study conception, visualization, design and methodology. Material preparation, data collection and curation, validation, analysis and software were performed by Huynh Thi Kim Duyen, Nguyen Quoc Thinh, Tran Minh Phu, Dang Thi Hoang Oanh, Marie-Louise Scippo, Mathias Devreese, Siska Croubels. The first draft of the manuscript was written by Huynh Thi Kim Duyen and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate The experiments and animals used in this work were followed and treated according to Decision No. 3965/QD-DHCT Date October 15 2021 "Can Tho University Regulation on Ethics in animal experimentation" URL: https://dra.ctu.edu.vn/images/upload/news/246.pdf.

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Competing interests The authors declare no competing interests.

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