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Impact of Copper Fungicide Use in Hop Production on the Total Metal Content and Stability of Wort and Dry-Hopped Beer

Benjamin J. Chrisfield, Helene Hopfer and Ryan J. Elias *

Department of Food Science, College of Agricultural Sciences, The Pennsylvania State University, University Park, PA 16802, USA; bzc5304@psu.edu (B.J.C.); hxh83@psu.edu (H.H.)

* Correspondence: elias@psu.edu; Tel.: +1-(814)-865-5371; Fax: +1-(814)-863-6132

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Abstract: Transition metals, including copper, iron, and manganese, are known to catalyze the generation of reactive oxygen species (ROS) in beer leading to reduced product stability. Metals in beer are generally derived from raw ingredients. The present study aims to evaluate the impact of brewing and dry-hopping using hops treated with copper-based fungicides (CBFs) on the final transition metal content of model buffer solutions and pilot-scale systems of wort and beer. Copper levels in model wort and beer solutions were elevated (105.6% and 230.4% increase, respectively) when CBF-treated hops were used. In laboratory-prepared wort, elevated copper concentrations were not observed when CBF-treated hops were used for boiling. Dry hopping of beer using CBF-treated hops led to significant increases in total copper content (ca. 75 µg/kg vs. ca. 40–50 µg/kg in the control-hopped beer) when yeast was absent from the treated beer, but not when yeast was present. It was observed that manganese levels were significantly elevated in all hopped beers (ca. 495–550 µg/kg vs. 90–125 µg/kg in the unhopped control), regardless of hop treatment. A hop varietal thiol, 4-Mercapto-4-methylpentan-2-one, was spiked into treated beers, and the rate of oxidative loss was monitored during aging. Rates of thiol loss in treated beer samples did not differ across CBF treatments but were significantly lower in unhopped controls in the absence of yeast ($p < 0.0001$) and correlated significantly with total manganese content of the beers ($R^2 = 0.4228$, $p = 0.0006$). The rate of staling in hopped beers as measured by the rate of 1-hydroxyethyl radical generation did not differ among hop treatments, suggesting that excess copper content contributed from the hops does not negatively impact the oxidative stability of the beers. These findings suggest that brewers can use CBF-treated hops without any negative implications for the shelf stability of their beers and do not contraindicate the use of CBF in hops production when necessary.

Keywords: hops; beer; brewing; dry hopping; copper-based fungicides; transition metals; oxidation

1. Introduction

Maximizing beer stability during brewing and packaging is paramount for brewers as they are often unable to control storage time or conditions once the finished product leaves the brewery. Common defects related to beer aging include loss of flavor and aroma, oxidative staling, decreased foam stability, and haze formation [1]. While the overall decline of quality in packaged beer during aging cannot be attributed to a single factor, many of the underlying mechanisms are driven by the generation of reactive oxygen species (ROS) [2]. Ground state (triplet) oxygen is inherently non-reactive and requires activation by electron transfer, achieving a higher, more reactive spin state [3]. This activation can be directly catalyzed by transition metal ions such as Fe^{2+}, Mn^{2+}, or Cu^{1+} or by-products of the Fenton or Haber–Weiss reactions catalyzed by the same metals [4]. Therefore, while controlling the total oxygen in finished beer is important, minimizing the levels of transition metal catalysts throughout the brewing process is equally important.

Numerous patents exist for technologies aimed at reducing the transition metal content of beer, but this can be challenging as metal content is dependent on multiple factors such as the metals concentrations naturally found in the ingredients used and the potential for metal leaching from brewery equipment [5–8]. Transition metals are often present in beer at levels sufficient to catalyze these oxidative reactions (>50 µg/L), resulting in a more rapid loss of product quality over time [9–11].

Metal concentrations in brewing ingredients like hops and malt are dependent on growing conditions and processing and are generally outside the brewer's control. The largest reservoir of potential transition metals in the brewing is the malted barley or other cereal grains, as they are used in much larger quantities relative to hops on a weight basis [8]. A study by Wietstock and Kunz found that despite the fact that grains contain relatively high concentrations of transition metals (4 mg/kg Cu, 34 mg/kg Fe, 18 mg/kg Zn, and 1133 mg/kg Mg), they are tightly bound to the grain, and the majority (61.8% Cu, 95.2% Fe, 86.2% Zn, and 77.6% Mg) remain in the spent grains which are separated from wort during lautering [8]. While hops are used in smaller quantities compared to grains on a weight basis, they contain higher concentrations of transition metals, which have been demonstrated to contribute significantly to the final metal load of finished beer [5,8]. Transition metal contributions from hops are of greater potential concern in dry-hopped beers where hops are added directly to fermented beer in quantities that generally exceed those used in kettle hopping [12].

When hops are added to the wort, metals can be complexed and removed from the wort through co-precipitation with proteins and phenolics in trub during the boil or through complexation and sequestration by yeast during fermentation, both of which are removed during the brewing process by filtration [8,13,14]. Higher concentrations of transition metals have been found in dry-hopped beer, and few remedial opportunities remain between dry hopping and final packaging, leading to a loss of stability of the finished product [11]. Therefore, the concentrations of transition metals present in hop products used for dry hopping must be minimized, and their potential for extraction in finished beer must be established to reduce the potential for accelerated oxidation in the package.

The metal content of hops is established during cultivation. The use of copper-based fungicides (CBFs) on hop plants as a means to prevent fungal growth has been shown to significantly increase the copper content of hops flowers at harvest [15]. Copper is a multi-site antifungal agent, meaning the development of resistance is low and is therefore commonly used in conjunction with synthetic, single-site fungicides to prevent the development of fungicide resistance over time [16,17]. A variety of copper-containing fungicides are approved for use on hops [5]. "Bordeaux Mixture," made by combining copper (II) sulfate ($CuSO_4$) and slaked lime ($Ca(OH)_2$), has historically been used in German hop yards, and hop cones from those fields exhibit significantly elevated levels of total copper compared to hops grown in the Pacific Northwest where the use of CBFs is less prevalent [18]. Copper contents of German-grown cultivars have been shown to range from ca. 80–300 mg/kg, while US-grown varieties contained less than 20 mg/kg [18]. Even single-season treatments of hop plants with copper sulfate can increase the copper content of hops by over 800% (7.1 mg/kg to 67.9 mg/kg) [15]. The use of CBFs is often required in growing regions such as the Northeastern United States, where fungal disease pressure is more severe due to increased rainfall and relative humidity throughout the growing season. The potential impact of CBF-derived copper in hops on the total metal content of finished beer and its impact on oxidative stability, however, has not been thoroughly evaluated.

In the present study, copper, iron, and manganese concentrations were measured during kettle boiling and dry hopping in both model buffers and pilot-scale wort and beer dosed with CBF-treated hops. The impact of the presence of yeast cells on metal concentrations of finished beer during dry-hopping was also evaluated. Hop varietal thiols such as 4-mercapto-4-methylpentan-2-one (4-MMP), 3-mercaptohexanol (3-MH), and 3-mercaptohexyl acetate (3-MHA) contribute characteristic hop aromas including grapefruit, passion fruit, and black currant [19]. These volatile aroma compounds are oxidatively labile and have been shown to react stoichiometrically with cupric ions resulting in their oxidation and the formation of disulfide bonds and the loss of the desirable aromas [20]. The impact of CBF treatments on the rates of thiol loss in dry-hopped beers was therefore evaluated. As mentioned

previously, transition metals, including copper, iron, and manganese, are known to catalyze the generation of ROS and resulting in the production of staling compounds. The rates of ROS generation can be estimated through the spin trapping of the 1-hydroxyethyl radical (1-HER), which results from the abstraction hydrogen from ethanol by hydroxyl radicals formed by metal-catalyzed hydrogen peroxide reduction (i.e., the Fenton reaction) [21]. The rate of 1-HER generation has been established as an estimation of beer stability [22]. The 1-HER generation was measured in dry-hopped beers to evaluate the potential impact of CBF treatments on beer stability. The aim of the current investigation was to evaluate metal transfer from hops treated with CBFs into brewing solutions during beer production and determine the impact of total metal content on the oxidative stability of beer.

