JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Hepatic Retention and Toxicological Responses during Feeding and Depuration Periods in Atlantic Salmon (*Salmo salar*) Fed Graded Levels of the Synthetic Antioxidant, Butylated Hydroxytoluene

EIVIND HOLAAS,[†] VICTORIA B. BOHNE,[‡,§] KRISTIN HAMRE,[‡] AND AUGUSTINE ARUKWE*,[†]

Department of Biology, Norwegian University of Science and Technology (NTNU), Høgskoleringen 5, 7491 Trondheim, Norway, National Institute of Nutrition and Seafood Research (NIFES), Safe Seafood, P.O. Box 2029, Nordnes, 5817 Bergen, Norway, and NOFIMA Ingredients, Bergen, Kjerreidviken 16, 5141 Fyllingsdalen, Norway

The human safety aspects of seafood production require the expansion of vital knowledge of both nutrients and possible contaminants along the entire production chain. Thus, production of safer seafood can be achieved by using feed materials that are low in contaminants, while maintaining balanced nutrition, in order to secure optimal fish and consumer health. Our understanding of primary responses of fish health and production related diseases, as well as biological processes that influence carry-over and lowering of contaminants in farmed fish, will contribute to a sustainable production of safer seafood products. Therefore, we have studied the liver deposition and toxicological effects in salmon fed graded levels of BHT during a 12-week feeding followed by a 2-week depuration period using chemical, molecular, and catalytic assays. In general, our data showed that BHT was significantly retained in the liver and selectively modulated toxicological responses in the xenobiotic biotransformation pathways during the feeding period. Specifically, BHT produced consistent dose- and time-specific gene expression patterns for AhR2α, AhR2β, CYP1A1, CYP3A, UGT1, and GSTπ. The effect of BHT on the gene expression of biotransformation enzyme did not parallel enzyme activity levels, suggesting a possible inhibition by parent BHT or its metabolites. As a safety precaution, the production of farmed Atlantic salmon in Norway requires a mandatory 2-week depuration period prior to slaughtering and market delivery to ensure the elimination of veterinary medicaments, additives, and other undesirable components. Comparison of feeding and depuration periods showed that BHT was highly retained in fish liver, as only 8−13% of fed BHT was eliminated during the 2-week depuration period. This is just a part of the total concentration in the whole fish, since BHT may have been distributed and accumulated in other organs. Since BHT or its metabolites putatively inhibited biotransformation enzymes and affected metabolism of the compound, they may have potential for toxicological and adverse health effects for both fish and fish consumers through carry-over processes from the fish products.

KEYWORDS: Synthetic antioxidants; BHT; biotransformation; retention; food safety; salmon

INTRODUCTION

In recent years, the aquaculture industry has experienced a steady growth that has been attributed to the provision of high quality products and expansion into a variety of markets. The growth of Atlantic salmon in seawater increases with increasing lipid levels in the diet up to approximately 40% (*1*). This has led to an increase of lipid content in fish feed during the last 30 years. Since tissues and fish feed are rich in polyunsaturated fatty acids (PUFAs), they require more use of antioxidants to prevent oxidation and rancidity (*2*). In the Norwegian aquaculture industry, the use of synthetic antioxidants as preservatives in fish feed- and ingredients against the auto-oxidation of unsaturated lipids is common (*3*). Butylated hydroxytoluene (BHT: 2,6-di-*tert*-butyl-4-methylphenol) is a water-insoluble, white, crystalline solid antioxidant that is soluble in food oils and fats. It is also soluble in organic and hydrocarbon solvents and is an antioxidant for foods, animal feed, petrol products, synthetic rubbers, plastics, animal and vegetable oils, and

* Corresponding author. Phone: +47 73 596265; fax: +47 73 591309, e-mail: arukwe@bio.ntnu.no.
[†] Norwegian University of Science and Technology (NTNU).
[‡] National Institute of Nutrition and Seafood Research (NIFES).
[§] NOFIMA Ingredients.

10.1021/jf8025524 CCC: $40.75
Published on Web 11/13/2008

soaps (*4, 5*). While it is effective in animal fats, BHT is not as effective in vegetable oils and soaps and is known for its high-temperature stability and its carry-through effect in fats (*4, 5*). The compound is important as a food antioxidant because it is readily soluble in glycerides, insoluble in water, and susceptible to loss by volatilization and distillation under certain food processing conditions, such as frying (*4, 5*).

Although the United States Food and Drug Administration (USFDA) generally recognize the safety of BHT, several reports have established that this antioxidant is a causative agent in a number of toxic and carcinogenic processes in animals, including pulmonary toxicity in mice (*6*), hemorrhagic death in rats (*7*), tumor promotion in mouse lungs (*8*), hepatotoxcity in rats (*9*), and nephrotoxicity in mice and rats (*10*). Most of these adverse effects appear to be dependent, at least in part, on the biotransformation of BHT through the cytochrome P450 (CYP) system, to one or more reactive quinone methides (QMs) intermediates (*11*). In humans, the intake of BHT can also result in health problems for oversensitive individuals, evaluated as allergic reactions (*12*).

Toxicological responses such as the expression of biotransformation enzymes, including CYP1A1 and phase II enzymes (uridine diphosphate glucuronosyltransferase, UGT and glutathione S-transferase, GST) are regulated though the aryl hydrocarbon receptors (AhRs). The AhRs are members of the ligand-dependent basic-helix−loop−helix-PER-ARNT-SIM (bHLH-PAS) group of transcription factors through which agonists cause altered gene expression and toxicity (*13, 14*). Upon binding to a ligand, the AhR dimerizes with AhR nuclear translocator (Arnt) and the complex translocates to the nucleus where it transactivates mRNA transcription of genes containing xenobiotic responsive elements (XRE) in their upstream regions. Several genes involved in metabolism and degradation of lipophilic and persistent compounds contain XREs in their promoter, including CYP enzymes, UGT, and AhR-repressor (AhRR) (*15*). Because of their roles in the detoxification and activation of exogenous and endogenous compounds, alteration of the expression of hepatic CYPs and phase-II enzymes markedly affects the potential risks and benefits of xenobiotics and is important from a toxicological and physiological point of view (*16*).

