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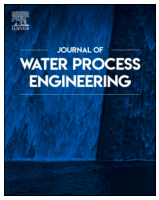

Comparison of the inactivation of *Uronema marinum* in mariculture by UV/chlorine, UV/monochloramine, and UV/chlorine dioxide: Efficiency, mechanism and feasibility

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ABSTRACT

Despite a growing interest in advanced oxidation processes (AOPs) for the inactivation of harmful microorganisms, there is relatively little research on the inactivation of harmful protozoa in aquaculture water. The research investigated the effectiveness and mechanisms of UV/chlorine, UV/monochloramine (UV/NH_2Cl) and UV/chlorine dioxide (UV/ClO_2) inactivating *Uronema marinum* (*U. marinum*), and assessed the feasibility of these processes in marine aquaculture. Three AOPs effectively inactivated *U. marinum* in the following sequence: UV/chlorine > UV/NH_2Cl > UV/ClO_2. Furthermore, the concentrations of free radicals (•OH, •Cl, and •ClO) generated in the three AOPs exhibited the same order. In the investigation of the inactivation mechanism of *U. marinum*, transcriptomic analysis results indicate that the effects of chlorine-based AOPs on the transcription, translation, and lipid metabolism of *U. marinum* may be critical factors of the inactivation of *U. marinum*. Additionally, the feasibility of chlorine-based AOPs for inactivating protozoa in marine aquaculture was assessed through pathological analysis of fish. The results indicate that compared to UV/chlorine and UV/NH_2Cl treatments, tilapia cultured in seawater treated with UV/ClO_2 had lower mortality rates and minimal damage. This study provides valuable fundamental information for the selection and operation of AOPs to deactivate harmful microorganisms in marine aquaculture water.

1. Introduction

Chlorine-based advanced oxidation processes (AOPs) typically refer to the process of UV photolysis of chlorine, chloramine (NH_2Cl), or chlorine dioxide (ClO_2) (hereinafter referred to as UV/chlorine, UV/NH_2Cl, and UV/ClO_2, respectively) [1–6]. In microbial inactivation, chlorine-based AOPs exhibit higher efficiency compared to chlorination and UV alone, with lower residual chlorine content and the absence of photoreactivation repair phenomenon, respectively [7,8]. Moreover, in contrast to other AOPs (UV/sodium persulfate, UV/hydrogen peroxide, etc.), the residual chlorine in the chlorine-based AOPs system can offer sustained bactericidal efficacy in the treatment of marine aquaculture water [9].

Chlorine-based AOPs have been studied in the laboratory and are expected to be applied in water disinfection [9–11]. The main reason should be attributed to various highly reactive species formed in these AOPs systems, which enhance the inactivation of harmful microorganisms, particularly chlorine-resistant bacteria [12–14]. Primary radicals, such as •OH and •Cl, are produced by UV/chlorine (Eq. S1 in Table S1). The formation process of secondary free radicals such as $Cl_2^{\bullet-}$ and •ClO is shown in Eq. S2–S6 in Table S1 [15]. The UV photolysis of NH_2Cl produces •Cl and amidogen radical (•NH_2) (Eq. S7 in Table S1), •OH can be formed by •Cl, while •NH_2 is relatively unreactive (Eq. S9 in Table S1) [16–18]. As shown in Eq. S13–S17 in Table S1, •ClO, •Cl, •OH, and O_3 are generated in UV/ClO_2 [19]. Cl^-, HCO_3^- and Br^- are common ions present in seawater that can influence the formation and transformation of reactive radicals in chlorine-based AOP systems. In the UV/ClO_2 system, it was found that •Cl and •OH concentrations decreased as Cl^-

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concentrations increased [19]. UV/chlorine systems oxidise Br^- to bromine which can consume •Cl and •OH [20]. When 1 mM HCO_3^- was added to the UV/ClO_2 system, both •Cl and •OH concentrations were reduced by 25 % and 24 %, respectively [19]. The Cl^-, HCO_3^- and Br^- concentrations in seawater can reach as high as 19.0 g L^{-1}, 152.0 mg L^{-1} and 65.0 mg L^{-1}, respectively [6]. However, the comparison of reactive formation in seawater by the UV/chlorine, UV/NH_2Cl, and UV/ClO_2 has not been well addressed.

UV/chlorine, UV/ClO_2, and UV/NH_2Cl have been demonstrated to be effective in deactivating various harmful microorganisms in water. For example, at similar UV (360 mJ cm^{-2}) irradiation and chlorination (1 mg L^{-1}), the inactivation of *Pseudomonas aeruginosa* with UV and chlorination alone ranged from 6.0 log and 5.6 log after 30 min, respectively. However, UV/chlorine AOP inactivated almost all *P. aeruginosa* within 10 min (over 7.0 log) [8]. At a UV dose of 80 mJ cm^{-2} and 2.0 mg L^{-1} NH_2Cl, the inactivation of *Aspergillus niger, Aspergillus flavus*, and *Aspergillus fumigatus* by the treatment of UV/NH_2Cl could reach 3.33 log, 2.84 log, and 4.75 log, respectively, significantly higher than those treated with UV or NH_2Cl alone [21]. The inactivation of *Staphylococcus aureus* (*S. aureus*) by UV/chlorine treatment reached 7 log at the UV was 18.0 mJ cm^{-2} and the chlorine content was 2.0 mg L^{-1} [22]. Additionally, the inactivation mechanisms of reactive radicals on microorganisms have been extensively researched. Researchers have shown that the critical factor of virus inactivation following UV/chlorine AOP is the destruction of the viral genome caused by both RCS (Reactive Chlorine Species, including •ClO, •Cl, etc.) and •OH radicals [23]. Another study confirmed that the reactive radicals formed in the UV/chlorine AOP significantly decreased metabolic activity and inhibited photoactivation, thereby enhancing the damage to cell membranes of *Staphylococcus aureus* [22]. Besides, the microbial species may impact the inactivating effectiveness of RCS and •OH. In UV/chlorine treatment, •OH contributed to eliminating tetracycline-resistant bacteria by 48 % and amoxicillin-resistant bacteria by 19 %, while RCS had a negligible effect [24]. According to reports, the main protozoan groups including *amoeba*, *flagellates*, *apicomplexans*, *microsporidians*, and *ciliates* can cause serious morbidity and mortality in farmed fish [25,26]. Although chlorination is widely used to disinfect aquaculture water, theoretical research on the application of chlorine-based AOPs in aquaculture disinfection remains limited [27]. In addition, research on the inactivation of harmful protozoa in marine aquaculture by chlorine-based AOPs is limited.

Uronema marinum (*U. marinum*) is a tissue phagocytic ciliate that can cause ciliophora disease in marine fish. According to reports, ciliophora disease has a high incidence in the intensive marine aquaculture of flatfish (such as *olive flounder, Paralichthys olivaceus*, etc.) [28]. Therefore, *U. marinum* was selected as a representative harmful marine protozoan to explore the inactivation efficiencies and mechanisms of UV/chlorine, UV/NH_2Cl, and UV/ClO_2. The aim of this study is to 1) investigate the inactivation dynamic kinetics of *U. marinum* under UV/chlorine, UV/ClO_2, and UV/NH_2Cl systems to compare their inactivation abilities; 2) research the production of reactive radicals in the three AOPs in seawater and the influencing factors to explore the contribution of •OH, •Cl, and •ClO to inactivation; 3) explore the mechanism of *U. marinum* inactivation by transcriptomics; 4) determine the contents of the halogenated methanes (THMs) and haloacetic acids (HAAs) generated in the chlorine-based AOPs to study the ecological toxicity of these processes; 5) investigate and compare the feasibility of chlorine-based AOPs in aquaculture through fish pathology methods. The research is expected to provide valuable foundational information for selecting and applying chlorine-based AOPs to inactivate harmful protozoa in mariculture.