2. Materials and Methods

2.1. Materials

Sodium acetate trihydrate was purchased from VWR International (Radnor, PA, USA) and 200 proof ethanol from Decon Labs, Inc. (King of Prussia, PA, USA). Pilsen dry malt extract (DME) was purchased from Briess Malt & Ingredients (Chilton, WI, USA), Cascade HopShot hop resin from Northern Brewer (Roseville, MN, USA), and 1056 American Ale yeast from Wyeast (Hood River, OR, USA). Sodium azide was obtained from Mallinckrodt Chemicals (Dublin, Ireland). Nitric acid was purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ, USA) and was twice-distilled in two Savillex DST-1000 acid purification systems (Savillex, Eden Prairie, MN, USA) prior to use. N-tert-Butyl-α-phenylnitrone (PBN) and 4-mercapto-4-methylpentan-2-one (4-MMP) were purchased from MilliporeSigma (Burlington, MA, USA). Hydrogen peroxide for trace metal analysis was obtained from VWR International (Radnor, PA, USA). Ultra-high purity argon (99.999%), nitrogen (99.995%), and carbon dioxide (99.5%) were supplied by Penn State General Stores (University Park, PA, USA). All hops were grown at Penn State's Russell E. Larson Research Farm (Pennsylvania Furnace, PA, USA) in 2018 and 2019.

2.2. Hop Treatments

Hop treatments consisted of no (control), low frequency (low copper), or high frequency (high copper), application of the copper (II) hydroxide fungicide "Champ WG" (Nufarm, Laverton, Australia). High copper-treated plants were sprayed with a foliar application of copper (II) hydroxide formulation once every 2 weeks using a tow sprayer while low copper plants were sprayed once every 4 weeks. Both the control and copper-treated plants were sprayed on a regular basis with a conventional copper-free fungicide to prevent loss of hops due to fungal infection in the control groups. Supplier-labeled application rates for use on hops were followed, and the final application was administered one month prior to harvest at the end of August. Hops used for the initial evaluation of copper transfer in model solutions were whole cone hops treated and harvested in 2018 from single replicates consisting of the pooled cones of 7 bines treated with control and high copper fungicide application protocols. Hops used in the evaluation of metal transfer during boiling and dry hopping consisted of hop powders prepared from equal mixtures of freeze-dried cones from 6 different field replicates of the same fungicide treatments and were harvested during the 2019 growing season. Control, low copper, and high copper treatments were evaluated for total metal transfer during wort boiling and dry hopping in the finished beer. All hopping experiments were performed in triplicate.

2.3. Hop Boiling in Model Buffer

A model wort buffer consisting of a sodium acetate solution (0.01 M; pH 5.2) in ultrapure water was prepared to approximate the pH of a typical wort after lautering. This buffered solution will be referred to as "model wort." One liter of model wort was transferred into a tared Erlenmeyer flask, and its initial mass was recorded. The model solution was brought to a boil on a hot plate. Once boiling, hops were added at a rate of 3 g/L in a nylon mesh bag and allowed to boil for one hour with agitation by a magnetic

stir bar. The mass of hops was recorded prior to addition to the boil. Model wort was allowed to cool at room temperature for one hour, and the final weight was recorded. A 50 mL sample was collected in a 50 mL polypropylene centrifuge tube (VWR International, Radnor, PA, USA) and spun down at 3220× *g* for 10 min to remove hop particulate. Aliquots (ca. 1 mL) for metals analysis were passed through polytetrafluoroethylene (PTFE) (13 mm; 0.45 µm) syringe filters (CELLTREAT Scientific Products, Pepperell, MA, USA). Wort samples were maintained at −80 °C prior to analysis. Recorded masses were used to adjust measured copper concentrations to correct for evaporative losses that occurred during the boil. Boiling experiments were conducted in triplicate for control and high copper hops collected in the 2018 season.

2.4. Dry Hopping in Model Buffer

A buffered solution consisting of a 0.01 M sodium acetate buffer at pH 4.5 prepared in an aqueous solution of 5% (*v*/*v*) ethanol was used as a "model beer" (MB) to evaluate copper transfer in a simplified system. Dry, whole hop cones were added to 750 mL of MB in a 1 L media bottle at a rate of 10 g/L to simulate dry hopping of a finished beer. MB was purged with nitrogen for 10 min prior to hop addition, and headspace was displaced with nitrogen after hops were added. Media bottles were capped, sealed with Parafilm (Bemis, Neenah, WI, USA), and stored in the absence of light for 14 days at 25 °C. At the end of the dry hopping period, 50 mL beer samples were removed from the media bottle and centrifuged at 3220× *g* for 10 min to remove hop particulate and then passed through PTFE syringe filters (13 mm; 0.45 µm) prior to storage at −80 °C. Model dry hopping was conducted in triplicate for control and high copper hop treatments.

2.5. Laboratory-Scale Wort Boiling

Wort was prepared by dissolving 100 g of DME in 750 g of ultrapure water in a 1 L Erlenmeyer flask, fitted with a 24/40 ground glass joint, to achieve a specific gravity of approximately 1.05 based on DME product specifications. The actual masses of water and DME were recorded so that metal concentrations could be normalized to actual DME concentrations. Hops samples (3.00 g) from the 2019 season were sealed in 120 µm nylon mesh bags using an impulse heat sealer (American International Electric, City of Industry, CA, USA) and added to the water and DME. An Allihn condenser cooled with circulating water from an ice bath was secured to the top of the boiling flask to minimize wort evaporation. Hopped wort was brought to a boil on a hot plate with magnetic stirring. Wort was boiled for one hour and then cooled on the benchtop for 10 min. Wort was transferred to a one-liter media bottle, and the hops were removed. The wort was placed in an ice bath for 20 min to maximize trub precipitation. Wort was decanted, leaving ca. 80 mL of wort in the media bottle. The remaining suspension of trub in wort was transferred to two 50 mL centrifuge tubes and spun down at 3220× *g* for 10 min. The wort in the supernatant was collected and stored at −80 °C prior to analysis. Trub pellets were combined and freeze-dried using a Labconco FreeZone 2.5 lyophilizer (Kansas City, MO, USA) for 48 h and then stored at −80 °C. Trub pellets were crushed in a pestle and mortar and mixed thoroughly prior to acid digestion for metals analysis to ensure sample homogeneity.