The synthetic antioxidant BHT has the potential for toxicological and health effects for both fish and fish consumers through carry-over processes from the fish feed, and these toxicological aspects have not been systematically investigated. Therefore the main objective of this study is to study the toxicological effects of BHT in salmon fed graded levels during a 12-week feeding followed by a 2-week depuration period, using chemical, molecular, and catalytic assays. Our hypothesis is that Atlantic salmon fed graded levels of BHT will accumulate BHT in the liver and show dose and time-dependent toxicological responses through the modulation of biotransformation enzymes. Thus, the differences in phase I and II biotransformation patterns will be indicative and predictive of toxicological and susceptibility to BHT, thus posing potential adverse health effects to both fish and human consumers of fish products.

MATERIALS AND METHODS

Chemicals and Reagents. Butylated hydroxytoluene (BHT; >97.5% purity), dimethyl sulfoxide (DMSO), ethoxyresorufin, benzyloxyresorufin, and NADPH were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Trizol reagent and TA cloning kit were purchased from Gibco-Invitrogen Life Technologies (Carlsbad, CA). Iscript cDNA Synthesis Kit and iTaqSybr Green supermix with ROX were purchased from Bio-Rad laboratories (Hercules, CA) and Generuler 100bp DNA ladder and dNTPs from Fermentas GmbH (Germany). Microtiter plates (MaxiSorp) were purchased from Nunc (Roskilde, Denmark). All other chemicals were of the highest commercially available grade.

Table 1. Formulation of and Proximate Composition (g/kg diet) of Experimental Fish Diets

	diet ingredient
BHT-free fishmeal[a]	66
BHT-free NorSea oil	34
Mais Suprex	15
vitamin mix[b]	1.4
mineral mix[c]	0.45
astaxantin	0.11
ash	8.7
water	6.3
protein	43.3
lipid	30.1
carbohydrate	11.1
energy (kJ g^{-1})	24.2

[a] BHT-free feed pellets were produced from BHT-free fishmeal and coated with BHT-free oil, both stabilized with commercial mixture of naturally antioxidants (Vitalox, Helm). [b] Vitamin mixture (g/kg dw) was composed as follows 1 g of retinyl acetate, 0.4 g of cholecalciferol, 20 g of α-tocopherol acetate, 0.5 g of menadione, 28.57 g of ascorbic acid, 162 mg of choline, 4.55 g of thiamine mononitrate, 0.63 g of riboflavin, 0.5 g of pyridoxine, 5 g of niacin, 0.5 g of folic acid, 5 g of biotin, 0.1 g of cobalamin, 2 g of Ca-pantothenate and 769.25 g of casein (filler); [c] Mineral mixture (g/kg dw) was composed as follows 506 g of Mg (as $MgSO_4 \cdot 7H_2O$), 44 g of Zn (as $O_4SZn \cdot 7H_2O$), 24.9 g of $FeSO_4 \cdot 7H_2O$), 4.6 g of Mn (as $MnSO_4 \cdot 7H_2O$), 2 g of Cu (as $CuSO_4 \cdot 7H_2O$), and 418.5 g of casein (filler).

Table 2. Total Consumed Butylated Hydroxytoluene (BHT) Expressed as Mean Fish Biomass of Three Experimental Tanks and Standard Deviation (SD) in Parentheses

	treatment group[a]			
	0	15	150	1500
amount of total consumed BHT (mg/per fish) during 12 weeks	0	2.4	24.7	239.7
(mg/per fish) during 12 weeks		(0.2)	(1.1)	(10.9)

[a] Based on BHT amount added to the fish feed. The stated levels of BHT in diets were adjusted by mixing of commercially available BHT (>97.5% purity) into coating BHT-free oil (NorsEcoOil, Sildoljefabrikk A/S).

Diets, Fish, Rearing Conditions, and Feeding Experiment. The diet formulation (**Table 1**) and ecological BHT-free fishmeal (NorsEcoLT, Sildoljefabrikk A/S) was used to produce four experimental diets at NOFIMA Ingredients, Bergen, Norway. The diets were given increasing levels of BHT are shown in **Table 2**. The feeds were produced in one batch, as 4 mm extruded pellets and coated with BHT-free fish oil supplemented with graded levels of BHT. The diets were stored at −20 °C through the entire experimental period. The feed requirement was calculated with start fish weight of 0.2 kg. predicted daily growth rate of 1%. and feed conversion factor of 1 (dry weight feed/fresh weight fish). The feeding experiment was conducted with a total of 900 individual of Atlantic salmon produced from a March 2001 egg batch at Aqua-Gen AS (Kyrksaeterøra, Norway). The experimental fish were half-year smolt with a mean weight of 200 g. The fish were kept in an indoor tank (5 m diameter, 1.5 m height) with seawater for at least two months for acclimatization to the environment. To ensure equal toxicological status of individuals at the beginning of the exposure, fish were fed experimental diet (**Table 2**) free of BHT for one month before the experiment started. After acclimatization, fish were stocked in fifteen 1.5 × 1.5 × 0.5 m glass fiber tanks with 60 fish per tank and four experimental diets (**Table 2**) with graded levels of BHT randomly assigned to the fifteen tanks (i.e., in triplicate). The salinity and temperature during the experiment were monitored and maintained at 27 ± 2‰ and 9.5 ± 0.8 °C, respectively. The daily light and dark cycle was artificially adjusted to follow the natural photoperiod for Matre (near Bergen, Norway, 60°N), and feeding was performed automatically every 5 min from 8 a.m. to 4 p.m. in the winter months,