2. Materials and methods

2.1. Chemicals

Please refer to Table S2 for details on the chemicals and materials used in this study. The preparation details of chlorine, NH_2Cl, and ClO_2 stock solutions are shown in Text S1.

2.2. Aquatic harmful protozoa

U. marinum was chosen as a representative of harmful marine protozoa for the experiment. Detailed information on *U. marinum* refer to Text S2 and Fig. S1. The liquid culture medium was established for expanding cultivation in the laboratory. Individual cells were separated from the original population using a pipette. Contaminated microorganisms were reduced by multiple washes in sterile seawater. Cultivate clean single cells in the seawater of wheat grains and count every 24 h until the cell density reaches approximately 100 cells μL^{-1} [29]. Transfer ciliates and nutrients from the culture dish to a conical flask containing sterile seawater to expand the culture. *U. marinum* is cultured in seawater with a temperature of 20–25 °C, salinity of 35 ‰, and pH = 8 [30]. Bacteria serve as the food source for ciliates, and grains naturally harbor bacteria that can support their nutrition. Incubate sterile seawater containing 150 grains of wheat per litre continuously at 25 °C for 24 h to obtain wheat leachate as a nutrient solution [31]. The *U. marinum* used in each experimental group originates from the same batch of culture medium, and the magnetic stirrer was used to maintain a consistent concentration of bacteria in the reaction solution [32]. For detailed information on the *U. marinum* counting method, please refer to Text S2.

2.3. UV exposure

The reactor, equipped with a constant voltage UV lamp, quartz reaction tube, and magnetic stirrer (diameter 2.5 cm, length 18 cm), was used for inactivation experiments. A low-pressure mercury lamp, which primarily emits 254 nm light, was used as the light source, and the UV fluence was 1.68 mW cm^{-2}. Details on the measurement of incident fluence rate are shown in the Text S3.

2.4. Experimental procedures

An oxidant stock solution (chlorine, NH_2Cl, and ClO_2) was added to 40 mL of seawater containing *U. marinum* to achieve an oxidant content of 25 μM. The use of a 25 μM oxidant dose was practical and close to actual operational conditions (detailed explanations in Text S4 and Table S3). Ciliate suspensions containing oxidants (40 mL) were placed in quartz reaction tubes positioned parallel to the incident light on a stir plate for reaction. The seawater used in the experiment was prepared with sea salt and deionised water, achieving a salinity of 35 ‰ and a pH of 8, making it the most closely matched to the parameters of actual marine aquaculture water. The main components of sterile seawater include NaCl, $MgSO_4{\cdot}7H_2O$, $MgCl_2{\cdot}6H_2O$, $CaCl_2$, $NaHCO_3$, KCl, NaBr, H_3BO_3, Na_2SiO_3, H_3PO_4, Al_2Cl_6, NH_3, and $LiNO_3$ (Detailed contents of each substance can be found in Table S4). Unless otherwise stated, most experiments were conducted in seawater (35 ‰) with an initial density of 5000 cells mL^{-1}, 25 μM oxidant, and UV fluence of 1.68 mW cm^{-2}. The density was chosen based on the studies about infection and inactivation of *U. marinum* [32,33]. In the inactivation kinetics experiment, the inactivation time was set at 120 s to facilitate a more precise comparison of the efficiency of various techniques. The kinetics studies investigated the impact of seawater salinity (12, 17, 35 ‰) and NOM (Natural Organic Matter) concentration (0, 1.5, 3.0 mg L^{-1}) (Detailed information in Text S2). The kinetics curve of *U. marinum* inactivation is similar to those described in previous literature, with an m value of 2 or 3 during the microorganism's inactivation process. The rate constant of

inactivation is fitted using the Hom model and can be expressed as ($R^2 > 0.900$):

$$log\left(\frac{N_0}{N_t}\right) = \frac{k}{2.303}c^n t^m = k_{obs}t^m$$

where k ($\mu M^{-n}\ s^{-2}$), c (μM), n, k_{obs}(s^{-2}), and t (s) represent the experimental reaction rate constant, disinfectant concentration, empirical constant, inactivation kinetic constant, and time (s), respectively (Detailed information in Text S2) [34].

Add 25 μM of nitrobenzene (NB), benzoic acid (BA), and 1,4-dimethylbenzene (DMOB) to the solution before the reaction, and calculate the steady-state concentrations of •OH, •Cl, and •ClO in the chlorine-based AOPs system by detecting the degradation rates of these substances. The concentrations of NB, BA, and DMOB were measured using high-performance liquid chromatography (HPLC) equipped with a C18 column (Agilent Poroshell 120 EC) and UV detector (1260 Infinity II, Agilent) (Details refer to Text S5) [35–39].

In the experiments on the inactivation mechanism, extracellular DNA concentration was detected using the ND1000 Nanodrop spectrophotometer (Nanodrop Technologies, USA), with a reaction time set to 10 min to thoroughly investigate the effects of various processes on DNA [8,22]. In transcriptomic analysis, total RNA was extracted from the sample using the Trizol reagent kit. To meet the quality inspection standards, and keep all other conditions constant, the initial microbial concentration was increased to 1×10^8 cells mL^{-1}, and the reaction time was extended to 5 min. After passing the quality assessment of total RNA, enriched mRNA fragments were fragmented using random primers and reverse transcribed into cDNA. The constructed library was sequenced using Illumina Novaseq 6000. Differential expression genes (DEGs) were analyzed using the KEGG database.

Samples were collected after two minutes of reaction in the three AOPs, and the concentrations of THMs and HAAs were analyzed after quenching [14] (refer to detailed information on quenching and sample pre-processing methods in Text S6).

To compare the application of UV, chlorination, and chlorine-based AOPs in practical marine aquaculture, experiments were conducted at a laboratory scale using tilapia fry as a representative species of marine aquaculture. The seawater containing *U. marinum* was disinfected (treatment time = 5 min, initial concentration = 5000 cells mL^{-1}, salinity = 17 ‰), after which the fish fry were placed in the disinfected seawater for cultivation. After 48 h of cultivation, a small portion of the living body was taken for pathological section analysis of the fish gills. The survival rate of juvenile fish was calculated within 168 h of cultivation. To mitigate the impact of tilapia excrement, juvenile fish were fed twice a day during the experiment, with an average of 1–2 g per fish per feeding. Residual food and excrement were removed using gauze nets and capillary straws 1 h and 4 h after feeding to minimize the effects of tilapia excreta (refer to detailed information in Text S7).

3. Results and discussion

3.1. Comparison of U. marinum inactivation in the three AOPs

Fig. 1a illustrates the inactivation of *U. marinum* by UV, chlorination and chlorine-based AOPs. Obviously, the AOPs exhibited better inactivation efficiency than both chlorination and UV treatment alone. At a UV dose of 200 mJ cm^{-2}, chlorine, NH_2Cl, ClO_2 and UV treatments resulted in *U. marinum* inactivation rates of 0.43 log, 0.26 log, 0.20 log, and 0.44 log, respectively. Fig. 1b illustrates that UV/chlorine, UV/NH_2Cl, and UV/ClO_2 significantly enhanced inactivation by 1.50 log, 1.20 log, and 0.86 log, respectively. The k_{obs} followed the order of UV/chlorine ($10.69 \times 10^{-5}\ s^{-2}$) > UV/$NH_2Cl$ ($6.91 \times 10^{-5}\ s^{-2}$) > UV/$ClO_2$ ($4.56 \times 10^{-5}\ s^{-2}$) > chlorine ($3.77 \times 10^{-5}\ s^{-2}$) > UV ($3.50 \times 10^{-5}\ s^{-2}$) > NH_2Cl ($1.81 \times 10^{-5}\ s^{-2}$) > ClO_2 ($1.58 \times 10^{-5}\ s^{-2}$) (Table S5). The various oxidative free radicals generated within the three AOPs systems are the reasons for the synergistic effects of these systems, such as •OH and RCS produced through the photocatalytic degradation of oxidants, as shown in Table S1 [8].