2.6. Pilot-Scale Dry Hopping

Beer intended for use in dry-hopping experiments was prepared using DME and hop extract in 20 L of water in order to achieve a wort with a specific gravity of approximately 1.05 and 50 international bitterness units (IBU). Wort was boiled for one hour in a LEE Industries CWD, 15-gal jacketed steam kettle (Philipsburg, PA, USA) and then cooled to 20 °C by running cold water through the kettle jacket. Wort was blanketed with gaseous CO_2 during cooling. In order to remove residual trub prior to fermentation, the wort was racked into a 5-gal, stainless steel corny keg and then passed through a 2 µm Draft Brewer BeerBrite® candlestick filter (Northern Brewer, Roseville, MN, USA) using pressurized CO_2 directly into a 7-gal stainless-steel conical fermenter (Blichmann Engineering, Lafayette, IN, USA). American ale yeast was pitched at a rate of 1×10^7 cells/mL. Active yeast cell counts were assumed based on the manufacturer's reported yeast concentration and viability. Primary

fermentation was allowed to occur at room temperature (~21 °C) for 7 days, after which the fermenter was transferred to cold storage (4 °C) in order to cold-settle and clarify the beer. The beer was then racked into a 5-gal, stainless steel corny keg, and filtered under pressure into a 5-gal glass carboy through a 2 µm filter to remove yeast. The fermenter, keg, and carboy were all purged with CO_2 to minimize the oxygenation of the beer. The yeast suspension remaining in the conical fermenter was collected and distributed into 50 mL conical, polypropylene tubes, and centrifuged at 3220× *g* for 20 min. Isolated yeast pellets were freeze-dried, powdered with pestle and mortar, mixed, and then stored at −80 °C prior to being added back to the beer during dry hopping. Beer from the 5-gal carboy was aliquoted into 24 Erlenmeyer flasks (500 mL capacity) under argon using a peristaltic pump. Flasks were filled on an analytical balance to ensure each contained exactly 400.00 g of filtered beer. Beers were dry-hopped with 3.00 g of freeze-dried, powdered hops from either the control, low copper, or high copper hop treatment groups harvested in 2019, in a sealed, 120 µm nylon mesh bag. There was a total of six replicates for each hop treatment. The remaining six flasks were not hopped and served as negative controls. Four milliliters of 2% (*w*/*v*) sodium azide solution was added to all beers to prevent the refermentation of beer due to sugars liberated by hop enzymes during dry-hopping [23].

In order to evaluate the impact of the presence of yeast on the metal content of beer during dry hopping, 2.00 g of lyophilized yeast recovered from primary fermentation was added to three of the six beers in each hopped or negative control treatment. All beers were blanketed with argon, sealed with rubber stoppers and parafilm, and then stored in darkness at room temperature for 14 days. After dry hopping, two aliquots (50 mL) of beer were collected from each flask into 50 mL polypropylene centrifuge tubes and spun down at 3220× *g* for 10 min to remove hop particulate or sediment. Samples for metals analysis were further filtered through PTFE syringe filters (13 mm, 0.45 µm). All samples were stored at −80 °C prior to analysis.

2.7. Metal Analysis by Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

Elemental analyses of hop cones were conducted by the Agricultural Analytical Services Laboratory (The Pennsylvania State University, University Park, PA, USA) according to established methods [24]. All other samples underwent nitric acid digestion prior to analysis by ICP-MS. For liquid samples (i.e., model wort, model beer, wort, and beer), 500 µL samples were used. For trub, 50 mg samples were used. All samples were refluxed with 2 mL of 4 N nitric acid in sealed Teflon vessels overnight on a hot plate at approximately 90 °C. After initial digestion, approximately 250 µL of 30% H_2O_2 was added to each vessel, and samples were left open to dry on the hot plate at 70 °C. Residues were resuspended in 10 mL of 2% (*v*/*v*) nitric acid and refluxed overnight before being transferred to 15 mL tubes for analysis. Quantification was performed by the Laboratory for Isotopes and Metals in the Environment, a multiuser facility housed within the College of Earth and Mineral Sciences at Penn State (University Park, PA, USA). The lab utilized an iCAP RQ ICP-MS (Thermo Scientific, Waltham, MA, USA). Calibration standards were created from a serial dilution of standard solution ICP-MSCS-M purchased from High Purity Standards (North Charleston, SC, USA) and ranged from 0.1 to 100 µg/L for all metals analyzed. Standard reference material (SRM) 1640a was measured between each set of replicates to ensure the accuracy of measured analytes. The analyses of all samples were performed in triplicate.

2.8. Measurement of Oxidative Stability of Hopped Beers by Electron Paramagnetic Resonance

Oxidative resistance was measured by an electron paramagnetic resonance (EPR)—spin trapping-based lag time assay according to the official American Society of Brewing Chemists methods, and those methods reported by Uchida et al. with minor modifications [22,25]. All EPR experiments were performed at room temperature on a Magnettech MiniScope MS 400 X-band EPR spectrometer (Magnettech, Berlin, Germany) controlled by MinicopeCtrl software (Magnettch GmbH, ver 1.0.01542). EPR instrument parameters for the measurement of PBN-1-hydroxyethyl radical (1-HER) spin adduct were as follows: magnetic field, 334.66 ± 0.867 mT; modulation, 0.1 mT; microwave attenuation, 5.0 db;

sweep time, 15 s; number of passes, 4; smoothing, 0.2 s; and gain, 500. The field sweep was narrowed to focus fully on the first upfield doublet of peaks of the PBN-1-HER adduct spectrum. The amount of 1-HER generated during aging was represented by the amplitude of the first peak in the PBN spin adduct spectrum as calculated by the ESR-MPlot software (Magnettech GmbH, ver 1.0.0.489) at each time point over the course of the accelerated oxidation study. Differences in oxidative stability were determined by comparing the area under the curve (AUC) for the adduct intensity over time, calculated as the sum of all peak intensities time T0 through T160.

PBN stock solutions (2.5 M) were freshly prepared daily by dissolving 886 mg of crystalline PBN in 1 mL of absolute ethanol and 1 mL of ultrapure water in an amber vial, with subsequent vortexing until the PBN was fully dissolved. PBN solutions were kept in an ice water bath prior to addition to samples. A single aliquot was used for each set of EPR measurements. At the start of each measurement, an aliquot of lager was thawed and in a sonicating water bath in the absence of light. Beer (5 mL) was transferred to a 15 mL, screw-top centrifuge tube. Samples were vortexed for 30 s and then placed in an ice-water bath in the absence of light. At T0, a tube would be removed from the ice bath, and 100 µL of PBN solution was added, the beer was vortexed for 10 s, and the initial measurement (T0) was taken. The tube would then be placed in a 60 °C water bath in the absence of light to accelerate the rate of oxidation. Measurements were taken every 20 min for 160 min after T0 for a total of 9 measurements per tube. Triplicates of all treatments were measured. The area under the curve (AUC) was determined for each sample by summing the intensities of the spin adduct signal. Oxidative stability was assessed by comparing the average AUC across all treatments.

