Research Article

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Interactions between accumulated copper, bacterial community structure and histamine levels in crayfish meat during storage

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Abstract

BACKGROUND: Pollution in aquaculture areas may negatively impact edible species and threaten seafood quality and safety. The aim of this study was to determine the interaction between copper and bacteria in the aquatic habitat and their impact upon crustaceans. Marbled crayfish was chosen as a model of aquatic crustaceans and the influence of metal contamination on bacterial community structure in water used to culture crayfish and in crayfish themselves was investigated. Histamine, an allergen commonly formed by certain groups of bacteria in crustacean edible tissue during storage, was also determined.

RESULTS: Copper exposure increased its concentration in crayfish meat by 17.4%, but the copper concentration remained within acceptable food safety limits. Elevated copper levels affected the bacterial community both in the water used to cultivate crayfish and in the marbled crayfish themselves. Cluster analysis of 16S rRNA-gene based microbial community fingerprints revealed that copper impacted the bacterial community in the water and in the crayfish meat. However, copper exposure reduced the formation of histamine in crayfish meat during storage by 66.3%.

CONCLUSION: Copper from the habitat appears to reduce histamine accumulation in crayfish meat during storage by affecting the bacterial community structure of the cultivation water and most likely also in the intestine of the crayfish. From a food safety point of view, copper treatment during the aqua culturing of crustaceans has a positive impact on the postharvest stage.

Keywords: copper; bacterial community structure; histamine; crayfish; storage

INTRODUCTION

World aquaculture has grown tremendously in quantity, but the quality and safety aspects of aquaculture products are still a concern due to the risk of food-borne illnesses.[1] Chemical residues and bacterial contamination are two common problems threatening the quality and safety of aquaculture products.

Crustaceans are important seafood species (widely defined as edible aquatic species living in sea water, brackish water or fresh water), being the second most valuable seafood commodity.[2] Although they have high economic value, crustaceans are highly perishable food. The relatively high moisture and free amino acid content make crustacean meat quite susceptible to deteriorative bacteria.[3]

The bacterial density and diversity of freshly caught seafood are closely related to its habitat.[1,4] Bacterial growth during storage is often unavoidable, and especially occurs at inadequate storage temperatures, leading to decay of seafood. In the case of storage of crustaceans, which are histidine rich, the deteriorative process is often associated with histamine formation.[5] Hygienic qualities, handling and storage (time and temperature) are factors determining the level of histamine in seafood.[6–8] Histamine in fresh seafood stored for 12 h at room temperature reached >75 μg g^{-1} (wet weight)[7] and accumulate to over 500 μg g^{-1} (wet weight) in commercial canned seafood.[8] Several bacteria possess histidine decarboxylase, an enzyme which transforms the free amino acid histidine into histamine.[9] Ingestion of food containing histamine can cause histamine poisoning with allergic-like symptoms such as nausea, vomiting, diarrhea, hives, itching, red rash and hypotension.[10] In crustaceans, histamine may not only be produced by bacteria, but is also naturally present and acts as a neurotransmitter.[11,12] The United States Food and Drug Administration (US FDA) has set the maximum level of histamine in seafood as 50 μg g^{-1} (wet weight).[13]

In aquacultural practices, the use of copper sulfate is often unavoidable to control the overgrowth of algae. Especially at night, algae can suppress the availability of oxygen in

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water, which can be lethal for aquacultural species. On the other hand, copper itself can be considered a heavy metal pollutant. Elevated copper concentrations in aquatic environments are found worldwide, including the environments in which aquaculture normally takes place.[14–18] The copper concentrations in crustaceans increase with the length of exposure. Copper accumulation patterns in crustaceans also depend on the organ (hepatopancreas > gills > exoskeleton > muscle).[19,20] With regard to food safety assurance, the Australian National Health and Medical Research Council (ANHMRC) recommends a maximum copper level of 10 μg g^{-1} wet weight in crustaceans.[21]

Copper can have an inhibitory effect on the growth of bacteria.[22,23] Bacteria do not respond equally to copper, and can for instance develop resistance to copper.[24–27] By nature, the interaction between metals and microbes is a very complex phenomenon. Some metals are essential to certain microbes, whereas others are toxic even at low concentrations. Further, a mixture of metals (copper, nickel, chrome and iron) was found to reduce the microbial diversity in the digestive tract of aquatic crustaceans (noble and signal crayfish).[28] However, it is not yet clear if elevated levels of copper in water may interfere with the microbial community in water and in the intestine of crayfish, or if any toxic effect of copper on the microbial communities in crayfish may reduce the formation of histamine during storage. Here, we investigated the interaction between accumulated copper, microbial community structure and histamine formation in marbled crayfish during storage.