3.2. Effect of the salinity and NOM

Detected and compared the effect of salinity on the inactivation by three AOPs. As shown in Fig. 2a, when the salinity of seawater decreased from 35 ‰ to 12 ‰, the k_{obs} increased from $10.69 \times 10^{-5}\ s^{-2}$, $6.91 \times 10^{-5}\ s^{-2}$, and $4.56 \times 10^{-5}\ s^{-2}$ to $17.96 \times 10^{-5}\ s^{-2}$, $10.30 \times 10^{-5}\ s^{-2}$, and $9.31 \times 10^{-5}\ s^{-2}$ by the treatment of UV/chlorine, UV/NH_2Cl, and UV/ClO_2, respectively (Table S5). The result may be attributed to the reaction between anions in seawater and major reactive radicals (including •OH and RCS). For instance, Cl^- promotes the formation of $•Cl_2^-$ ($k = 6.5 \times 10^9\ M^{-1}\ s^{-1}$) (Table S1), which does not contribute to the inactivation of microorganisms [8,38]. The concentrations of •OH and •Cl were both reduced by the reaction with HCO_3^- with reaction rate of $8.5 \times 10^6\ M^{-1}\ s^{-1}$ and $2.2 \times 10^8\ M^{-1}\ s^{-1}$(Table S6), respectively, while the scavenging effect of HCO_3^- toward •ClO was barely ($<6.0 \times 10^2\ M^{-1}\ s^{-1}$) [19].

Furthermore, experiments were conducted using humic acid (HA) as a NOM to investigate the effect of NOM on the inactivation efficiency of chlorine-based AOPs in natural seawater. As shown in Fig. 2b, the k_{obs} decreased from $10.69 \times 10^{-5}\ s^{-2}$, $6.91 \times 10^{-5}\ s^{-2}$, and $4.56 \times 10^{-5}\ s^{-2}$ to $1.78 \times 10^{-5}\ s^{-2}$, $1.19 \times 10^{-5}\ s^{-2}$, and $0.93 \times 10^{-5}\ s^{-2}$ with the addition of 3.0 mg L^{-1} HA in UV/chlorine, UV/NH_2Cl, and UV/ClO_2, respectively (Table S5). Obviously, the presence of NOM significantly inhibited the inactivation of *U. marinum*. The reduction in inactivation efficiency was attributed to the decreased formation of reactive radicals

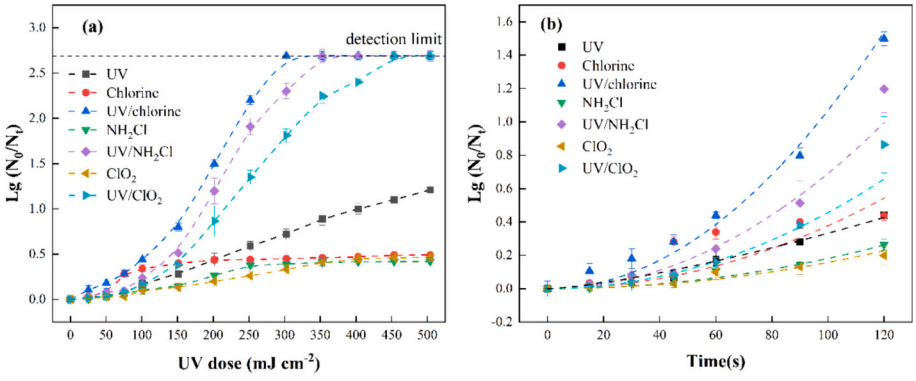

Fig. 1. Inactivation of *U. marinum* under different process treatments. (a) inactivation curve at 5 min of reaction; (b) inactivation kinetic curve fitted by the Hom model. Baseline conditions: Initial concentration of *U. marinum* = 5000 cells mL^{-1}, [chlorine] = [NH_2Cl] = [ClO_2] = 25 μM, PH = 8, salinity = 35 ‰, and UV fluence of 1.68 mW cm^{-2}.

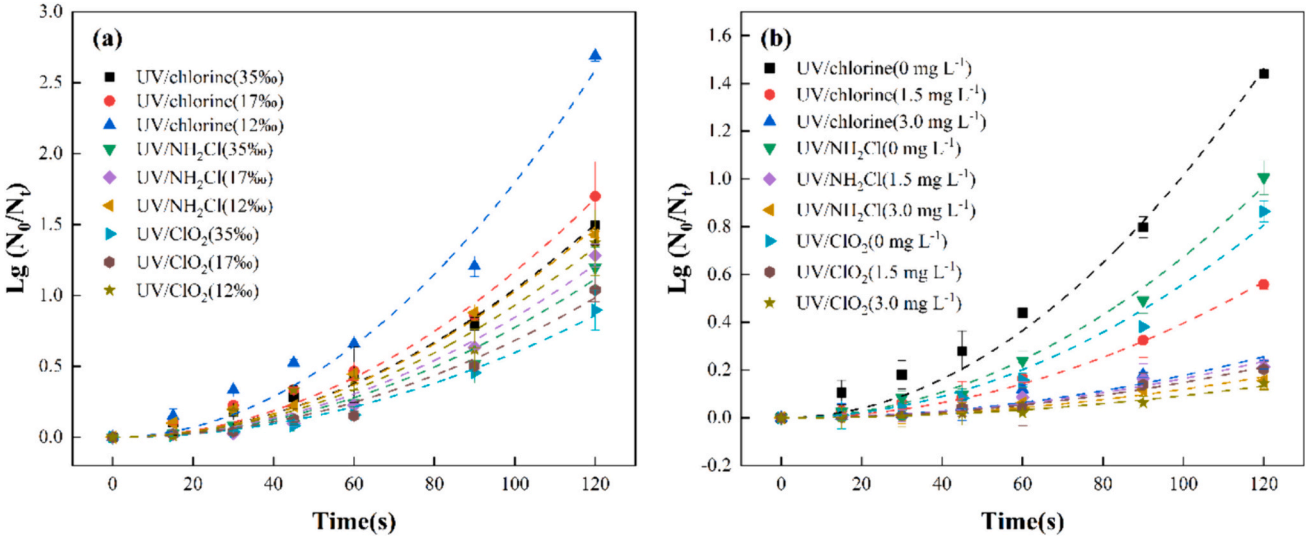

Fig. 2. The impact of various water quality parameters on the inactivation efficiency of *U. marinum* by chlorine-based AOPs. (a) salinity; (c) concentrations of HA. Baseline conditions: Initial concentration of *U. marinum* = 5000 cells mL^{-1}, [chlorine] = [NH_2Cl] = [ClO_2] = 25 μM, PH = 8, salinity = 35 ‰, and UV fluence of 1.68 mW cm^{-2}.

by chlorine-based AOPs. Previous research has reported that NOM acts as a scavenger for reactive radicals, reacting rapidly with •OH and RCS, as shown in Table S7 [19]. Therefore, the effect of salinity and NOM on the concentrations of free radicals in the chlorine-based AOPs was also investigated.