2.9. Loss of 4-Mercapto-4-Methylpentan-2-One (4-MMP) During Accelerated Aging

Several varietal thiols from hops contribute desirable, characteristic aromas to beer yet are oxidatively labile and have been shown to be directly oxidized by cupric ions in other systems [20]. The thiol, 4-MMP, is one of several hop varietal thiols found in hopped beer, and its loss due to oxidation by copper species has been reported previously in hops and wine and was therefore chosen as a model thiol for this study [15,20]. Hopped beers were spiked with 4-MMP at a concentration of 150 µM to study the loss of thiols over time as one indicator of the oxidative stability of the treated beers that may potentially be impacted by the total transition metal content. The method of thiol analysis was adapted from techniques published by Capone et al. for the analysis of endogenous thiols in white wine [26]. Immediately after spiking the beers and mixing by vortex, aliquots were derivatized to form stable, UV-active thiopyridine derivatives that could be quantified by high-performance liquid chromatography (HPLC), establishing the initial thiol content for each beer. Aliquots of beer were transferred into amber, 2 mL, low-adsorption, air-tight sample vials in a volume sufficient to cause the meniscus of the sample to crown over the lip of the sample tube (~2100 µL) in order to exclude air when sealed with the vial cap. In an effort to further reduce the potential for oxygen ingress, the vials were vacuum-sealed in FoodSaver nylon-strengthened, polyethylene bags and sealed using a commercial vacuum sealer (Newell Brands, Atlanta, GA, USA). A sufficient number of samples were prepared so that one vial for each treatment replicate could be sacrificed at every given time point, preventing the ingress of excess oxygen over time. Samples were placed in a laboratory incubator maintained at 30 °C in the absence of light to accelerate aging reactions. The thiol content of each sample was measured at 0, 1, 3, 6, 24, 48, 72, 120, 240, and 480 h from the time of thiol addition, for a total of 10 time points. Thiol loss relative to initial thiol content was plotted against time. The linear portion of thiol loss was determined to be from 24 to 240 h ($R^2 \geq 0.975$ across all samples). These data points were fitted with linear trendlines to determine the rate of thiol loss represented by the slope of the line. The rates of thiol loss (i.e., slopes) were compared across treatment groups.

For the quantification of thiols at a given time point, a 400 µL aliquot was removed and mixed with 100 µL of 10 mM 4,4′-dithiodipyridine, a thiol derivatizing agent, and allowed to derivatize for 30 min at room temperature. Samples were then filtered through PTFE syringe filters (13 mm, 0.45 µm) and transferred into 2 mL, HPLC sample vials with 300 µL inserts. Thiols were separated using a

Shimadzu 10AD-series reverse-phase HPLC (Shimadzu Corporation, Kyoto, Japan) equipped with an Eclipse Plus C18, 2.1 × 150 mm, 5 µm column (Agilent Technologies, Inc., Santa Clara, CA, USA) and quantified by the area of their absorption peaks at 330 nm using a photodiode array detector. The aqueous solvent (A) consisted of 0.5% (*v*/*v*) formic acid in ultrapure water. The organic phase (B) was HPLC grade acetonitrile containing 0.5% (*v*/*v*) formic acid. The gradient elution program was as follows: 0 min, 20% Solvent B; 2.5 min, 20% Solvent B; 3 min, 80% Solvent B; 4.5 min, 80% Solvent B; 5 min, 20% Solvent B; 7 min 20% Solvent B. The flow rate was 0.7 mL/min throughout.

2.10. Datasets and Statistical Analysis

All relevant data can be accessed through ScholarSphere (The Pennsylvania State University, University Park, PA, USA) at https://doi.org/10.26207/dwmj-ww11. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA). The student's *t*-test was used to determine differences in copper concentrations of model solutions. Differences in metal concentrations among treatments in the laboratory and pilot-scale samples were determined using a one-way analysis of variance (ANOVA) with Tukey's honestly significant differences (HSD) post hoc test. The Brown–Forsythe test was used to ensure equal sample variances ($\alpha = 0.05$). Only differences in treatments among a given metal type were evaluated. Simple linear regression was used to compare metal content to the rates of thiol loss. *p*-values less than 0.05 were considered statistically significant.

3. Results

3.1. Metal Content of Hop Cones

Total copper, iron, and manganese contents were determined in acid-digested hops using inductively-coupled plasma mass spectrometry (ICP-MS), as described above. Concentrations were reported on a dry weight basis and are listed in Table 1. Elevated copper concentrations (>15 mg/kg) were observed in all hops treated with CBFs throughout the course of the growing season, regardless of year. These elevated copper levels are consistent with those reported by Morimoto et al. in hop trials where "Bordeaux Mixture" (copper sulfate and slaked lime) was applied to hops during the growing season, although slightly lower than the 5-fold difference in copper ion concentration observed in that study [15]. The copper content of the control hops (7.22–8.59 mg/kg), as well as the manganese content of all hops (54.72–66.69 mg/kg), was similar to other reported values for the same variety [11,15,18]. Iron and manganese content of hops from different treatments were relatively similar, with only the iron content of the control hops from 2019 being slightly elevated (80 mg/kg vs. 50.99–66.29 mg/kg). The differences were disregarded for the sake of data interpretation as hops from the 2018 season were used only in buffer studies, and those from the 2019 season were used only in pilot brewing studies. No cross-harvest comparisons are made in the experiments performed. The total iron content of the hops was somewhat lower compared to reported values, which ranged from 200–400 mg/kg [11,18].

Table 1. Total Metal Content (mg/kg) of Hops Used in Boiling and Dry Hopping as Determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

	Harvest Year					
	2018			**2019**		
	Cu	**Fe**	**Mn**	**Cu**	**Fe**	**Mn**
Control	7.22 (1.51)	50.99 (4.68)	59.10 (7.96)	8.39 (0.93)	76.48 (22.11)	65.37 (13.46)
Low Copper	-	-	-	18.04 (4.40)	60.83 (17.52)	61.32 (13.19)
High Copper	18.71 (5.37)	53.72 (5.66)	58.83 (7.08)	19.67 (2.66)	49.08 (12.21)	57.03 (15.63)

All values reported as mean (standard deviation) in mg/kg total metal content. For the 2018 harvest $n = 10$ for each treatment and for the 2019 harvest, $n = 6$ for each treatment.

3.2. Effect of Copper-Treated Hops on Final Copper Content in Model Wort Buffer and Wort after Boiling

The impact of CBF-treated hops on the final copper content of a buffer adjusted to wort pH and laboratory-prepared wort after boiling was evaluated. Control hops and hops treated with CBFs were boiled for one hour in model wort, a solution consisting of acetate buffer adjusted to pH 5.2 to approximate the typical pH of wort. The copper content of the hopped model wort was determined by ICP-MS. The total copper concentrations of model wort boiled with CBF-treated hops were found to be significantly elevated compared to those boiled with control hops ($p < 0.001$). Copper concentrations of model wort doubled (37.39 µg/kg vs. 18.19 µg/kg) when copper-treated hops were used in place of control hops (Figure 1). This finding establishes the fact that hop-derived copper is soluble under wort pH and that increased copper concentrations in hop flowers from CBF applications increase the amount of copper that may be solubilized in the wort during the boil.

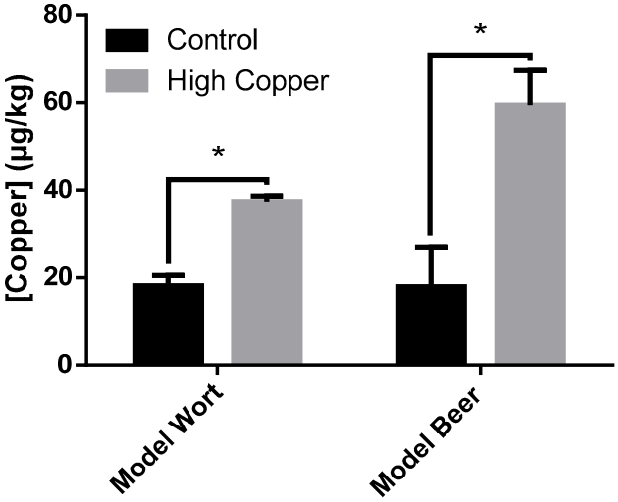


Figure 1. Total copper content of hopped model brewing solutions (µg/kg) as determined by ICP-MS. Error bars represent standard deviations ($n = 3$). (*) indicates significant differences ($p < 0.05$).