EXPERIMENTAL

Source of marbled crayfish and acclimatization

Marbled crayfish (*Procambarus fallax* forma *virginalis*; Malacostraca, Decapoda, Astacida) were used as a model of aquatic crustaceans. Marbled crayfish are parthenogenetic and have genetic uniformity.[29] Marbled crayfish were provided by Alterra, Wageningen University and the Research Centre, Netherlands. Prior to copper exposure experiments, all crayfish were acclimated for 1 week in continuously aerated, filtered copper-free tap water (pH 7.3 ± 0.1; $CaCO_3$ hardness 2–4 mmol L^{-1}) in 20 L tanks. Copper-free water was obtained from a system delivering tap water through plastic rather than metal piping on the university's premises. An aquarium filter system was applied to remove crayfish feces. Acclimatization was performed under constant laboratory conditions (temperature 20 ± 1 °C; day/night cycle of 12 h light and 12 h dark) and the animals were fed with commercial crayfish feed pellets (Tetra Wafer Mix, Tetra Europe, Melle, Germany) three times a week, prior to replacing the tank water. At the start of the experiments, the animals measured 3.7–5.8 cm and weighed 1.6–4.7 g.

Copper exposure experiment

A 0.5 mg Cu L^{-1} solution was prepared from $CuSO_4.5H_2O$ (p.a., Merck, Darmstadt, Germany) in copper-free water. Seventy animals were randomly divided over two groups and exposed for 14 days, to either copper-free water (control treatment) or water containing 0.5 mg Cu L^{-1}. Incubation conditions were as described for the acclimatization period, in ten 3 L aerated plastic tanks with seven animals in each.

Crayfish meat preparation and storage

After 14 days of exposure, all crayfish were aseptically killed by decapitation using sterile scissors. The headless specimens (edible parts; crayfish meat and tail) were individually placed in a sterile Petri dish and stored in a refrigerator (5 ± 1 °C). At various times of storage (0, 2, 4, 5, 6, 7 and 10 days) five specimens from each treatment were collected and frozen at −20 °C until analysis.

Tank water sampling

After 14 days of exposure, the tank water of both treatments were individually collected and analyzed. The number of microbial cells in the water samples was measured using a Coulter counter (Multisizer™ 3 Coulter counter, Beckman Coulter Inc., Brea, CA, USA). To collect microbes from the tank water for molecular analysis, 500 mL tank water was vacuum filtered over a 45 mm diameter, 0.2 μm pore size filter (Millipore, EMD Millipore Corporation, Billerica, MA, USA). All filters were aseptically collected and kept in a freezer (−20 °C) until analysis.

Analysis of copper concentrations

Copper concentrations were measured as described previously.[12] All crayfish samples (weighing around 0.25 g fresh weight) were freeze-dried for 48 h and digested in 2.0 mL of a 4:1 mixture of concentrated HNO_3 (Sigma-Aldrich Chemie, Zwijndrecht, Netherlands) and HCl (Riedel-de-Haën, Seeize, Germany), in a closed Teflon 'bomb' (VU University Amsterdam, Netherlands) at 140 °C for 7 h. The digests were then diluted with HNO_3 0.1 mol L^{-1} to 10 mL and the copper concentrations were determined using flame atomic absorption spectrophotometry (AAnalyst 100, PerkinElmer Inc., Waltham, MA, USA). The copper concentrations in the cultivation water were directly measured before and after renewing the water. Quality control of the analysis was maintained by digesting certified reference material Dolt-2 (CNRC, Ontario, Canada). The recoveries of copper in the reference material averaged 113% of the certified reference value. The detection limit was 0.003 mg L^{-1}.