3.3. Effects of salinity and NOM on reactive species concentrations

To investigate the reactivity of radicals in the inactivation of *U. marinum*, the key radicals (•OH and RCS) formed in the chlorine-based AOPs were detected. $•Cl_2^-$ was neglected because it was reported not to be contributed to the inactivation of microorganisms [8]. As shown in Table 1, the contents of •OH, •Cl, and •ClO formed in the AOPs followed UV/chlorine > UV/NH_2Cl > UV/ClO_2 which corresponded to the inactivation rates of *U. marinum*. The reason for the lowest concentration of reactive radicals in UV/ClO_2 may be attributed to organic matter and components in water and microorganisms, which could react with ClO_2 to produce ClO_2^-, a species that rapidly reacts with •OH, •Cl, and •ClO. It should be emphasised that the concentrations of •ClO in the three AOPs were much higher (1–2 orders of magnitudes) than both •OH and •Cl. On the other hand, •OH, •Cl, and •ClO have been proven to react rapidly with natural organic matter (NOM) with a second-order rate constant of 2.5 × 10^4, 1.4 × 10^4, and 4.5 × 10^4 (mg C $L^{-1})^{-1}$ s^{-1}, respectively [19]. Proteins, polysaccharides, fats, and nuclear acids are important organic components that constitute microorganisms within NOM [39,40]. Among the reactive radicals generated in the chlorine-based AOPs system, •ClO may play a crucial role in inactivating *U. marinum*, given its highest concentration and the fastest reaction rate with NOM [11]. The results are similar to previous studies which focused on the degradation of micropollutants by chlorine-based AOPs [41,42].

Table 1
The steady-state concentrations of reactive species generated in UV/chlorine, UV/NH_2Cl, and UV/ClO_2 systems.

	•OH (×10^{-14} M)	•Cl (×10^{-15} M)	•ClO (×10^{-13} M)
UV/Chlorine	3.08 (±0.086)	4.89 (±0.439)	3.20 (±0.067)
UV/NH_2Cl	2.31 (±0.013)	2.05 (±0.164)	2.23 (±0.033)
UV/ClO_2	2.26 (±0.158)	4.05 (±0.141)	1.09 (±0.181)
UV/Chlorine[a]	6.92 (±0.127)	4.57 (±0.187)	3.38 (±0.183)
UV/NH_2Cl[a]	3.33 (±0.134)	1.15 (±0.429)	2.44 (±0.133)
UV/ClO_2[a]	3.38 (±0.179)	5.15 (±0.638)	1.09 (±0.166)
UV/Chlorine[b]	9.74 (±0.292)	8.95 (±0.140)	5.56 (±0.173)
UV/NH_2Cl[b]	6.92 (±0.194)	9.02 (±0.477)	3.49 (±0.143)
UV/ClO_2[b]	4.10 (±0.236)	5.20 (±0.590)	3.14 (±0.201)
UV/Chlorine[c]	2.31 (±0.204)	5.98 (±0.122)	0.61 (±0.037)
UV/NH_2Cl[c]	1.54 (±0.062)	1.88 (±0.295)	1.80 (±0.075)
UV/ClO_2[c]	1.28 (±0.063)	1.38 (±0.179)	0.69 (±0.171)
UV/Chlorine[d]	1.11 (±0.043)	1.19 (±0.162)	0.43 (±0.033)
UV/NH_2Cl[d]	1.03 (±0.018)	0.88 (±0.096)	0.96 (±0.049)
UV/ClO_2[d]	1.10 (±0.103)	0.70 (±0.019)	0.58 (±0.022)

Baseline condition: [chlorine] = [NH_2Cl] = [ClO_2] = 25 μM, PH = 8, salinity = 35 ‰, UV fluence of 1.68 mW cm^{-2} and treatment time was 120 s. a. salinity = 17 ‰. b. salinity = 12 ‰. c. [HA] = 1.5 mg L^{-1}. d. [HA] = 3.0 mg L^{-1}.

Effects of salinity and NOM on the concentrations of reactive species were also determined. As shown in Table 1, the concentrations of radicals decreased with increasing water salinity from 12 ‰ to 17 ‰ and 35 ‰ in the three AOPs. Under the salinity of 35 ‰ which was consistent with standard seawater, the concentrations of •OH, •Cl, and •ClO decreased to 3.08 (±0.086) × 10^{-14} M, 4.89 (±0.439) × 10^{-15} M, and 3.20 (±0.067) × 10^{-13} M, 2.31 (±0.013) × 10^{-14} M, 2.05(±0.164) × 10^{-15} M, and 2.23(±0.033) × 10^{-13} M, 2.26(±0.158) × 10^{-14} M, 4.05 (±0.141) × 10^{-15} M, and 1.09 × 10^{-13} M by the system of UV/chlorine, UV/NH_2Cl, and UV/ClO_2, respectively. The phenomenon should be attributed to the anions in seawater including Cl^- (19,700 mg L^{-1}), SO_4^{2-} (3200 mg L^{-1}) and HCO_3^- (152 mg L^{-1}), etc. In seawater with high Cl^- content, SO_4^{2-} is considered to have minimal impact on free radicals [43,44]. Cl^- has been proven to affect the formation of Cl• as shown in Table S1, the effect was more significant than that on •OH and •ClO. In contrast, HCO_3^- barely affected the concentrations of •ClO while markedly reducing the contents of •OH and •Cl [19]. Tea polyphenols are used as quenchers to detect the effect of secondary free radical $•CO_3^-$ on inactivation, which is generated by the reaction of HCO_3^- with •OH and •Cl [6]. The results indicate that $•CO_3^-$ has minimal impact on the inactivation efficiency, attributed to its low concentration and slow reaction rate with NOM (as shown in Fig. S2 and Table S1) [11]. The concentrations of radicals all decreased with increasing HA contents from 0 to 1.5 and 3.0 mg L^{-1} in the three AOPs. In the presence of 3.0 mg L^{-1} of HA, the concentrations of •OH, •Cl, and •ClO in the UV/chlorine, UV/NH_2Cl, and UV/ClO_2 systems decreased by 64 %, 76 %, and 69 %; 56 %, 57 %, and 57 %; and 52 %, 83 %, and 47 %, respectively. This was attributed to the fact that as a NOM, HA acted as a free radical scavenger in the AOPs. The results correspond with the inactivation experiment, indicating that the reactive radicals formed in the chlorine-based AOPs system are the main substances for inactivating *U. marinum*.

3.4. Inactivation mechanisms

The concentration of extracellular DNA (eDNA) of *U. marinum* after chlorine-based AOPs treatments was investigated. As shown in Fig. 3, the concentration of eDNA remained relatively constant at 7.26 (±0.02) ng μL^{-1} during 10 min of UV treatment. For the UV/chlorine, UV/NH_2Cl, and UV/ClO_2 treatments, the contents of eDNA peaked at 7.64, 7.69, and 7.67 ng μL^{-1} at 2 min, and then gradually decreased to 6.12, 6.40, and 6.66 ng μL^{-1} at 10 min, respectively. Similar results were obtained in the experiments involving UV/chlorine inactivating *Pseudomonas aeruginosa* [8]. •OH and RCS generated by chlorine-based AOPs have been reported to effectively damage cell membranes and degrade DNA in microorganisms [8,22]. For chlorine-based AOPs, intracellular DNA (iDNA) was released into the solution and then retained in the form of eDNA, resulting in an increase in eDNA concentration instead of complete degradation after 2 min treatment. Due to the ideal biological inactivation that could be achieved at low chlorine content (25 μM), eDNA cannot be completely removed at low ratios of free radicals to eDNA. Nevertheless, •OH and RCS exhibited efficient reactivity with DNA bases, resulting in the reduction of eDNA concentration during subsequent treatments [31].