Although the use of buffered system allows for the direct monitoring of hop-derived copper content, model solutions lack the complexity of grain-derived wort, which contains significant concentrations of low- and high-molecular-weight carbohydrates, proteins, trace elements, and small molecules. Increased copper concentrations in wort may negatively impact the stability of a finished beer as increased copper concentrations have been linked to increased rates of ROS generation measured in beer by means of chemiluminescence [27] and EPR measurement of radical generation during forced aging of beer [28]. The transfer of hop-derived metals during the boiling of laboratory-prepared wort was also evaluated. Control and CBF-treated hops were evaluated in wort prepared with DME. Copper, iron, and manganese concentrations were determined using ICP-MS in boiled wort as well as trub isolated from the boil. In contrast to model experiments, no significant differences were found in the copper concentrations of the worts (ca. 30 µg/kg, $p = 0.893$) regardless of the hops used in the boil (Figure 2A). Similarly, no significant differences were observed in the total iron (ca. 135 µg/kg, $p = 0.498$) or manganese (ca. 190 µg/kg, $p = 0.155$) content of the worts. Trub isolated from the wort contained large concentrations of copper (ca. 240 mg/kg), indicating that copper was removed from the solution during trub precipitation (Figure 2B). This is consistent with the findings of Wietstock and Kunz, whose analysis revealed that copper was the most abundant metal ion found in the hot break after wort boiling [8]. A large amount of copper from the DME makes a direct measurement of hop-derived copper impractical, but the data indicate that the copper contributed from hops did not appreciably affect the copper content of finished wort. It is of note that manganese concentrations were relatively high in the wort relative to copper and iron (ca. 190 µg/kg vs. 30 µg/kg and 135 µg/kg, respectively), while lower in the trub (ca. 9 mg/kg vs. 240 µg/kg and 110 µg/kg, respectively, Figure 2).

This would indicate that manganese was not bound by precipitated sediment during the boil as was the case with copper and iron. Similar observations were found by Zufall and Tyrell when exogenous copper (100 µg/kg), iron (600 µg/kg), and manganese (200 µg/kg) salts were spiked in the wort during beer production [27]. When added to kettle-full wort prior to boiling, a higher proportion of added manganese was found in the finished beer compared to copper and iron, where only a quarter of the initial copper and no significant portion of added iron was found in the finished beer.

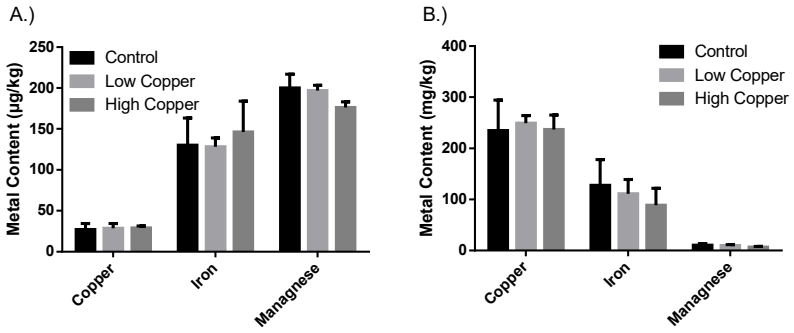


Figure 2. Total metal content of wort in µg/kg (**A**) and recovered trub in mg/kg (**B**) as determined by ICP-MS. Error bars represent standard deviation (n = 3). Multiple comparisons within each metal revealed no significant differences ($p > 0.05$).

3.3. Copper Transfer from CBF-Treated Hops into Dry-Hopped Model and Pilot-Scale Beer

The transfer of copper from CBF-treated and conventionally grown hops was evaluated in model beer (MB), a buffered solution that approximated the pH and ethanol content of beer. After a 14-day incubation at a hop dosage of 10 g/L, MB solutions hopped with CBF-treated hops had significantly higher concentrations (p = 0.009) of total copper compared to MB hopped with control hop cones grown without CBFs. On average, MB hopped with high copper hops exhibited an increase in total copper concentrations of 230.4% compared to the control (59.41 mg/kg vs. 17.98 mg/kg, Figure 1). This finding established the fact that the copper in CBF-treated hops is capable of being extracted into solution under beer-like conditions during dry hopping.

Just as the composition and complexity of wort-like buffer and grain-derived wort solutions differ, so too does the composition and complexity of model beer buffer and beer. While the model beer buffer approximates the pH and ethanol content of beer, it fails to mimic the presence of proteins, melanoidins, phenolics, yeast, and numerous other molecules that are present in beer at varying concentrations depending on the style, age, and processing parameters.

A pilot-scale beer was produced using DME and hop extract to better understand the impact of other beer components on the partitioning of hop-derived copper during dry hopping. While the use of DME in a small-scale production fails to fully reflect the realities of commercial-scale brewing, it simplifies the brewing process, allows for tight control of beer parameters, and enables rapid, reproducible beer production with more readily accessible equipment. Dry hopping was conducted both in the presence and absence of yeast due to the fact that yeast and yeast hulls, or lees, are known to uptake and release metal ions during and after fermentation [29]. Concentrations of copper, iron, and manganese in all beers were evaluated after a 14-day dry hopping period as these transition metals are most closely associated with the generation of ROS in beer during aging. While it is unlikely for the iron and manganese content of these hops to be affected by the application of CBFs, their measurement was included to better evaluate the total transition metal load of the prepared wort and beer.

In the absence of yeast, copper concentrations of beers dry-hopped with copper-treated cones were significantly elevated compared to the control-hopped and unhopped control beers (ca. 75 µg/kg vs. ca. 40–50 µg/kg, p = 0.001, Figure 3A). No significant difference was found in the control-hopped beer (ca. 50 µg/kg) compared to the unhopped control (ca. 40 µg/kg). From this data, we can infer that

the excess copper imparted to the hops from CBFs makes its way into the finished beer during dry hopping and elevates final copper concentrations in beer.

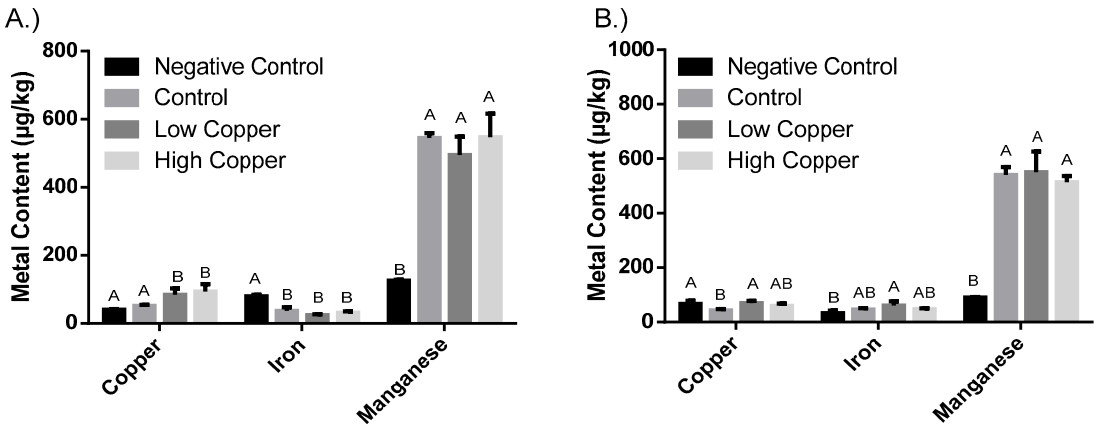


Figure 3. Total metal content (µg/kg) of all dry-hopped and unhopped beers in the absence of yeast (**A**) and in the presence of yeast (**B**). Error bars represent standard deviation (n = 3). One-way ANOVA was used to determine significant differences ($p < 0.05$) among treatments within a yeast treatment for a given metal. Significant differences between means were determined by Tukey's honestly significant differences, and these differences are indicated by the letters above each bar (HSD, α-level = 0.05). Treatment means that do not share the same letter within a given metal are significantly different from one another.