Table 3. Primer Pair Sequences, Accession Numbers, Amplicon Size, and Annealing Temperature Conditions for Genes of Interest Used for Real-Time PCR

target gene	primer sequence[a] forward	primer sequence[a] reverse	amplicon size (nucleotides)	annealing temp (°C)	GenBank accession no.
CYP3A	ACTAGAGAGGGTCGCCAAGA	TACTGAACCGCTCTGGTTTG	146	60	DQ361036
AhR2α	AGGGGCGTCTGAAGTTCC	GTGAACAGGCCCAACCTG	82	60	AY219864
AhR2β	GACCCCCAGGACCAGAGT	GTTGTCCTGGATGACGGC	96	65	AY219865
CYP1A1	GAGTTTGGGCAGGTGGTG	TGGTGCGGTTTGGTAGGT	76	60	AF364076
UGT1	ATAAGGACCGTCCCATCGAG	ATCCAGTTGAGGTCGTGAGC	113	55	DY802180
GSTπ	CGCATTGACATGATGTGTGA	TGTCGAGGTGGTTAGGAAGG	121	57	DQ367889

[a] Sequences are given in the 5′→3′ order.

and from 8 a.m. to 8 p.m. from April to July. Unconsumed feed pellets were collected and weighted, and the daily feed and BHT intake was calculated.

During the 12-week feeding and 2-week depuration period, five fish from each tank were randomly selected and anesthetized in benzocain (ethyl aminobenzoate) bath, prepared from 5 mL of stock solution of 0.1% benzocaine in 96% ethanol (EtOH) in 10 L of seawater. The gall bladder was carefully removed, and bile was collected. The liver was dissected, weighed, and rinsed in physiological saline. The liver was divided into two, placed in plastic tubes, and frozen in liquid nitrogen for the different analysis. The samples were collected at day 0 (control), 3, 14, and 84 of feeding and at day 3, 7, and 14 in the depuration period. The samples were stored at −80 °C in air and light tight containers until analyzed. The BHT concentration was measured in three of the five fish from each tank, while molecular and biochemical analysis was performed on all five fish from each tank.

HPLC Analysis of Liver BHT Levels. Exactly 0.1−1 g of liver was weighed into 15 mL dark glass tubes with screw caps, and liver homogenates were carefully prepared to prevent the samples from air- and light-mediated oxidation of the BHT. Next, 7.5 mL of acetonitrile mixed with solid ascorbic acid (0.1 g/100 mL) was added to the homogenate. BHT was extracted by vigorous shaking in 20 min. Phase separation was forwarded by centrifugation for 5 min at 3000 rpm. The acetonitrile phase was collected in syringe and filtered through a 0.45 μm syringe filter prior to chromatography. If the concentration of BHT in the samples was below the quantification limit (see below), the known volume of filtered extract was up-concentrated by flushing with nitrogen to dryness followed by reconstitution in a smaller known volume of acetonitrile/ascorbic acid. Quantification of BHT was based on the reverse-phase HPLC conditions as previously described for ethoxyquin (EQ) (*17, 18*). Briefly, separation of the compounds was achieved on tandem-coupled phenyl-hexyl and C18 columns by two-phase gradient elution with acetonitrile/ascorbic acid/acetic acid/diethylamine organized in a 23.5 min sequence. BHT was detected at 282 and 307 nm for excitation and emission, respectively. The detection limit of matrix-spiked BHT compounds was 0.013 mg/kg, and the quantification limit was 0.04 mg/kg. BHT extraction from the fish feed was performed following the procedure of the Association of Official Agricultural Chemists (AOAC) International for EQ (*17*) and further analyzed by the same method as used on liver extracts. All analyses of salmon liver and feed were performed in duplicates.

Preparation of Postmitochondrial Supernant (PMS), Enzyme Activity, and Protein Analysis. Liver postmitochondrial fraction (PMF) was prepared by centrifugation as described previously (*19*). Liver samples (50 mg) homogenate was first centrifuged for 20 min at 12000*g* at 4 °C. The pellets were discarded leaving the postmitochondrial- or S-12 supernatant. The S-12 supernatants were collected and stored in aliquots at −80 °C until analyzed. The 7-ethoxyresorufin O-deethylase (EROD) assay was according to Burke and Meyer (*20*). UGT activity toward *p*-nitrophenol (*p*NP) was measured in PMS samples as described by Andersson et al. (*21*) and GST activity in hepatic cytosolic samples were measured using 1-chloro-2,4-dinitrobenzene (CNDB) as substrate as described by Habig et al. (*22*). As a quality control in all enzyme assays, two known samples were assayed in parallel with all assay series, in order to ensure the consistency of the results obtained with unknown samples. All enzyme activities were assayed at room temperature (25 °C). Total protein concentration was determined with the method of Bradford (*23*), using bovine serum albumin (BSA) as standard.

RNA Purification and cDNA Synthesis. Total RNA was purified from tissues homogenized in Trizol reagent according to established procedures. Quantification, purity, and RNA integrity were evaluated by absorbance at 260 and 280 nm using a NanoDrop ND-1000 UV−visible spectrophotometer (NanoDrop Technologies, Wilmington, DE) and formaldehyde agarose gel electrophoresis. Total cDNA for the real-time polymerase chain reaction (PCR) were generated from 1 μg of total RNA from all samples using a mixture of random and poly-T primers from iScript cDNA synthesis kit as described by the manufacturer (Bio-Rad). Prior to quantification, each cDNA sample was diluted 1:6 with RNase-free distilled water.