Furthermore, the eDNA concentrations after 10 min treatment followed UV/ClO_2 > UV/NH_2Cl > UV/chlorine, which correlated with the RCS concentrations in the order of UV/chlorine > UV/NH_2Cl > UV/ClO_2. The reason should be ascribed to that the impact of •OH on DNA can be negligible due to the rapid consumption of •OH [40]. On the contrary, RCS may contribute more to gene degradation than •OH, as RCS have higher reactivity toward substances with electron-donating groups (alkyl/alkoxy aromatic hydrocarbons, olefins, etc.) [8]. The above result indicates that the reactive species produced by chlorine-based AOPs could cause DNA leakage and degradation in cells, which may contribute to the inactivation of *U. marinum*.

The impact mechanisms of different processing techniques on *U. marinum* were analyzed from a molecular biology perspective. DEGs analysis through the KEGG database revealed the main enrichment pathways, as shown in Fig. 4. As shown in Fig. 4a, under UV treatment, the DEGs of *U. marinum* primarily enriched in the ribosome pathway associated with translation, exhibiting upregulated expression of several genes related to key enzymes (EF-TU, SecY, IF2). EF-TU, SecY, and IF2 play essential roles in ribosomal function and transport. Previous studies have shown that UV irradiation can effectively degrade specific subunit complexes of ribosomes, thereby influencing cellular processes related to ribosomal function [46]. The aberrant expression of these genes may be attributed to UV-induced damage to the ribosomes.

Previous studies have demonstrated that free radicals can impact microorganisms by disrupting substances within them, such as lipid peroxidation and degradation of proteins/enzymes [45,47–49]. Chlorine-based AOPs significantly enriched a large number of DEGs in pathways associated with lipid metabolism compared to UV treatment alone (Figs. 4b–3d). In the glycerophospholipid metabolism pathway associated with lipid metabolism, UV/chlorine and UV/NH_2Cl exhibited upregulated expression of various genes related to key enzymes (PEMT, PCYT2, EPT1, etc.). The upregulation of these DEGs in the glycerophospholipid metabolism pathway indicates an enhancement in the biosynthesis processes of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) within *U. marinum* cells, thereby promoting intracellular phospholipid synthesis. The result may be attributed to the oxidisation of intracellular phospholipids within *U. marinum* under the UV/chlorine and UV/NH_2Cl systems, consequently intensifying the synthesis of phospholipids within the cells.

Furthermore, under UV/ClO_2 treatment, DEGs were enriched in the fatty acid degradation pathway related to lipid metabolism (ACADM, ACADL, fadN, fadB, MFP2, etc.) (Fig. 4d). Fatty acids are critical components of cell membranes, signalling molecules, and energy reserves. The abnormality in the fatty acid degradation pathway may be a result of the peroxidation of fatty acids in *U. marinum* induced by UV/ClO_2. In

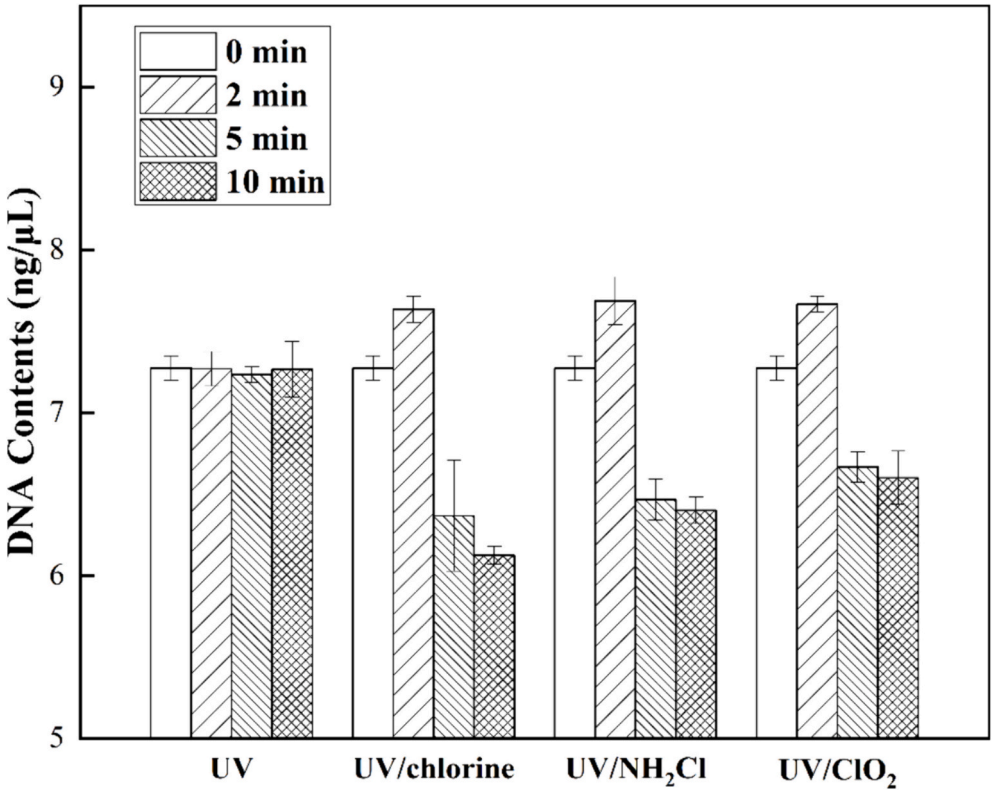

Fig. 3. Contents of eDNA after UV, UV/chlorine, UV/ClO_2, and UV/NH_2Cl treatments. (U. marinum = 1×10^6 cells·mL^{-1}, [chlorine] = 25 μM, UV fluence = 1.68 mW cm^{-2}, T = 298.15 K, pH = 8).

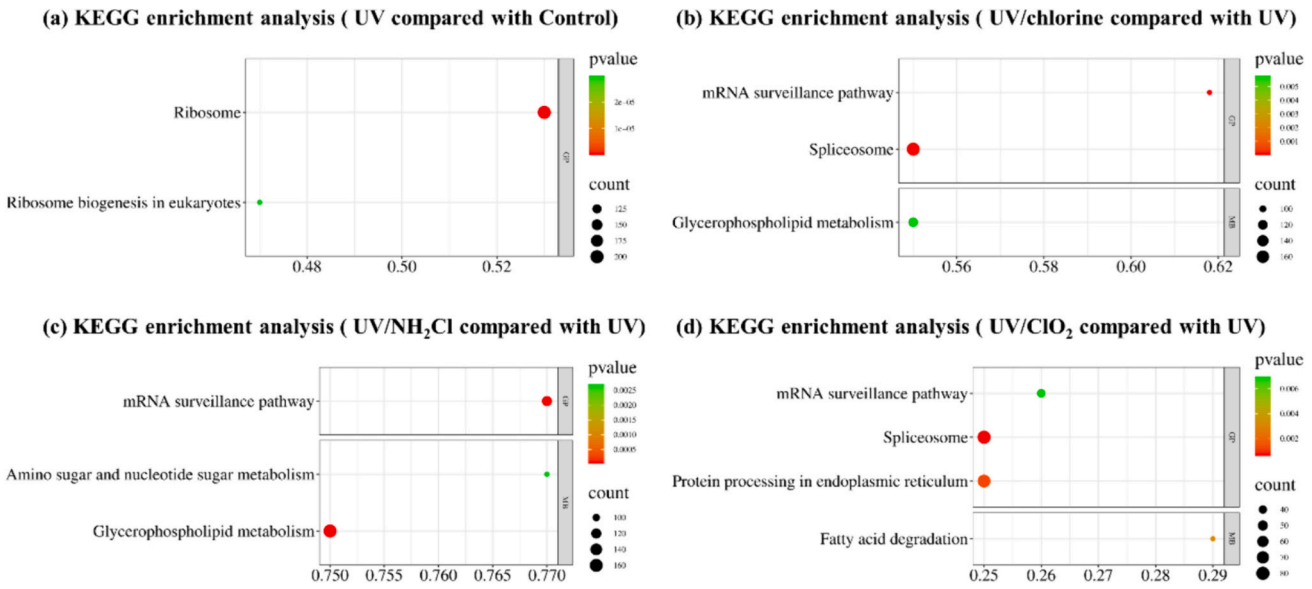

Fig. 4. KEGG pathway enrichment analysis of *U. marinum* which was treated by (a) UV, (b) UV/chlorine, (c) UV/NH_2Cl, (d) UV/ClO_2. (*U. marinum* = 1×10^8 cells·mL^{-1}, chlorine = 25 μM, UV fluence of 1.68 mW/cm^2, treatment time = 300 s, T = 298.15 K, pH = 8) (GP: Genetic Information Processing; MB: Metabolism).

addition, *U. marinum* exhibited an enrichment of DEGs in the spliceosome pathway related to transcription (Prp43, Snu114, Bn2, Prp5, etc.) under UV/chlorine and UV/ClO_2 treatments (Fig. 4a–c). The result indicates that the synthesis of the spliceosome during cellular transcription processes is affected.