Elevated levels of copper in dry-hopped beers are a concern for two significant reasons related to beer quality. Firstly, as discussed previously, copper ions act similarly to iron ions in their ability to catalyze the reduction of hydrogen peroxide to hydroxyl radicals in beer, leading to the subsequent generation of oxidation products such as carbonyl compounds and haze-forming polyphenolic polymers as well as the loss of iso-α-acids [1,4]. Secondly, cupric ions have been shown to complex and oxidize thiols [20]. Unlike the generation of ROS in beer, which can also be attributed to iron and manganese ions, the direct oxidation of thiols is unique to copper. Various hop-derived polyfunctional thiols commonly referred to as "varietal thiols," impart desirable aroma traits such as passionfruit, grapefruit, or black currant and are present in perceivable concentrations in a variety of heavily hopped and hop-forward beer styles [30–32]. Several types of hops used in dry-hopping have appreciable amounts of these thiols, and their precursors [33–35], and preservation of those aromas in a dry-hopped beer is vital to brewers [36]. Therefore, dry hopping with large quantities of hops treated with CBFs with the goal of increasing characteristic thiol aromas may be futile as the increased copper load may result in a more rapid loss of these compounds. Previous research on metal species found in beers indicates that copper is bound in relatively large (4–6 kDa), negatively charged complexes [37], which may impact the reactivity of the copper ions. While the total copper content of the beers was elevated, their activity towards thiols may depend on their speciation and complexation.

When yeast was added to dry-hopped beers, the copper content no longer varied significantly between beers hopped with copper-treated cones and the negative control (Figure 3B). The control-hopped beers (ca. 42 µg/kg) contained significantly less copper than the low copper treatment (ca. 69 µg/kg) and negative control (ca. 67 µg/kg) beers, but not the high copper-treated samples (ca. 60 µg/kg). The similar metal content of the copper treatment hopped beers, and the negative control may indicate a copper ion-sequestering effect of yeast cells in the finished beers or an impact of hop compounds not found in the negative control. As mentioned previously, live yeast, as well as yeast hulls, are capable of modulating the content of different metal ions in solution. This effect is dependent upon the stage of fermentation, yeast health, pH, and the identity and concentrations of ions in solution [38]. A study by Mochaba et al. found that yeast removed significant amounts of copper from wort during fermentation [14], although the majority of yeast, in this case, were viable cells. Sodium azide was used to ensure yeast cell death

in the present study in order to prevent refermentation or "hop creep" during dry hopping. Work with yeast biomass demonstrated the ability of non-viable yeast cells to remove copper ions from solution at beer-relevant pH [13]. It was observed that copper concentrations in the unhopped beers were significantly higher than the beers hopped with the control in the presence of yeast. The elevated copper concentrations in the negative controls may be due to the equilibration of copper in the yeast and the copper already present in the beer. The yeast isolated from the initial fermentation was not prepared in any way that would remove metal ions absorbed during fermentation. The reduced copper content in the control samples may be attributed to an effect of copper equilibration of yeast cells with the beer coupled with complexation and precipitation of copper by hop constituents such as bitter acids. While these data indicate that the presence of yeast during dry-hopping may mitigate the increased copper content contributed by CBF-treated hops, further investigation into the importance of yeast quality, concentration, and beer conditions during dry hopping for the optimization of this effect are needed.

No significant differences in iron concentrations were found between different hop treatments in the absence (ca. 24–37 μg/kg) or presence (ca. 47–60 μg/kg) of yeast, but all hopped beers had significantly lower iron levels compared to the negative control (ca. 80 μg/kg) in the absence of yeast, while significant differences in iron content were found only between the low copper treatment and the negative control in the presence of yeast (ca. 60 μg/kg vs. 33 μg/kg, respectively, Figure 3). Decreased concentrations of iron in hopped samples would indicate some interaction between iron found in the beer post-fermentation and hop-derived components. Hops contain a number of compounds reported to chelate and complex iron, mainly phenolic compounds and hop bitter acids [39–41]. Precipitates were observed in dry-hopped beers but were absent from negative controls. This may be indicative of transition metal binding with hop acids as reported by Wietstock et al. [8] who found that the addition of α-acids, β-acids, and iso-α-acids to beer resulted in complexes with iron and copper ions that could then be removed from solution by filtration through a 0.45 μm filter. Chelation of both bi- and trivalent iron ions by myricetin and kaempferol, polyphenols present in hops, was reported by Mira et al. [41] at a moderately acidic pH of 5.5, but it is unlikely that these relatively small complexes, alone, would be removed by the filtration protocols used in the present study. Further investigation into the nature of the precipitates, as well as the metal content of the spent hops, is required to explain the observed reduction in iron content of the hopped beers.

Manganese content of all dry-hopped beers was significantly elevated regardless of hop treatment when compared to the negative control but did not differ significantly between treatments (Figure 3). Based on the similar concentrations of manganese in the hops, it is not surprising that hop treatment had no effect on the final manganese content of the beers. Levels of manganese in hopped beers were nearly five times greater than those found in unhopped beer both in the absence (ca. 495–548 μg/kg vs. 125 μg/kg) and presence (ca. 513–550 μg/kg vs. 90 μg/kg) of yeast. This finding is consistent with the work by Porter and Bamforth, who found a similar magnitude of increase in the concentration of manganese in a dry hopping experiment of the same duration [11]. Several sources report the potential negative impact of elevated manganese in finished beer related to increased rates of ROS generation and staling [11,27,28]. Manganese removal during beer production is difficult as it tends to remain in solution through the boil as shown previously [27], is poorly bound by yeast during fermentation [14], and readily released into the beer during dry-hopping [11]. Other than reducing the manganese load in raw materials, no recommendations have been made to remove manganese from finished beer.

3.4. Impact of CBF Treatments on Oxidative Stability as Measured by EPR

While the CBF hop treatments have, up to this point, been demonstrated to increase the total copper content of dry-hopped beers, the impact of this elevated copper concentration on beer oxidation reactions has not been evaluated. The rate of oxidation was estimated based on the EPR signal intensity of PBN-1-HER adducts formed during accelerated aging. The 1-HER is formed by the abstraction of a hydrogen atom by a hydroxyl radical, primarily from the C-1 carbon of the ethanol molecule (Scheme 1) [21], which can then be indirectly measured through the formation of a stabilized radical

adduct with the exogenous spin trap, PBN. The resulting PBN-1-HER adducts have relatively long half-lives compared to 1-HER and yield a characteristic six-line EPR spectrum with the approximate hyperfine coupling parameters α_N = 15.8 G and α_H = 3.5 G (Figure 4A) [42].