Real-Time Quantitative PCR. PCR primers for amplification of 76−146 bp gene-specific PCR-products were designed from conserved regions of the studied genes. The primer sequences, their amplicon size, and the optimal annealing temperatures are shown in **Table 3** and were performed according to established methods in our laboratory (*17, 24*). Sequences were confirmed using NCBI nucleotide BLAST software (http://www.ncbi.nlm.nih.gov/BLAST). The expression of individual gene targets was analyzed using the Mx3000P Real-Time PCR System (Stratagene, La Jolla, CA). Every 25-μL cDNA amplification reaction contained 12.5 μL of iTAQSYBR Green Supermix with ROX (Bio-Rad), 5 μL of diluted cDNA, and 200 nM of each forward and reverse primers. The three-step real-time PCR program included an enzyme activation step at 95 °C (5 min) and 40 cycles of 95 °C (30 s), 52−60 °C for 30 s, depending on the primers used (see **Table 3**) and 72 °C (30 s). Controls lacking cDNA template were included to determine the specificity of target cDNA amplification. The methodological details are as previously reported (*17, 24*). This absolute quantification method is a well-validated procedure in our laboratory, as we do not use the so-called housekeeping genes because of their parallel modulation pattern with experimental samples both in our laboratory (*25*) and elsewhere (*26*).

Statistical Analysis. All statistical tests were performed with the use of SPSS software (version 14.0) for Statistical Visualization. The data were tested day-wise by one-way analysis of variance (ANOVA) with 'treatment' as independent variable. A Tukey−Kramer HSD test was performed to determine the differences within treatment groups and control groups. The level of significance was set at $p < 0.05$.

RESULTS

Liver Retention of BHT in Salmon. HPLC analysis of liver BHT levels showed a direct and apparently linear relationship between doses of parent BHT in feed and that retained in the salmon liver (**Figure 1**) for the 1500 mg/kg feeding group. Unfortunately, we lost samples for BHT liver determination (not for other analysis) at the last feeding day (day 84). It should be noted that the last feeding day (day 84) represents the end of the feeding period and start of the depuration period (i.e., depuration day 0). Liver BHT levels in the low (15 mg BHT/kg) feeding group were below the HPLC detection limit during both the feeding and depuration periods and therefore not quantified. Salmon fed with 150 mg BHT/kg feed produced fluctuating liver BHT levels during the feeding period (**Figure 1A**). In contrast, an increase in the levels of BHT was recorded from day 0 to 14 in salmon liver fed the highest BHT doses of 1500 mg/kg feed, during the feeding period (**Figure 1B**). After

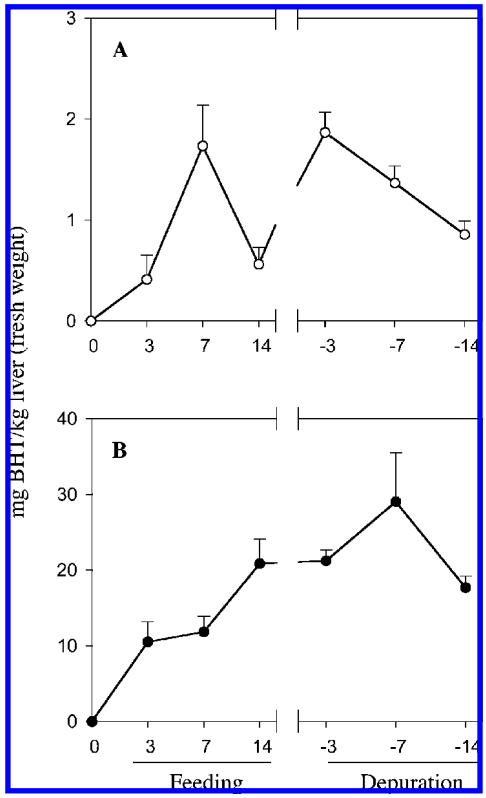

Figure 1. Levels of dietary BHT in Atlantic salmon liver fed 150 mg (A) and 1500 mg (B) BHT per kg fish feed during the feeding and depuration periods.

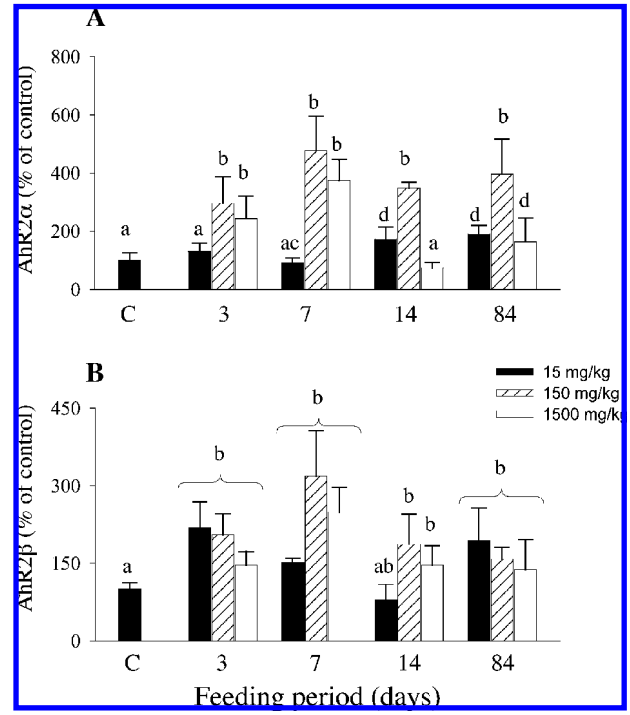

Figure 2. AhR2α (A) and AhR2β (B) mRNA levels during the feeding period with BHT. Aryl hydrocarbon receptor isoforms were analyzed using qPCR with gene-sequence primer pairs. Significant differences ($p < 0.05$) within the same treatment group at different time points are denoted with letters (according to results from nested analysis of variance (ANOVA), followed by Tukey's HSD. Data are given as mean values expressed as percentage (%) of control ± standard error of the mean (SEM).

day 84, there was a trend of decrease in BHT concentration during the depuration period in both feeding groups. Comparison of feeding and depuration periods showed that BHT was highly retained in fish liver as only respective 8−13% of fed BHT was eliminated during the 2-week depuration period.