Previous research has reported that free radicals can damage DNA by reacting with bases and sugars [50]. DEGs of *U. marinum* by chlorine-based AOPs treatments are also enriched in the pathway associated with translation processes (Fig. 4b–c). A substantial number of DEGs under chlorine-based AOPs treatments are enriched in mRNA surveillance pathway, upregulating genes that regulate various key enzymes (CBC, CFIm, CPSF, CstF, etc.). Aberrant mRNA in cells may potentially produce harmful truncated proteins, and the mRNA surveillance pathway plays a crucial role in both degrading abnormal mRNA and maintaining normal mRNA [51]. The upregulation of genes in mRNA surveillance pathways indicates the production of a significant amount of abnormal mRNA within the *U. marinum* system under chlorine-based AOPs treatments, possibly resulting from damage to the DNA, which serves as a template.

In summary, chlorine-based AOPs significantly impact transcription, translation, and lipid metabolism within *U. marinum*, crucially contributing to its deactivation. Specifically, UV/chlorine and UV/ClO_2 treatments primarily affect transcription, translation, and lipid metabolism processes, while the UV/NH_2Cl system predominantly influences translation and lipid metabolism (Fig. 5). The result may be attributed to the type of oxidant used in the chlorine-based AOPs system, as

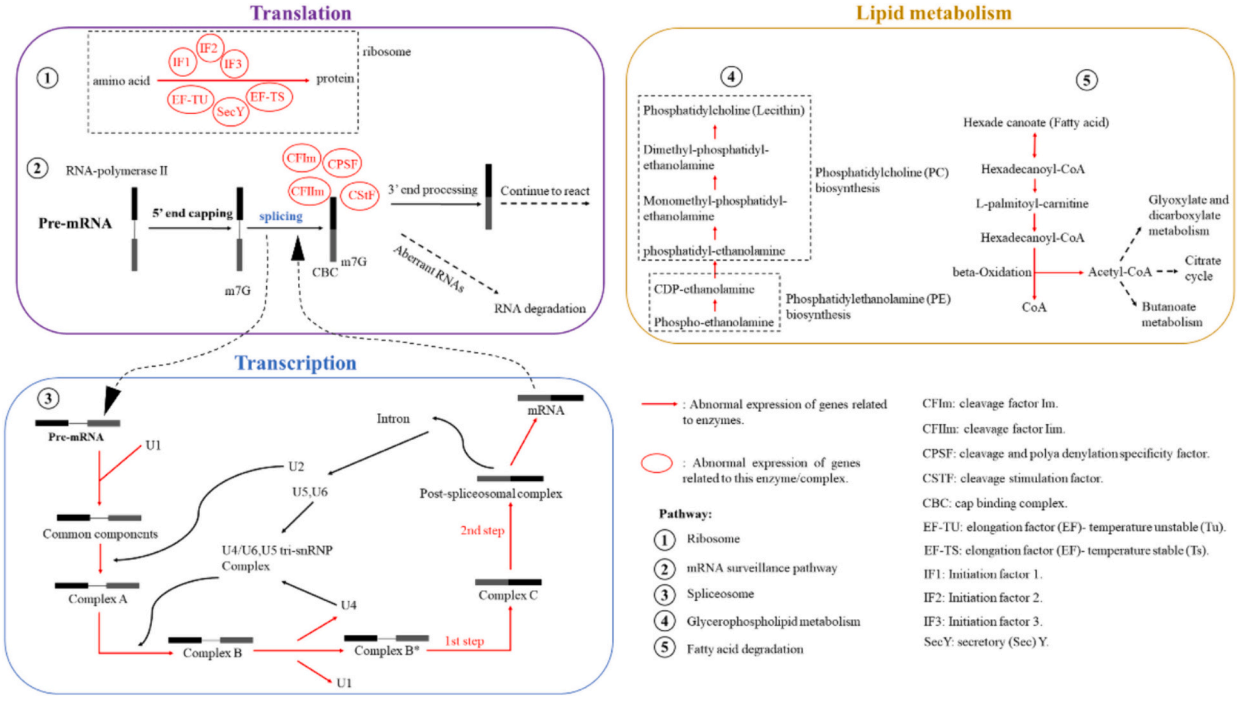

Fig. 5. The inactivation mechanism of Chlorinate-based AOPs on *U. marinum*.

U. marinum showed DEGs in transcription-related pathways following treatment with both chlorine and ClO_2 (details can be found in Text S8 and Fig. S3). Additionally, the pathways related to carbohydrate metabolism and protein synthesis observed in the UV/NH_2Cl and UV/ClO_2 systems may be attributed to the chlorine disinfectants present in each system, respectively. Further details refer to the supplementary materials (Text S8 and Fig. S3).

3.5. THMs and HAAs formation in the three AOPs

Halogenated disinfection by-products are inevitably formed in marine aquaculture water treatment practices, posing chemical risks to seafood and human health [27,52–56]. Two classical DBPs, THMs and HAAs, were detected. As shown in Fig. 6, THMs and HAAs in the three chlorine-based AOPs were higher than chlorination alone. For the AOPs, the concentration order of THMs was UV/chlorine (6.32 μg L^{-1}) > UV/NH_2Cl (4.32 μg L^{-1}) > UV/ClO_2 (3.86 μg L^{-1}). Due to the presence of bromine ions in marine aquaculture water (65.0 mg L^{-1}), monochlorodibromomethane (DBCM) had the highest concentration among THMs in all treatments. Trichloromethane (TCM) was only detected in the UV/chlorine treatment [27,57–59]. Four types of HAAs including dibromoacetic acid (DBAA), bromochloroacetic acid (BCAA), trichloroacetic acid (TCAA), and dichloroacetic acid (DCAA) were detected and the concentration order followed UV/chlorine (6.84 μg L^{-1}) > UV/NH_2Cl (5.77 μg L^{-1}) > UV/ClO_2 (5.58 μg L^{-1}). DCAA exhibited the highest content among all treatments. It was consistent with that reported in the degradation of micropollutants by UV/ClO_2 which the higher DBP concentration in the UV/ClO_2 was mainly due to the increased concentrations of DCAA [18]. The above results indicate that the more radicals generated, the more DBPs were produced. Previous research also reported that the DBP contents formed by UV/chlorine were higher than those formed by UV/NH_2Cl and UV/ClO_2, as the generation of free radicals can promote DBP formation [42]. However, the levels of both THMs and HAAs were much lower than the guidelines for drinking water set by the U.S. Environmental Protection Agency (80 μg L^{-1} for THMs and 60 μg L^{-1} for HAAs). Therefore, the generation of the selected regulated halogenated DBPs in the three chlorine-based AOPs is unlikely to be a concern [19].