Microbial DNA isolation

Microbial DNA in the crayfish meat was isolated using the PowerFood™ microbial DNA isolation kit (MO BIO Laboratories Inc., Solana Beach, CA, USA). A frozen sample of 0.25 g was placed in a bead beater tube, and one glass bead (4.0 mm diameter) and 1000 μL sterilized phosphate-buffered saline (10 g NaCl, 0.25 g KCl, 1.6 g Na_2HPO_4, 0.3 g KH_2PO_4 L^{-1} distilled H_2O) were added. A FastPrep Instrument (MP Biomedicals, Santa Ana, CA, USA) was then used for 40 s at a speed level of 4 m s^{-1}. The glass bead was included to degrade meat filaments without breaking the bacterial cells and to separate bacteria from the meat matrix. After 5 min of natural sedimentation, 250 μL supernatant was collected and centrifuged at 13 000 × *g* for 3 min. Residual liquid was removed and the microbial pellet at the bottom of the tube was resuspended in 450 μL PF1 solution from PowerFood™. Microbial DNA isolation was performed following the manufacturer's protocol (MO BIO Laboratories).

Microbial DNA from the Millipore filters containing microorganisms in the culture water was isolated using the FastDNA spin kit for soil (MP Biomedicals, Solon, OH, USA). The filter was aseptically cut into small pieces and further processed following the manufacturer's protocol. Two different bacterial DNA isolation kits were used intentionally, considering the differences in matrix characteristic of the samples. The PowerFood™ microbial DNA isolation kit is aimed at isolating DNA from foodstuffs very rich in organic, biological material, while the FAST DNA spin kit is developed for environmental, more inorganic samples, such as soils and filters containing both inorganic and organic materials.

Table 1. Primer sequences and conditions for amplifying bacterial 16S rRNA genes and histidine decarboxylase *hdc* genes of Gram-negative and Gram-positive bacteria present in water and crayfish sample

Primer	Sequence (5′ → 3′)	Primer concentration	Initial denaturation	Denaturation	Annealing	Elongation	Final elongation	Ref.
	16S rRNA genes							
F357GC	CGCCCGCCGCGCGC GGCGGGCGGG GCGGGGGCACGG GGGGCCTACGGG AGGCAGCAG	0.4 μmol L^{-1}	94 °C; 5 min	94 °C; 30 s	54 °C; 30 s	72 °C; 30 s	72 °C; 5 min	27
R518	ATTACCGCGGCTGC TGG							
	Gram-negative hdc genes							
hdc-f	TCHATYARYAACTGY GGTGACTGGRG	0.2 μmol L^{-1}	94 °C; 5 min	94 °C; 1 min	58 °C; 1 min	72 °C; 1 min	72 °C; 5 min	30
hdc-r	CCCACAKCATBARWG GDGTRTGRCC							
	Gram-positive hdc genes							
HDC-3	GATGGTATWGTTTCK TATGA	0.2 μmol L^{-1}	94 °C; 5 min	95 °C; 45 s	48 °C; 1 min	72 °C; 1 min	72 °C; 5 min	31
HDC-4	CCAAASWCCDGCAT CTTC							

PCR amplification of bacterial 16S rRNA genes

A set of primers targeting the bacterial 16S rRNA gene, F357GC and R518,[30] were used for polymerase chain reaction (PCR) amplification. Table 1 shows the sequence of primers and the amplification conditions we used prior to denaturing gradient gel electrophoresis (DGGE). For each PCR reaction, a total volume of 25 μL was used, consisting of 1 μL of 10 μmol L^{-1} forward primer; 1 μL of 10 μmol L^{-1} reverse primer; 1 μL of 10 mg mL^{-1} bovine serum albumin (BSA; New England BioLabs, Leusden, Netherlands); 12.5 μL GoTaq Colorless Master Mix 2× containing 1.25 units of Taq DNA polymerase (Promega, Madison, WI, USA); 8.5 μL DNase–RNase-free water (MP Biomedicals) and 1 μL microbial DNA template. DNA amplification was checked using electrophoresis on a 1% agarose gel in 1× TAE buffer. The observed size of the product of PCR amplification was ∼220 base pairs, indicating successful amplification of the bacterial DNA.