Biotransformation Gene and Enzyme Activity Levels during Feeding Period. The transcription of AhR2α and AhR2β mRNA was modulated during the feeding period in all treatment groups with variable BHT effect at different time intervals (**Figure 2A,B,** respectively). At feeding days 3 and 7, the AhR2α mRNA showed an apparent dose-dependent increase (**Figure 2A**). Salmon fed 15 mg BHT/kg fish feed did not show transcriptional changes in AhR2α mRNA expression pattern at days 3 and 7, while significant increases were observed at days 14 and 84 (**Figure 2A**). The group fed 150 mg BHT/kg fish feed produced a consistent and high AhR2α mRNA expression pattern throughout the feeding period, showing significant differences from control at day 14 and 84 (**Figure 2A**). At feeding day 14 and 84, the highest BHT dose (1500 mg/kg fish feed) produced reductions in AhR2α mRNA levels, compared to 15 and 150 mg/kg treatment groups (**Figure 2A**). For AhR2β mRNA, an apparent dose-dependent reduction (albeit significantly above control) and increase was observed at day 3 and 7, respectively, **Figure 2B**). At days 14 and 84, the AhR2β mRNA showed respective dose-specific increase and decrease, albeit significantly higher that control at 150 and 1500 mg BHT/kg (**Figure 2B**). In general, AhR2β showed minor differences in mRNA expression levels within treatment groups, compared to the AhR2α.

The CYP1A1 and CYP3A genes shared an almost identical pattern of expression during the feeding period (**Figure 3A**and 3**B**, respectively). At feeding day 3, the expression of CYP1A1 and CYP3A mRNA was BHT dose-specific with the 150 mg/kg treatment group showing the highest mRNA level. All BHT treatment groups produced significant differences compared with control at days 3 and 7 of exposure (**Figure 3A**,**B**, respectively). From day 7 to 14, CYP1A1 and CYP3A mRNA levels showed significant reductions in the groups fed 15 and 150 mg BHT/kg fish feed, while the mRNA levels remained unchanged in the highest BHT dose of 1500 mg/kg (**Figure 3A**,**B**, respectively). At the end of the feeding period (day 84), none of the BHT doses produced transcriptional changes for CYP1A1 and CYP3A mRNA, compared to controls. However, the reduced expression pattern was more severe for CYP1A1 in the medium and high BHT feeding groups (**Figure 3A**). In general, CYP1A1 mRNA showed higher expression level at all feeding days, compared to CYP3A.

The representatives of phase II enzyme genes, GSTπ and UGT1, showed unique and comparable expression patterns with phase I enzyme genes (**Figure 3C**,**D**). At feeding day 3, GSTπ mRNA showed significant increases, compared with control, in all BHT doses with the 150 mg/kg group showing the highest expression level (**Figure 3C**). At days 7 and 14, BHT produced an apparent dose-dependent increase in GSTπ mRNA expression (**Figure 3C**). Interestingly, the three BHT doses produced differential time-dependent effects on GSTπ mRNA expression between day 3 and 14 that was characterized as a decrease in the 15 and 150 mg/kg feeding groups and increase in the 1500 mg/kg feeding group, and with no difference between groups at day 84 (**Figure 3C**). For UGT1, the mRNA expression pattern was significantly higher than the control from day 3 to 14, and comparable with GSTπ (**Figure 3D**). At feeding day 3 and 7, UGT1 mRNA levels were the highest and almost the same in the 15 and 150 mg/kg feeding groups, while being low in the 1500 mg/kg feeding group (**Figure 3D**). At days 14, BHT produced an apparent dose-dependent increase in UGT1 mRNA expression (**Figure 3D**). Interestingly and similar to GSTπ, the

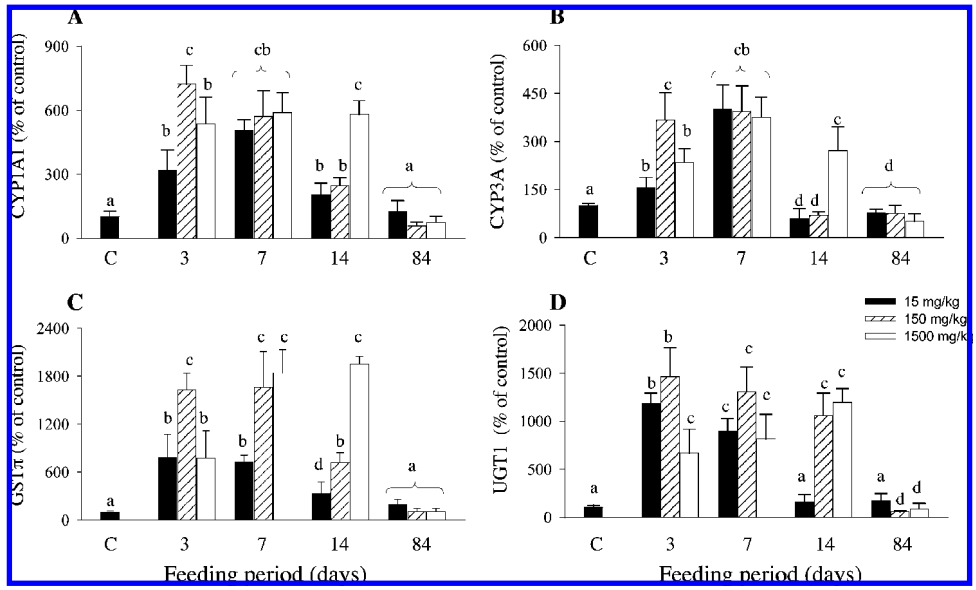

Figure 3. Modulation of CYP1A1 (A), CYP3A (B), GSTπ (C), and UGT1 (D) mRNA levels during the feeding period with BHT. Messenger RNA (mRNA) was analyzed using qPCR with gene-sequence primer pairs. Significant differences ($p < 0.05$) within the same treatment group at different time points are denoted with letters (according to results from nested analysis of variance (ANOVA), followed by Tukey's HSD). Data are given as mean values expressed as percentage (%) of control ± standard error of the mean (SEM).

three BHT doses produced differential time-dependent effects on UGT1 mRNA expression between day 3 and 14 that is characterized as a decrease in the 15 and 150 mg/kg feeding groups and an increase in the 1500 mg/kg feeding group, and indifferent at day 84 (**Figure 3D**).