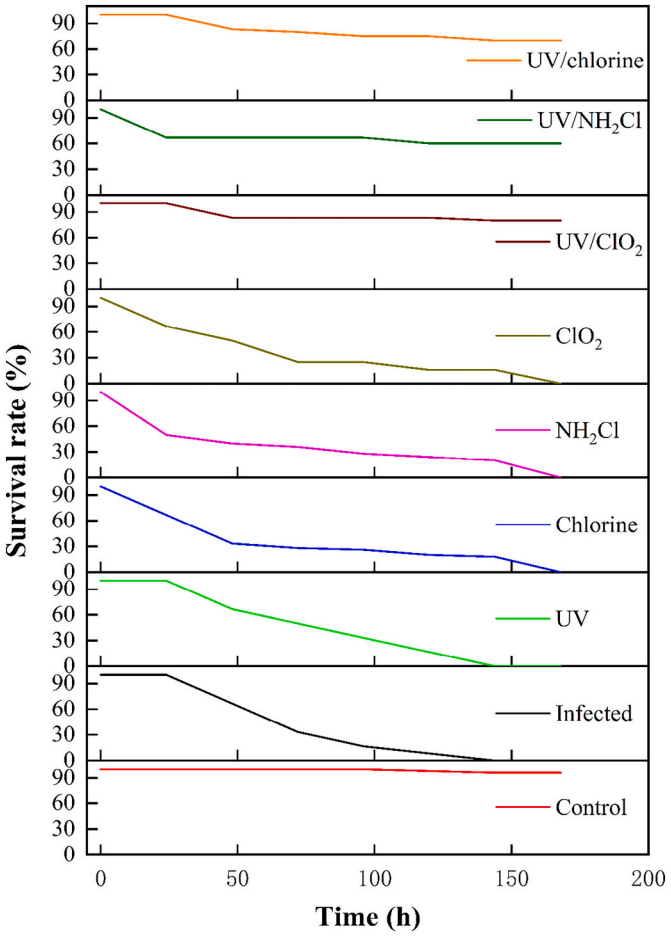

Fig. 7. Survival rate of tilapia in seawater treated with different processes. (Initial concentration of *U. marinum* = 5000 cell/mL, [chlorine] = [NH_2Cl] = [ClO_2] = 25 μM, PH = 8, salinity = 17 ‰, UV fluence of 1.68 mW cm^{-2}, disinfection time = 5 min, and culture time = 168 h.).

3.6. Application of chlorine-based AOPs for marine aquaculture

Tilapia was used as a representative to evaluate the safety of various disinfection methods. As shown in Fig. 7, the survival rate of tilapia was nearly zero in the untreated water, as well as in water treated with UV, chlorine, NH_2Cl, and ClO_2, after 168 h of cultivation. In contrast, chlorine-based AOPs demonstrated a significant advantage compared to UV and chlorination alone. The survival rates of tilapia in marine aquaculture water treated with UV/chlorine, UV/NH_2Cl, and UV/ClO_2 reached 70 %, 60 %, and 90 %, respectively. As a highly toxic and facultative ectoparasite, *U. marinum* can cause significant death in cultured fish by invading and damaging fish tissues [28]. The damage to

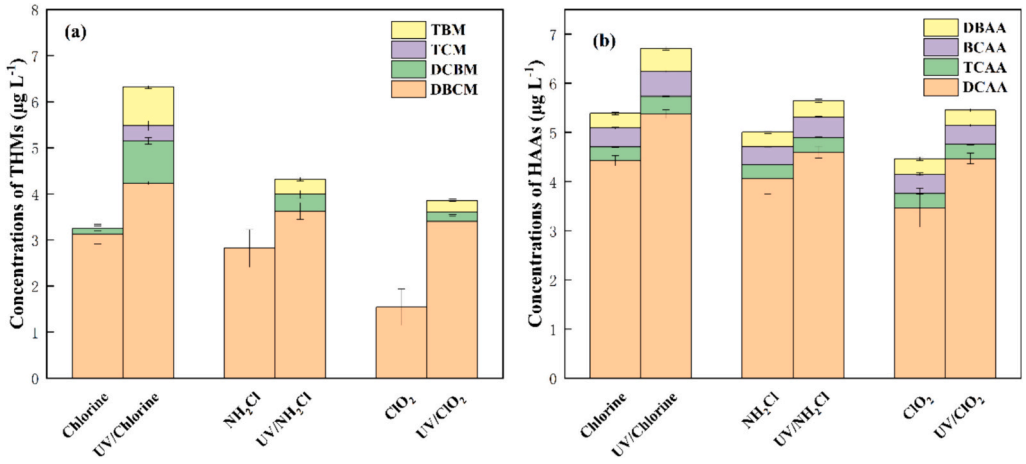

Fig. 6. The concentrations of DBPs in the three AOPs, (a) THMs, and (b) HAAs. Conditions: Initial concentration of *U. marinum* = 5000 cell/mL, [chlorine] = [NH_2Cl] = [ClO_2] = 25 μM, PH = 8, salinity = 35 ‰, and UV fluence of 1.68 mW cm^{-2}, treatment time = 120 s.

the gills of tilapia by *U. marinum* may be the primary factor for its death. Therefore, the research examined the histopathological changes in the gills of tilapia farmed in marine aquaculture water infected with *U. marinum* and treated by various processes. As shown in Fig. 8a, the overall structure of the fish gills is normal, with neatly arranged gill filaments and normal epithelial cells. However, the overall structure of the gills appeared abnormal with significant shrinkage and deformation of the gill filaments in tilapia cultured in the infected and UV-treated marine aquaculture water. *U. marinum* (indicated by green arrows), extensive congestion (indicated by red arrows), and inflammatory cell infiltration (indicated by black arrows) were also clearly observed in the gill tissue (Fig. 8b–c). The results indicate that UV cannot effectively prevent the infection of tilapia by *U. marinum* in marine aquaculture water.

Previous studies have shown that chlorine disinfectants can inactivate parasites by releasing reactive oxygen/chlorine species, but can cause extensive oxidative necrosis of fish gill filaments and plate-like epithelium [9,60]. Additionally, the release of reactive oxygen can stimulate excessive mucus secretion by fish gills to cope with acute ion efflux, aiding in the removal of parasites [61]. As shown in Fig. 8d–f, for tilapia cultured in chlorine, NH_2Cl, and ClO_2 treated marine aquaculture water, the gill tissues exhibited mild abnormality in overall structure, but the gill filaments remained organized, and no inflammatory cell infiltration was observed. Partial detachment and necrosis of respiratory epithelium were observed in the gill tissue (indicated by blue arrows), with a small amount of *U. marinum* (indicated by green arrows). The phenomena were consistent with the reported literature that chlorine disinfection resulted in dose-dependent gill pathology including epithelial protrusion, proliferation, hypertrophy, necrosis and lamellar fusion [36]. The results suggested that chlorine, NH_2Cl, and ClO_2 treatments could not completely inactivate *U. marinum* in marine aquaculture water, although *U. marinum* treated by chlorine has less damage to the gills of tilapia compared to UV treatment, the damage may still cause fatal damage to tilapia.