Denaturing gradient gel electrophoresis

DGGE was performed using the Bio-Rad D Code (Bio-Rad, Hercules, CA, USA). PCR products were loaded on to an 8% polyacrylamide gel with a denaturing gradient of 30–55% (100% denaturant was defined as 7 mol L^{-1} urea and 40% (v/v) formamide). The gels were electrophoresed in 1× TAE buffer (40 mmol L^{-1} Tris, 20 mmol L^{-1} acetic acid and 1 mmol L^{-1} Na-EDTA; pH 8.0) at 200 V and 60 °C for 4 h. A mixture of 12 different bacterial 16S rRNA gene fragments was used as marker. The gels were stained in 1× TAE buffer containing ethidium bromide (1 μg mL^{-1}) and were recorded with a charge-coupled device camera system. Gel images were converted, normalized and analyzed with GelCompar II software package (Applied Maths, Sint-Martens-Latem, Belgium). Similarity values between fingerprints were calculated using Pearson correlation and subjected to UPGMA (unweighted pair group method with arithmetic mean) cluster analysis.[31]

Sequencing of DGGE bands

One common DGGE band occurring intensively in crayfish meat samples was cut out with a sterile scalpel blade and diluted in 50 μL water. One microliter of the diluted DNA was re-amplified with primers F357 and R518 using the same PCR conditions as described above. PCR products that revealed a correctly sized band on agarose gel were aseptically cut out and purified using the Wizard SV gel and PCR clean-up system (Promega, Madison, WI, USA) according to the manufacturer's instructions. Sequencing was executed using Big Dye® Terminator v1.1 chemistry (Applied Biosystems, Carlsbad, CA, USA) on an ABI 3100 genetic analyzer (Foster City, CA, USA). BLAST (basic local alignment search tool) searching in GenBank and the Ribosomal Database Project (RDP) was performed to determine the closest known relatives of the obtained partial 16S rRNA gene sequences.[32]

Detection of *hdc* genes

Table 1 shows primer sequences and amplification conditions used to detect histidine decarboxylase (*hdc*) genes. Primers for the amplification of the *hdc* gene in Gram-negative bacteria were hdc-f and hdc-r, which amplify a DNA fragment of 709 bp.[33] Primers for the amplification of the *hdc* gene in Gram-positive bacteria were HDC-3 and HDC-4, which amplify a DNA fragment of 435 bp.[34] DNA isolated from *Photobacterium damselae* subsp. *damselae* (LMG 7892, BCCM, Gent, Belgium) and *Lactobacillus parabuchnerii* (DSM 5987, DSMZ, Germany) served as positive control of Gram-negative and Gram-positive histamine-producing bacteria, respectively.

Histamine analysis

Histamine was extracted from the wet crayfish meat samples. A precise weight (between 0.10 and 0.25 g fresh weight) of crayfish sample was crushed to homogeneity, diluted eight times with water and centrifuged (10 000 × *g*; 5 min). The histamine concentrations in the extract were analyzed using Histamarine

enzyme immunoassay kit IM2369 (Immunotech SAS, Marseille, France). Twenty microliters of the collected supernatant was added to 180 µL acylation buffer that was provided in the kit. Principal analysis is a competitive direct enzyme-linked immunosorbent assay (ELISA) in a micro-well format. A series of standard solutions containing predefined amounts of histamine (1–500 mg kg^{-1}) was used. Absorbance was read at 410 nm using a Versamax 340–750 nm plate reader (MTX Lab systems Inc., Vienna, VA, USA). The limit of detection (LOD) of the method was 1 mg kg^{-1}.

Data analysis

The effect of elevated copper concentration in the tank water on copper concentration in crayfish meat was analyzed using an unpaired *t*-test. The *t*-test was also used for determining the significance level between the number of microbial cells in the copper-exposed and copper-free tank water. Calculations and statistical analysis were performed with either Excel 2013 or the statistics software SPSS, version 17.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

The average weight of the animals increased slightly during the 14 days of exposure, but was not significantly different from the average weight at the start of the experiment, nor different between the two treatments. The averaged fresh weight of the marbled crayfish at the start of the exposure was 2.67 ± 0.89 g (mean $\pm$ SD; $n = 35$) and 2.73 ± 0.67 g (mean $\pm$ SD; $n = 35$), for copper-containing and control treatments, respectively. At the end of exposure they had reached 3.02 ± 1.00 g (mean $\pm$ SD; $n = 35$; copper exposed) and 3.12 ± 0.90 (mean $\pm$ SD; $n = 35$; copper free).