Scheme 1. The reaction of an ethanol molecule with hydroxyl radical leads to the formation of 1-HER.

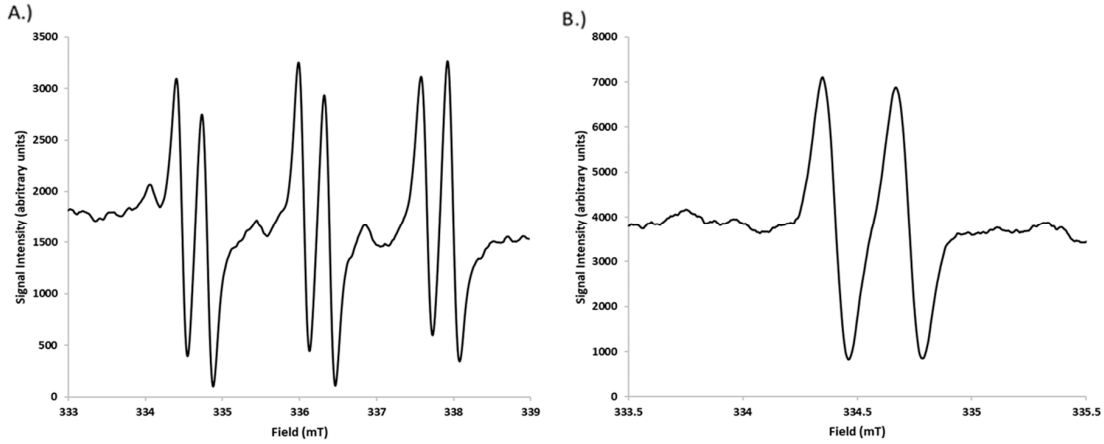
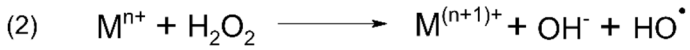


Figure 4. Sample electron paramagnetic resonance (EPR) signal of the N-tert-Butyl-α-phenylnitrone (PBN)-1-hydroxyethyl radical (PBN-1-HER) spin adduct (**A**) and the first doublet in the 1-HER-PBN adduct (**B**) measured to determine the rate of beer oxidation. Peak amplitude was expressed in arbitrary units and determined using ESR-MPlot software.

While transition metals are responsible for the generation of hydroxyl radicals via the Fenton reaction-mediated reduction of hydrogen peroxide (Scheme 2), the hydroxyl radical is extremely reactive and difficult to observe. This is due to the fact that hydroxyl radicals react with non-water beer components in proportion to their concentration [21]. As such, the primary target of these highly reactive radicals in beer is ethanol, resulting in 1-HER that can readily be trapped by PBN.



Scheme 2. The generation of hydroxyl radicals by the single electron reduction of hydrogen peroxide through a Fenton-like reaction catalyzed by the oxidation of transition metals (M) of oxidation state (n).

The 1-HER generation was calculated as the AUC for the PBN-1-HER spin adduct signal intensity (Figure 4B) over a period of 160 min. For the beers dry-hopped in the presence of yeast, no significant differences ($p > 0.05$) were found between the control (8891 ± 103.1 arb. units), low copper (8488 ± 358.9 arb. units), or high copper (8779 ± 1287 arb. units) treatments (Figure 5A). No significant difference ($p = 0.3005$) was observed in the AUC values for beers hopped with control (4,431 ± 244.5 arb. units), low copper (4051 ± 721.1 arb. units), or high copper hops (4367 ± 652.2 arb. units), nor the negative control (4938 ± 337.9 arb. units) in the absence of yeast during the dry hopping period (Figure 5B). The signal for the negative control of the yeast-present group, however, was significantly higher than the other treatments (28,319 ± 3182 arb. units, $p < 0.0001$). The increased oxidative instability of the negative control, in this case, may be due to the condition of the yeast used during dry hopping. When the signal intensities within each hop treatment are compared among yeast treatments using a two-way ANOVA with Sidak's multiple comparisons ($\alpha = 0.05$), the signal intensities are significantly elevated in the samples with yeast present during dry-hopping ($p < 0.0001$). It is generally accepted that yeast cells increase the stability of beer, acting in a reductive capacity [43,44]. In the present study, lyophilized yeast was added to beer containing sodium azide that was intended to render the yeast non-viable and prevent the refermentation of sugars that may be liberated by endogenous hop enzymes. Sodium azide is

known to function as a biocide through its inhibition of mitochondrial function [45]. This may have altered yeast viability in a manner that depleted its reducing capacity, resulting in the observed increase in 1-HER radical generation. The addition of hops to the samples with yeast present may have prevented or inhibited oxidation induced in the EPR assay through the contribution of antioxidative compounds, including hop phenolics and hop acids [46]. It is likely that the negative control, which was unhopped, would not benefit from the protective antioxidant effects of those hops and oxidize more rapidly.

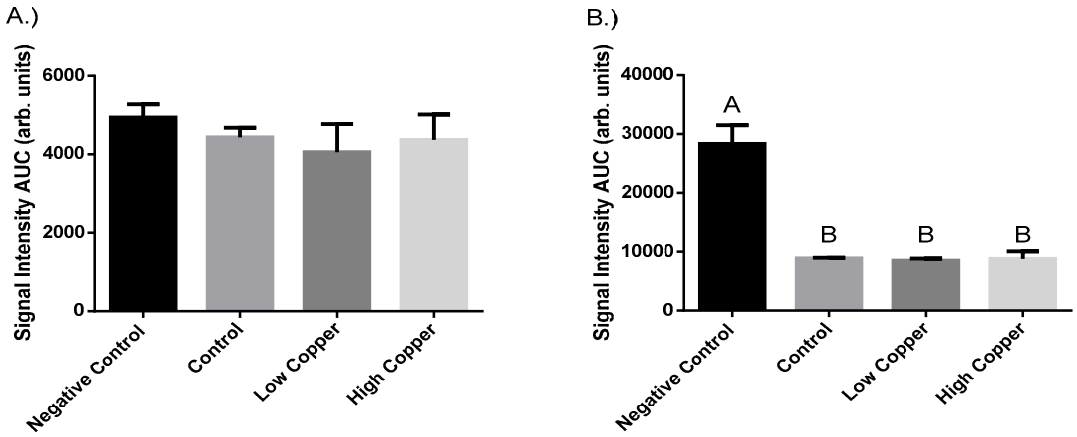


Figure 5. Total area under the curve (AUC) for 1-HER spin adduct signal intensity for beers hopped in the absence of yeast (**A**) and in the presence of yeast (**B**). One-way ANOVA was used to determine significant differences ($p < 0.05$) among treatments within a metal and yeast treatment. Significant differences between means were determined by Tukey's HSD (α-level = 0.05) and are indicated by differing letters (A or B) above each bar ($n = 3$). Treatment means that do not share the same letter are significantly different from one another.

Despite the result of the negative control, no significant differences were found between any of the hopped samples within a given yeast treatment. This would indicate that, despite the increased levels of total copper, the CBF treatments had no negative impact on the oxidative stability of the beers as measured by spin-trapping. Such a finding would lead us to believe that either the increase in copper levels was not substantial enough to negatively impact the oxidative stability of the beer, or that the copper is bound in some manner within the beer that is inhibiting its function as an oxidative catalyst. The former is unlikely, as previous research has closely linked increases in copper content to increases in the radical generation as measured by the present method [22].