As shown in Fig. 8g and h, for tilapia cultured in UV/chlorine and UV/NH_2Cl treated marine aquaculture water, the overall structure of the gills was normal, and there was no apparent infiltration of inflammatory cells. However, a small amount of sloughing and necrosis of the respiratory epithelium was observed in the tissues (indicated by blue arrows). This may be attributed to the oxidative effects of residual chlorine disinfectants and the free radicals generated by AOPs on the epithelial cells, as both of them have the ability to degrade proteins and lipids. Compared to UV and chlorine disinfectant treatments, AOPs demonstrated higher inactivation efficiency against *U. marinum* and caused less damage to fish gills. For tilapia cultured in UV/ClO_2 treated marine aquaculture water, the overall structure of the gills was normal, and the gill filaments were well-organized (Fig. 8i). The outer surface of the gill lamellae showed intact and smooth respiratory epithelial structures with no signs of sloughing or necrosis, and there was no evident infiltration of inflammatory cells in the tissues. The results indicated that UV/ClO_2 efficiently inactivates *U. marinum* and ensures the safety of fish, making it the most ideal disinfection method for marine aquaculture water.

To explore the potential application of chlorine-based AOPs in inactivating marine organisms, we conducted a preliminary cost analysis. According to the guidelines of the International Union of Pure and Applied Chemistry (IUPAC), the energy per order (EE/O) values and total costs of AOPs can be calculated using Eqs. (1) and (2), respectively [62].

$$EE/O = \frac{PT}{60 \times V \times log(N_0/N_t)} \quad (1)$$

$$C_T = 1.45 \times EE/O \times C_E + C_0 \quad (2)$$

*where P is the light power (W), t is the treatment time (min), V is the total treated bulk (L), N_0 is the original concentrations of ciliate *U. marinum* (cells mL^{-1}), N_t is the final concentrations of ciliate *U. marinum* (cells mL^{-1}), C_T is the total system cost ($\$ m^{-3}$), C_E is the electricity bills ($\$ KWh^{-1}$), and C_0 is the cost of oxidant ($\$ g^{-1}$).

According to Table 2., the total costs for UV, UV/chlorine, UV/NH_2Cl, and UV/ClO_2 are 0.283, 0.099, 0.115, and 0.148, respectively. The disinfection costs of chlorine-based AOPs are lower than those of UV treatment alone. Previous reports indicate that UV/H_2O_2, UV/PDS, and

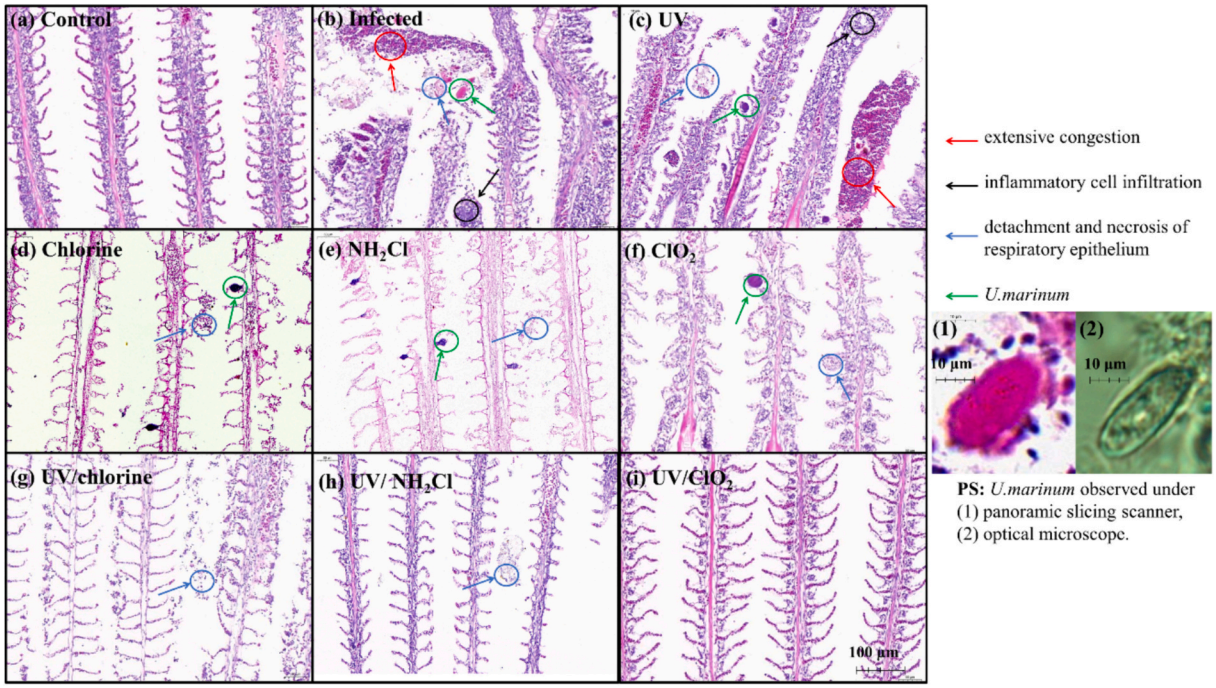

Fig. 8. Histopathological effects on the gills of *Tilapia* bathed in different treated seawater, the gills were used from fish that died after approximately 120 h of cultivation. Condition: Initial concentration of *U. marinum* = 5000 cell/mL, [chlorine] = [NH_2Cl] = [ClO_2] = 25 μM, PH = 8, salinity = 17 ‰, UV fluence = 1.68 mW/cm^2, disinfection time = 5 min, and culture time = 120 h.

Table 2
The costs of the chlorine-based AOPs system.

Treatment method	EE/O (kWh m^{-3} $order^{-1}$)	Electricity bills ($ KWh^{-1})	Oxidant cost ($ g^{-1})	Total ($ m^{-3})
UV	2.43	0.08	0	0.283
UV/chlorine	0.85	0.08	0.00007	0.099
UV/NH_2Cl	0.99	0.08	0.00014	0.115
UV/ClO_2	1.28	0.08	0.00025	0.148

UV/PMS for aquaculture water reached costs of 0.14, 0.15, and 0.15, respectively, at an oxidant concentration of 5 mg L^{-1} [62]. The costs of chlorine-based AOPs are comparable to other AOPs used for aquaculture water disinfection, indicating that chlorine-based AOPs are economically viable. However, precise economic evaluations are still needed following pilot testing and large-scale implementation.

4. Conclusions

This study provides a comparative investigation of chlorine-based AOPs regarding the generation of reactive radicals, inactivation of harmful protozoans, formation of byproducts, and feasibility in seawater. The outcomes of this research have practical implications, including:

(1) In contrast to the UV/NH_2Cl and UV/ClO_2 systems, the UV/chlorine system exhibits higher generation of RCS and •OH, leading to more efficient inactivation of *U. marinum*. However, the UV/chlorine system produces a greater quantity of DBPs than UV/NH_2Cl and UV/ClO_2. In addition, salinity and NOM in seawater can weaken the inactivation of harmful protozoa by inhibiting the generation of reactive radicals in chlorine-based AOPs. There is an inherent balance between the formation of reactive radicals and DBPs.
(2) Chlorination-based AOPs induce more pronounced damage to *U. marinum* cells compared to chlorination and UV. This may be attributed to the oxidation of DNA bases, peroxidation of lipids, and structural damage to enzymes caused by the RCS and •OH.
(3) In seawater containing *U. marinum*, juvenile tilapia experienced the least damage under UV/ClO_2 treatment, possibly due to the milder nature of ClO_2 compared to chlorine and NH_2Cl. Despite the less generation of free radicals in the UV/ClO_2 system, it still effectively deactivated harmful protozoa and produced fewer DBPs.

CRediT authorship contribution statement

Pin Gan: Writing – review & editing, Writing – original draft, Methodology, Investigation. **Xuan Chen:** Methodology, Investigation. **Yuanyuan Zhang:** Writing – review & editing, Writing – original draft, Funding acquisition. **Xudong Li:** Investigation. **Fangyu Liang:** Investigation. **Tianhao Wu:** Investigation. **Pengfei Xue:** Investigation. **Kefu Yu:** Funding acquisition.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jwpe.2024.105898.

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