Crayfish meat of the animals exposed to copper contained higher copper concentrations than the meat of crayfish exposed to copper-free water ($n = 10$; ANOVA, $p < 0.05$). Copper concentrations in the crayfish meat samples averaged 32.2 ± 5.6 µg g^{-1} dry weight (copper-exposed) and 26.6 ± 5.9 µg g^{-1} dry weight (copper-free). Copper concentrations in the culture water (average over the 14-day exposure period, $n = 12$) averaged 0.43 ± 0.04 mg L^{-1} for the incubation-amended copper 0.5 mg L^{-1}) and were below the detection limit of 0.003 mg L^{-1} for copper-free water.

The elevated copper in the tank water reduced the number of microbial cells significantly (*t*-test, $p < 0.05$) by one logarithmic level. Copper-exposed tank water contained on average 2.09 ± 0.05 10^6 cells mL^{-1} and copper-free tank water 1.48 ± 0.01 10^7 cells mL^{-1}. Bacterial community structures in both tank water samples and in stored crayfish meat samples were also affected by the copper exposure, as revealed by DGGE analysis of the amplified 16S rRNA gene fragments (Fig. 1). Fingerprints of the marker of 12 different bacterial 16S rRNA gene fragments of the DGGEs were well reproducible, at >85% (profiles not shown). Cluster analysis (Fig. 1) revealed clear differences between bacterial community structure in the copper-containing water (0.5 mg Cu L^{-1}) and in copper-free (control) water, with a similarity of $33.5\% \pm 19.1\%$ between the two treatments. The DGGE profiles revealed fewer bands (averaging 5.3 per profile) for copper-containing water than for copper-free water (averaging 13.3). The similarity in DGGE fingerprints of bacterial communities for three biological replicates of the control water was $66.5\% \pm 8.1\%$. More variation between biological replicates was observed in the copper-containing water, with a similarity among replicates of $46.1\% \pm 36.9\%$.

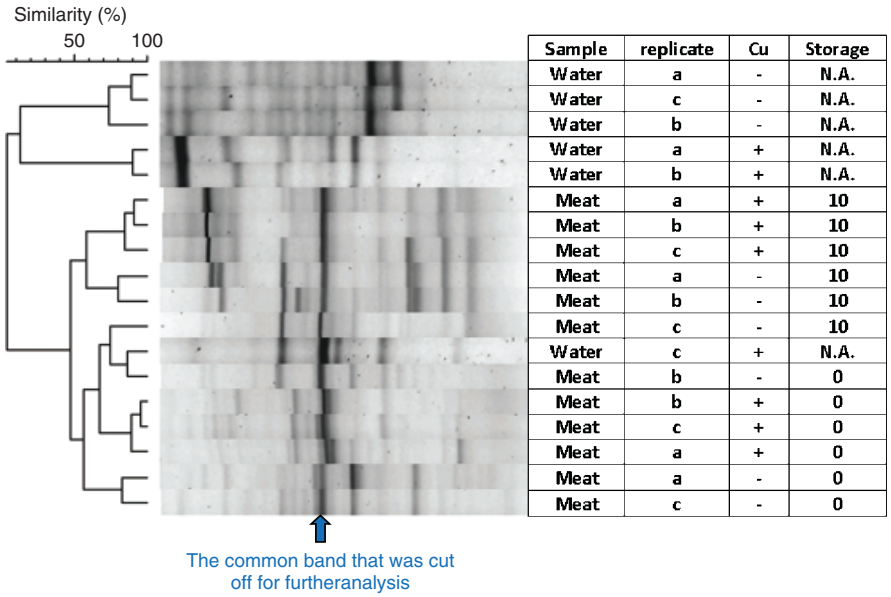
Bacterial community structures in fresh crayfish meat (0-day stored) were clearly different between the two treatments, in line with the observation that copper also affected community structure in the tank water. DGGE profiles of the crayfish meat in copper-free water (control) showed seven bands. Three of these bands were not strongly visible in the DGGE profiles derived from tank water from the control itself, which contained on average 14 bands. A similar observation appeared for crayfish meat previously exposed to copper-containing water. The DGGE analysis of meat samples of crayfish previously exposed in copper-containing waters revealed on average only 5.7 bands, while 9.0 bands were found in the corresponding water samples.