Previous research has established that very little copper found in beer is in an ionic form [47]. While the exact identity of the copper species is unknown, research by Svendsen and Lund demonstrated that beer components have the potential to complex exogenous copper into large, high-molecular-weight structures when it is added to beer in an ionic form [37]. Work by Sakellari et al. found that the copper-complexing capacity of beer as measured by differential pulse anodic stripping voltammetry was in excess of the total copper concentration in the beers measured indicating that all copper was bound in organic complexes [48]. It is, therefore, possible that the excess copper contributed by the hops in the current study has been bound by beer components, and its catalytic activity has been inhibited. Further work focusing on the speciation of the copper and the identification of the metal complexes present in these beers would be necessary to elucidate the cause of the inhibited oxidation reactions relative to the total metal content.

3.5. Varietal Thiol Loss in Dry-Hopped Beers

Thiols such as 4-MMP, 3-MH, and 3-MHA are derived from hops and contribute desirable aromas such as passionfruit, grapefruit, or black currant to hop-forward beers. Previous research in wine has established that copper can directly oxidize varietal thiols, leading to a loss of aroma over time [20].

A reduction in 4-MMP content of hops due to treatments with copper sulfate and slaked lime has been previously demonstrated [15]. Beers hopped with CBF-treated hops were spiked with 4-MMP to determine whether the increased levels of copper in the dry-hopped beers would lead to a more rapid loss of thiols during aging.

No significant differences were found in rates of thiol loss among dry-hopped samples in either of the yeast treatments (Figure 6A,B). This would indicate that the differences in copper content between treatments did not significantly impact the rate of 4-MMP loss over time. While inconsistent with the general hypothesis that increased copper would lead to increased rates of thiol loss, it is again possible that the additional copper is bound in a form that hinders the reaction of copper with the free thiols. The negative control samples in the absence of yeast were found to have significantly lower rates of thiol loss compared to the hopped samples (Figure 6A, $p < 0.0001$). This difference was not observed when beers were hopped in the presence of yeast (Figure 6B, $p = 0.0907$). The major difference in the measured parameters between the negative controls and all other samples would be in the total manganese content. Manganese may function as a prooxidant in beer, promoting Fenton-like reactions at beer pH and resulting in the oxidative loss of varietal thiols over time [49]. Previous research has demonstrated increased rates of thiol loss in wine in the presence of manganese, iron, and copper ions compared to manganese ions alone [50]. A positive linear correlation was found between manganese content and the rate of thiol loss regardless of treatment (Figure 6C, $p = 0.0006$). Unlike the EPR evaluation of oxidative stability, thiol loss was studied over a longer period in an oxygen-limited environment. Further analysis of aged samples is necessary to determine the root cause of thiol loss in the beers.

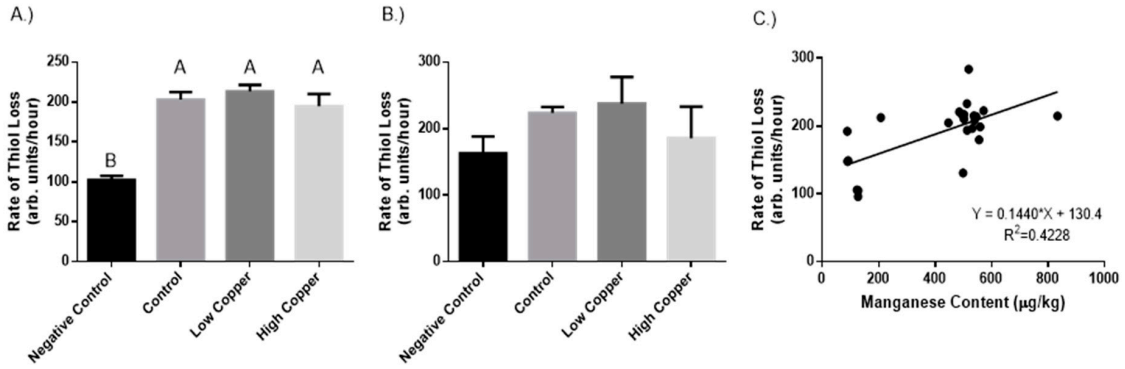


Figure 6. Rates of thiol loss for beers hopped in the absence of yeast (**A**) and in the presence of yeast (**B**) and the correlation between total manganese content and rate of thiol loss (**C**). One-way ANOVA was used to determine significant differences ($p < 0.05$) among treatments within a metal and yeast treatment. Significant differences between means were determined by Tukey's HSD, and these differences are indicated by the letters (A or B) above each bar. Treatment means that do not share the same letter are significantly different from one another. Simple linear regression was used to determine correlations between metal content and rates of thiol loss.

4. Conclusions

In conclusion, we have demonstrated that while excess copper found in CBF-treat hops is easily transferred into model brewing solutions, this effect is modulated in real wort and beer. In model wort, an increase of 105% in copper concentration was observed when CBF treated hops were used, but no differences in copper content were found under the same boiling conditions in real wort. This would indicate that the use of CBF-treated hops for bittering purposes would have no ill effect on the transition metal load of beer compared to hops grown without these fungicides. Copper was increased by 230% in model beer dry-hopped with copper-treated hops, and similar results were observed in beer dry-hopped in the absence of yeast. Due to the ability of copper ions to catalyze the generation of ROS and oxidize desirable thiols, the stability of beers dry-hopped with CBF-treated hops may be compromised. No significant differences in copper transfer between hop treatments in dry-hopping were observed in the presence of

yeast cells, indicating that the ability of yeast to modulate metal ion concentrations in beer may mitigate the impact of excess hop-derived copper. Elevated manganese (296–511% increase over negative controls) was observed in all dry-hopped beers regardless of dry-hopping conditions and may also negatively impact the stability of finished beer. EPR evaluation of oxidative stability revealed no significant differences in the rate of radical generation among the dry-hopped beer treatments within yeast treatments. This indicates that while the levels of copper were significantly increased, the rate of oxidative radical generation was not. Rates of spiked thiol loss revealed that, again, CBF treatment had no ill effect on thiol loss when compared to the hopped control. Interestingly, lower rates of thiol loss were found in the unhopped beers and were correlated with the total manganese content of those beers. Overall, it would appear that although the use of CBF-treated hops in dry-hopping leads to increased levels of copper, the overall oxidative stability is not impacted. From this, we would conclude that, although CBF-treated hops increase the final copper content of beers brewed with those hops, the increased levels of copper do not negatively impact the stability of these beers. The implication being that brewers do not necessarily need to concern themselves about the use of CBF-treated hops in their beers with respect to final product quality and stability. This is especially important for hop producers and brewers in areas with greater disease pressure, such as the northeastern United States, where the use of CBFs is more common than in more arid regions like the Pacific Northwest.

While these initial findings indicate that the use of CBF-treated hops does not negatively affect the oxidative stability of beer, further investigation into the long-term stability of beers produced with CBF-treated hops as measured by the generation of staling compounds and by the perception of trained sensory panels is necessary. The measurements of oxidative stability in this study were short-lived and did not evaluate all markers of product stability. Evaluation of different dry-hopping parameters that may influence the partitioning of copper and other transition metals during dry-hopping such as ethanol concentration, beer style, and yeast conditions is warranted.

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Conflicts of Interest: The sponsors had no role in the design, execution, interpretation, or writing of the study. The authors declare no conflicts of interest.

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