The enzyme activity with the substrates ethoxyresorufin (EROD: **Figure 4A**) and benzyloxyresorufin (BROD: **Figure 4B**) showed minor but nonsignificant differences between BHT treatment groups and control and maintained almost similar activity levels as the controls during the whole feeding period. Similarly, specific activity of GST (**Figure 4C**) and UGT (**Figure 4D**) enzymes did not show significant differences from the control in any of the treatment groups and maintained the same activity as the controls during the whole feeding period.

Biotransformation Gene and Enzyme Activity Levels during Depuration Period. It should be noted that the depuration day 0 represents the end (day 84) of the feeding experiment, and during this period AhR2α and AhR2β mRNA levels were not significantly altered in any of the feeding groups during the depuration period (**Figure 5A** and 5**B**, respectively). AhR2α mRNA levels significantly increased from depuration day 0 to day 3 in the highest BHT (1500 mg/kg) feeding group ((**Figure 5A**). There was a trend toward a decrease (significant at depuration day 7 and 14, compared to day 0) in AhR2α and AhR2β mRNA levels from depuration day 0 through to 14 in the low-feeding group (**Figure 5A** and 5**B**, respectively). During the depuration period, the expression patterns of CYP1A1 and CYP3A mRNA could generally be described as an increase in transcriptional expression at depuration day 3, and thereafter a decrease at day 7 and 14 (**Figure 6**A and B, respectively). At depuration day 3, CYP1A1 and CYP3A mRNA showed a significant dose-dependent increase in the 150 and 1500 mg BHT/kg treatment groups, compared to 15 mg/kg treatment group (**Figure 6A** and 6**B**, respectively). There was no dose-dependency in the increased CYP1A1 and CYP3A mRNA levels observed at depuration day 7. At the end of the depuration period (day 14), there was a significant BHT dose-dependent increase in CYP1A1 and CYP3A mRNA levels (**Figure 6A** and 6**B**, respectively).

The expression pattern of GSTπ and UGT1 mRNA during the depuration periods followed an almost identical pattern with CYP1A1 and CYP3A (**Figure 6C** and 6**D**, respectively) and could generally be described as an apparent dose-dependent increase at depuration day 3 and followed by a decrease at day 7 and 14 (**Figure 6C** and 6**D**, respectively). Again, specific enzyme activities for EROD, BROD, GST, and UGT did not show any significant differences from the control or depuration day 0 in any of the treatment groups and maintained the same activity as the control during the whole depuration period (data not shown).

DISCUSSION

To obtain safe seafood products and maintain high consumer reliance, research and documentation of trends in drug consumption patterns in addition to examination for drug residues and effects of such agents in farmed marine species is vital. Therefore, the present study was performed to evaluate the deposition and toxicological aspects of dietary BHT in salmon during a 12-week feeding followed by 2-week depuration periods with graded levels of BHT (a synthetic antioxidant). We showed that BHT was retained in the liver and selectively modulated toxicological responses in the xenobiotic biotransformation pathways. Specifically, BHT produced a consistent dose- and time-specific gene expression pattern for AhR2α, AhR2β, CYP1A1, CYP3A, UGT1, and GSTπ. The effect of BHT on the biotransformation enzyme gene expressions did not parallel enzyme activity levels. Biotransformation of BHT by these enzymes can result in the formation of reactive metabolites, which can be toxic for fish consumers.

BHT Kinetics in Salmon Liver. BHT is a highly lipophilic food additive (*4*) and therefore has the potential to accumulate in the salmon body. Because of its lipophilicity, BHT is likely to be rapidly and evenly distributed throughout the liver. Our data showed an apparent linear relationship between doses of parent BHT in the formulated feed and that retained in the liver during the feeding and depuration periods. An increase in the levels of BHT was registered in the muscle of the salmon during the feeding period with 150 mg/kg BHT (Bohne et al.,