During storage for 10 days, a few new bands appeared in the community fingerprints of the crayfish meat samples from both the copper-exposed and the copper-free (control) treatments. The similarities of DGGE profiles between fresh crayfish meat and stored over 10 days were $57.3\% \pm 6.7\%$ for the copper-exposed and $48.6\% \pm 16.6\%$ for the copper-free treatment. Between the copper-exposed and copper-free treatment, the similarity of bacterial community structure in crayfish meat after 10 days storage was $55.3\% \pm 11.6\%$. This value was slightly lower than for the crayfish meat at 0 day storage, which had a similarity of $62.9\% \pm 17.3\%$. Thus differences in bacterial community structure in relation to copper treatment were already visible in the crayfish meat at the start of storage. Also, in both treatments, bacterial community structure in the crayfish meat and in the crayfish tail after 10 days' storage showed very similar profiles of DGGE fingerprints (the similarity was $78.5\% \pm 6.4\%$; data not shown). One common band was present in all DGGE profiles from crayfish meat samples, irrespective of copper treatment or the duration of storage (see the arrow in the Fig. 1). This band was excised and sequenced. Based on BLAST searching, the sequence (162 nucleotides) was 99% identical to *Pseudomonas* sp.

Histidine decarboxylase-encoding genes of neither Gram-negative nor Gram-positive bacteria could be detected in any of the water or crayfish meat samples. However, the histamine concentration in crayfish meat increased with time of refrigerated storage, for both crayfish that were exposed to copper for 14 days as well as for crayfish from the copper-free water (Fig. 2). The highest histamine concentrations were observed in meat from crayfish that were exposed in copper-free water (control experiment). After 2 days of storage, histamine concentrations in crayfish meat samples were significantly different between the two treatments (*t*-test, $P < 0.05$), reaching 3.19 ± 0.46 mg kg^{-1} for copper-exposed and 8.91 ± 0.44 mg kg^{-1} for copper-free treatment. At the end of the storage (10 days), the histamine concentrations in crayfish meat samples from copper-exposed and copper-free treatment were also significantly different (*t*-test, $P < 0.01$). Meat from crayfish previously exposed in 0.5 mg Cu L^{-1} contained less histamine than meat from crayfish incubated in the copper-free water (control), reaching 7.28 ± 2.58 and 21.6 ± 6.63 (mean $\pm$ SD) mg kg^{-1} wet weight, respectively.

DISCUSSION

Generally, copper is an essential element for (micro)organisms[35] and not harmful at low concentrations (1–10 µmol L^{-1}). For crustaceans, copper even plays an important role in respiration, since it binds oxygen in hemocyanin.[36] In agricultural practice, however, copper and copper-containing compounds are widely used as bactericides,[37] algicides and fungicides, contributing to environmental copper pollution and exposure to elevated concentrations of copper (>10 µmol L^{-1}). This study contributes knowledge that links animal toxicology and food



Sample	replicate	Cu	Storage
Water	a	-	N.A.
Water	c	-	N.A.
Water	b	-	N.A.
Water	a	+	N.A.
Water	b	+	N.A.
Meat	a	+	10
Meat	b	+	10
Meat	c	+	10
Meat	a	-	10
Meat	b	-	10
Meat	c	-	10
Water	c	+	N.A.
Meat	b	-	0
Meat	b	+	0
Meat	c	+	0
Meat	a	+	0
Meat	a	-	0
Meat	c	-	0

Figure 1. Cluster analysis of DGGE profiles of bacteria (30–55% denaturant gradient) present in the water and marbled crayfish (*Procambarus fallax* forma *virginalis*) meat samples after Pearson correlation. The table next to the profiles designate the sample (water and crayfish meat), replicates (a, b and c), copper treatment (+ and −; means treatment with 0.5 mg Cu L^{-1} and control, respectively) and storage time (0 and 10 days). Water samples were not stored and directly measured on the harvesting day. Therefore, for water samples the storage time is written 'N.A.' (not applicable).

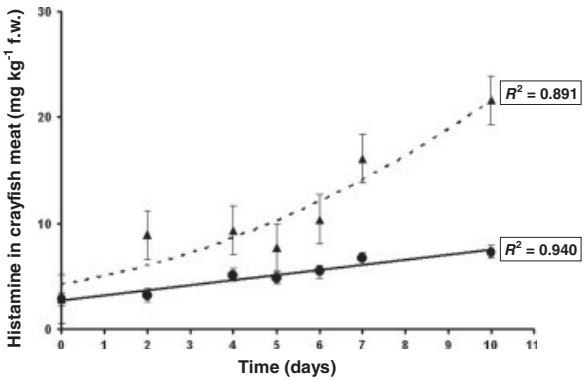


Figure 2. Histamine accumulation in marbled crayfish (*Procambarus fallax* forma *virginalis*) meat samples during refrigerated storage for 10 days, including the trend lines based on polynomial regression (linear for the 0.5 mg Cu L^{-1} samples, quadratic for the copper-free samples). Each data point represents the average value of histamine concentration ($n = 5$) with the standard error bars measured in meat of crayfish exposed for 14 days to copper 0.5 mg L^{-1} (●) or animals incubated in copper-free water (▲. Coefficients of determination (R^2) of the regressions are indicated on the graph.

safety/food microbiology, especially by looking at the correlation between elevated copper concentrations in water and histamine accumulation in marbled crayfish meat during storage. Further, this study provided evidence for copper-induced changes in the bacterial community structure in the water used to cultivate crayfish and in the crayfish meat. After 10 days of storage, the meat of crayfish previously grown at 0.5 mg Cu L^{-1} revealed fewer DGGE bands and contained lower histamine concentrations than the meat of crayfish from the copper-free control.

Copper exposure, copper accumulation and safety limits

A high copper concentration in the tank water used to cultivate crayfish generally leads to elevated copper concentrations in the organs or tissues of crustaceans,[38,39] and this was also observed in the present study. In crustaceans, excess copper mostly accumulates in the hepatopancreas and gills – both are considered non-edible parts – but not in the edible muscle.[19,20] Upon exposure to 0.5 mg Cu L^{-1}, copper concentration in crayfish meat increased by 17.4% compared to the control, to 32.2 µg g^{-1} dry weight. The Australian National Health and Medical Research Council recommends a maximum copper level in crustaceans of 10 µg g^{-1} wet weight.[18] In our study we measured copper in micrograms per gram dry weight. Considering that the crayfish meat contained on average 78% water in our study (data not shown), we converted the recommended upper level as stated in micrograms per gram wet weight[18] to a maximum upper level per gram dry weight: 45.5 µg g^{-1} dry weight,. Thus, when compared to the latter value, the copper concentration measured in this study does not exceed the recommended value and does not pose a threat to human health.

Interactions between copper, microbial communities and histamine levels

Despite the fact that copper concentrations in meat remained within food safety limits, effects of copper exposure were observed on microbial communities and histamine accumulation in stored crayfish meat. The histamine accumulation pattern appeared to correlate with the differences and changes in bacterial communities over time. Crayfish previously exposed to 0.5 mg Cu L^{-1} revealed fewer microbial species and contained lower histamine concentrations in their meat after refrigerated storage for 10 days. We observed a correlation between microbial communities in water and in crayfish meat. The results showed that copper exposure reduced microbial diversity in the water and most likely changed the bacterial community structure in the gut of crayfish.

The intestinal tract of crayfish, running along the dorsal side of the animal, contains high numbers of bacteria,[28] including putative deteriorative bacteria.[40] The edible, and stored, portion

of crayfish usually still contains the intestine; therefore the meat might be contaminated by intestinal material. During storage, deteriorative bacteria in the intestinal tract of crayfish can decompose amino acids into biogenic amines. Depending on the bacterial community composition, storage time and temperature, histamine that is derived from histidine can accumulate to levels exceeding the safety limit.[7,8] In the present study the crayfish were decapitated with sterilized scissors and further treated under aseptic conditions; therefore the risk of bacterial contamination from other sources during sample preparation was minimal.

It is likely that microbial communities in the water used to cultivate the crayfish influence the microbial composition of the crayfish intestine and that bacteria causing decay derive from the water, via intestinal communities. This can be concluded from the following arguments: (i) elevated copper dramatically changed the bacterial communities in the tank water; (ii) however, copper concentrations in the crayfish meat were not significantly affected by copper treatment and thus likely had a minor influence on the intestinal communities; (iii) nevertheless, clear differences in microbial communities in freshly stored meat were observed, and histamine levels in the crayfish meat during storage were different between the treatments.