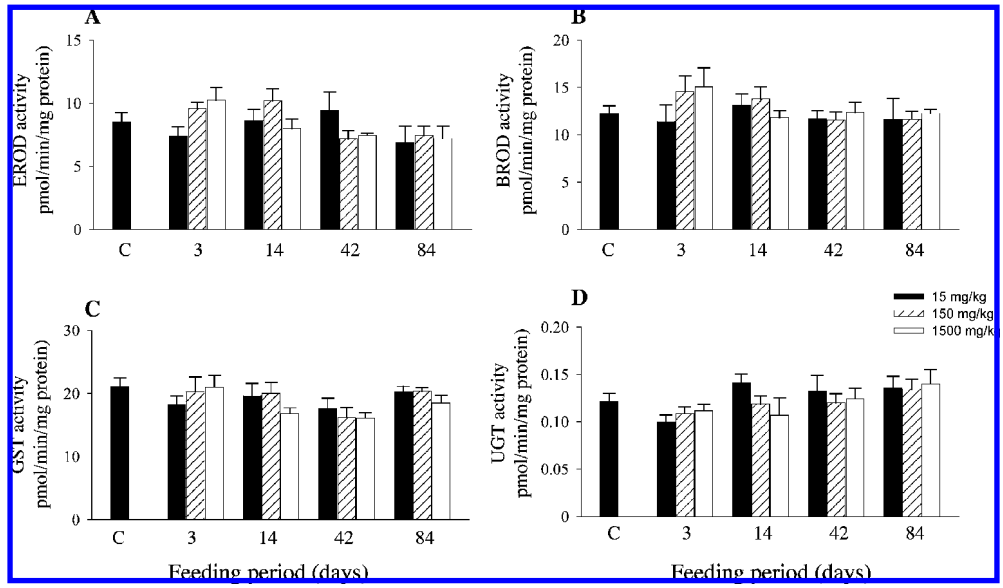

Figure 4. Hepatic activity levels for ethoxyresorufin O-deethylase (EROD: A), benzyloxyresorufin O-deethylase (BROD: B), glutathione S-transferase (GST: C), and uridine diphosphate glucuronosyl-transferase (UGT: D) activities of salmon during the feeding period with BHT. Data are given as mean activity values ± standard error of the mean (SEM).

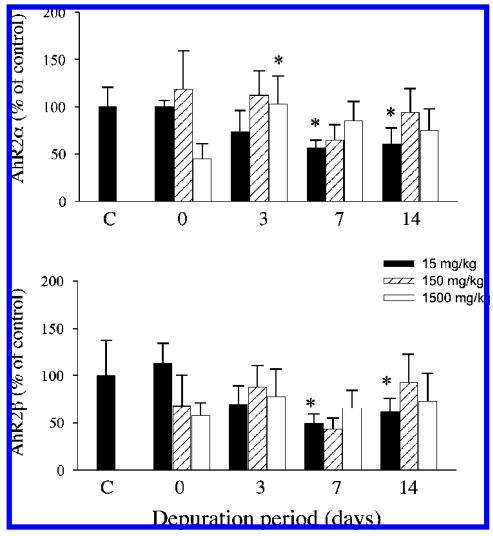

Figure 5. AhR2α (A) and AhR2β (B) mRNA levels at different time of the depuration period. Significant differences ($p < 0.05$) between depuration period day 0 for the corresponding feeding group at different time points is denoted with asterisk (according to results from nested analysis of variance (ANOVA), followed by Tukey's HSD). Data are given as mean values expressed as percentage (%) of control ± standard error of the mean (SEM).

manuscript in preparation). Comparison of feeding and depuration periods showed that BHT was highly retained in fish liver as only 8−13% of fed BHT was eliminated during the 2-week depuration period. Unfortunately, due to the availability of limited analytical methods for salmon, we were not able to run HPLC analysis of the potential BHT metabolites. To obtain metabolite information, it was necessary to make an extended use of the literature on mammalian species. For example, studies have shown that P450 and peroxidase catalyzed oxidation of BHT produces electrophilic quinone methides (QMs). QMs are strongly electrophilic and readily form adducts with proteins (*27*).

Biotransformation Enzyme Genes and Activity Levels during the Feeding Period. In this study, both AhR2α and AhR2β mRNA was expressed in all treatment groups and days of exposure showing a differential pattern at day 3 of the feeding period and with 15 mg BHT/kg during the entire feeding period. The 150 mg BHT/kg feeding group produced the highest AhR2α mRNA levels at all feeding days, compared to the other feeding groups. The diversity of the AhR genes are more pronounced in nonmammalian vertebrates compared to mammalian species as revealed through comparative genomic analyses (*28*). In salmonid species (salmon and rainbow trout), AhR2α, AhR2β, AhR2γ, and AhR2δ represent the four distinct AhR2 genes. In addition, the salmon genome contains two genes of the AhR1 variant, which are presumably nonfunctional (*29*). It has been suggested that rainbow trout AhR2α and AhR2β differ in their promoter preference and may regulate distinct sets of genes (*30*). In accordance with our data, it has been shown in a previous study that administration of BHT to rat, mouse, and rainbow trout produced the induction of liver AhR isoforms (*31*). In this study, we were unable to quantify the AhR2γ and AhR2δ isoforms probably because of low abundance levels (*24*). However, the different expression patterns between the AhR2 isoforms observed in this study suggest functional differences between the two receptor isotypes and may postulate different biological tasks within the cell.

The expression of CYP1A1 mRNA and enzyme activity are elevated in amphibians, teleosts, and avian and mammalian species after exposure to coplanar halogenated organic compounds (*32*). Comparatively, less is known about CYP1A1 induction by non-dioxin-like compounds. Previous studies have shown that BHT-induced hepatic CYP enzyme activity in rainbow trout (*Salmo gairdneri*) (*33*) and mRNA levels in rat (*34*). Our results are in accordance with these results, showing significant and consistent increases in the amount of AhR isoforms, CYP1A1, CYP3A, GSTπ, and UGT1 transcripts during the feeding period (except at day 84). Interestingly and contrary to the gene expression, specific CYP1A1 (EROD), GSTπ, and UGT1 enzyme activity was not significantly altered (compared to the control) and remained unchanged in all treatment groups during the feeding period. Several potential modes of action have been suggested and could account for the loss of catalytic function and inactivation of CYP forms, GST and UGT. These include (a) binding of BHT or its metabolites to these enzyme resulting in direct destruction of