The effect of copper on microbial community structure in water may depend on several factors: among others, the exposure concentration,[41] the chemical form of the toxic substance[42–44] and species-specific attributes to deal with copper.[45] Many species of bacteria show resistance to copper: among others, *Pseudomonas* syringae,[25] *Xanthomonas campestris* pv. *Juglandis*[46] and *Escherichia coli* strains.[47] In this study, *Pseudomonas* sp. occurred in all crayfish meat samples, from both treatments and throughout storage. Previously, *Pseudomonas* strains isolated from water and sediment revealed copper resistance.[24,48] Furthermore, some species of *Pseudomonas* such as *Ps. fluorescens*, *Ps. putida* and non-fluorescent *Pseudomonas* spp. showed the ability to produce low concentrations of histamine.[49] However, we did not culture microorganisms to establish relationships between identity and copper resistance, nor determined their histamine production potential. Considering (i) the relative low copper exposure concentrations applied in this study compared to the observed potentials to resist copper;[24,25,45,46,48] (ii) the fact that the copper concentration in meat is only slightly enhanced after copper exposure; and (iii) that the bioavailable copper concentration in the digestive tract – the major habit for microorganisms – was not determined, it is difficult to evaluate the importance of resistance of bacteria to copper in relation to the observed community changes in water and meat. The effect of copper may also simply relates to the fitness of bacteria (e.g. affecting growth rates or bacterial functions). The difference in fitness between species in relation to copper may lead to a reorganization of the bacterial community, without requiring resistance mechanisms.

The toxic effect of the cupric ion (Cu^{2+}) on microorganisms in water is also influenced by dissolved organic material such as humic substances,[50] aerobic–anaerobic conditions[42] and salinity,[51] all which may vary depending on the geographical region and aquaculture practices. While a positive effect of copper exposure on the crayfish meat was observed in this study, it might be interesting to look further at the effect of certain environmental conditions on the microbial communities as well as histamine accumulation.

Application of molecular tools to deteriorative processes

Cultivation-independent DNA-based fingerprinting of bacterial communities, followed by cluster analysis, is a reproducible, rapid and relatively unbiased technique to describe the dynamics in microbial communities and their responses to experimental treatments.[31] Isolation and analysis of DNA directly from food samples (without cultivation of microorganisms) has an advantage over the analysis of cultivable bacteria. Only a minor fraction of microorganisms can be cultured under laboratory conditions, even when using enriched or selective media.[30] By using specific primers and gene encoding, functional characteristics can be determined easily. However, although histamine production was observed, *hdc* genes could not be detected in our study. Therefore, it is likely that microbes harboring *hdc* genes that deviate from common *hdc* genes were present. The primers currently available for detecting *hdc* genes have been developed primarily for bacteria with an ability to produce high concentrations of histamine, such as *Photobacterium damselae* subsp. *damselae* or *Lactobacillus parabuchnerii* – the positive control of Gram-negative and Gram-positive histamine-producing bacteria, respectively, used in this study. It might be that microbes that produce lower quantities differ in the genes involved in histamine production. Alternatively, histamine producers might have been present below the detection limit of the approach ($>1.6\ 10^3$ gene copies per gram dry weight). Thus there is a need to attempt to culture these species and to use more detailed, culture-independent approaches, such as metagenomics,[52–54] to gain better insight into the full repertoire of microorganisms and genes involved in histamine production. Based on the results of these studies, in the future new PCR amplification approaches to detect genes involved in histamine production might be developed and applied. At the same time, these culturing and molecular approaches may also provide insight into the microbial potential to deal with copper.

CONCLUSIONS

Exposure to 0.5 mg Cu L^{-1} for 14 days had a minor, acceptable effect on copper concentration in the meat of marbled crayfish. However, it had an impact on the bacterial community structure in both the water used to culture crayfish and in the crayfish meat. Furthermore, copper exposure led to reduced histamine accumulation in crayfish meat during storage. This suggests that excess copper in aquaculture ponds may have a positive effect on the shelf life and safety of crustacean meat.

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