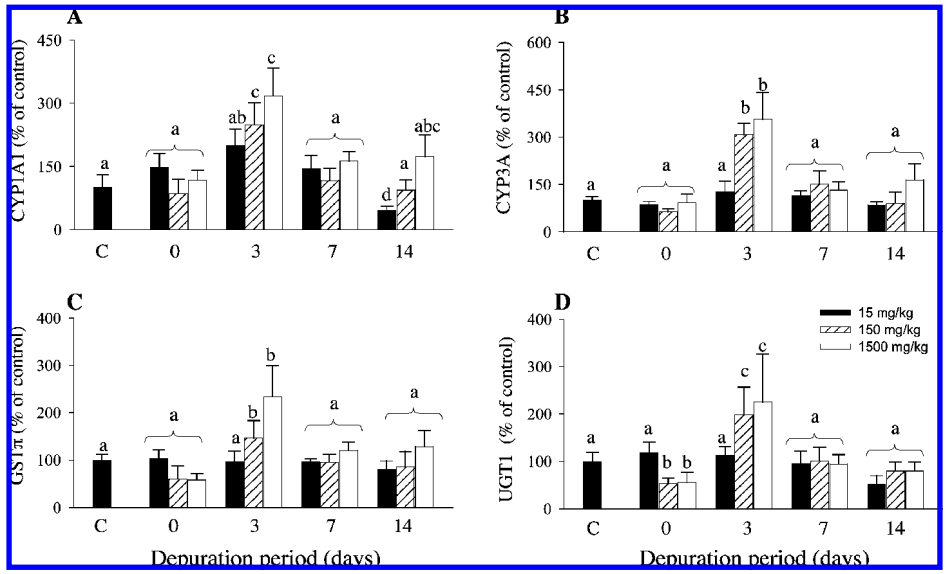

Figure 6. Alteration of CYP1A1 (A), CYP3A (B), GSTπ (C), and UGT1 (D) mRNA levels at different times of the depuration period. Significant differences ($p < 0.05$) within the same treatment group at different time points are denoted with letters (according to results from nested analysis of variance (ANOVA), followed by Tukey's HSD). Data are given as mean values expressed as percentage (%) of control ± standard error of the mean (SEM).

the native enzyme, with subsequent loss of enzyme activity, and (b) inhibition of the NADPH cytochrome P450 reductase that affects the electron transport for substrate oxidation (*35*). Previously, it was demonstrated by Klein and co-workers (*36*) that dietary BHT significantly decreased activities of EROD and MROD in turkey liver. In another study, administration of BHT (25, 100, and 500 mg/kg/day) to rats produced minor changes in EROD activity that was not dose related (*34*). In another study by Williams and co-workers (*37*), it was shown that both butylated hydroxyanisole (BHA) and BHT inhibited intercellular molecular exchange between cultured liver cells, suggesting that these compounds may have an epigenetic effect on cell membrane function that could be the basis for their tumor-promoting activity. In a more recent study, it was shown that BHA and BHT at a dietary content of 100−125 ppm inhibited the initiation phase of 2-acetylaminofluorene (AAF) or aflatoxin B1 (AFB1) hepatocarcinogenesis and therefore may act to block intracellular effects of the carcinogen (*38*). Therefore, the mechanisms of BHT effects are not well understood and still subject to further studies and discussion. However, it is possible that inhibitory effects of BHT on salmon hepatic phase I and II enzymes at the activity levels and not at the transcriptional levels may be an integral aspect of its protective antioxidant function.

Another interesting effect of BHT in the present study was observed in the phase II gene expressions. Phase II enzyme genes showed a parallel and higher expression pattern, compared to the phase I enzyme genes. This may be explained by the suggestion that phase II genes are believed to be activated by monofunctional inducers which trigger cellular signals that activate gene transcription through an antioxidant or electrophile response element (ARE/EpRE) in responsive genes (*39, 40*). In contrast, the phase I enzymes are not believed to be induced by monofunctional inducers, and CYP genes have not been found to contain functional ARE/EpREs. BHT has been shown to act as a monofunctional inducer (*41*). Interestingly, it was found in a study with rats that several CYP enzymes were induced by monofunctional inducers (BHA, EQ, and olitipraz), suggesting a possibility that some phase I enzymes may also be regulated by a mechanism involving ARE/EpRE elements (*42*). Another interesting aspect of GSTπ and UGT1 mRNA levels is that both showed a trend toward reduction (albeit above control levels) between feeding day 3 and 14 in the low (15 mg/kg) and medium (150 mg/kg) doses but was increased in the high dose (1500 mg/kg) during the same time period. This may be because the low and medium doses were metabolized more rapidly, compared to the highest dose. The UGT is a multigene family of enzymes that has been shown to metabolize a broad spectrum of both endogenous and exogenous substances such as bilirubin, testosterone, estrogens, 4-nitrophenole, and morphine (*43*). In fish, multiple forms of the enzymes have been purified (*44*). In addition, nucleotide sequencing data of zebrafish (*Danio rerio*) have detected at least 10 different isotypes of the UGT gene (*45*). The primer sequences used in our real-time PCR assay was designed based on an up-regulated UGT sequence in a suppression subtractive hybridization (SSH) salmon cDNA library in our laboratory (*46*), and the primers spanned the conserved region of fish UGTs.

In this study, UGT mRNA showed a time-specific increase during the feeding period. At the end of the feeding period (day 84), the three doses did not show any transcriptional changes for either GSTπ or UGT1 levels, compared to controls, indicating that these mRNA species may have reached a steady state level. For example, the site of UGT-mediated glucuronidation is generally an electron-rich nucleophilic heteroatom (O, N, or S). Therefore, phenols are one of the preferred substrates for glucuronidation (*47*). Most recent findings suggest and support the key role of the antioxidant/electrophile response element (ARE/EpRE) in the regulation of expression of some phase II genes such as GST and UGT by the monofunctional phenolic antioxidants and/or their metabolite(s) (*41*). Being a phenolic compound, the chemical properties of BHT may explain the high expression levels of phase II enzyme genes during the feeding period. Contrary to gene expression, specific UGT activity toward *p*-nitrophenol was not significantly altered, compared to the control, and remained unchanged during the feeding period. Our findings are not in accordance with previous studies showing that phenolic antioxidants increased the activity of UGT in the liver of rodents (*48*) for *p*-nitrophenol. Similar to UGT, GST is a cytosolic conjugation enzyme belonging to a multigene super family of five classes which has different substrate preferences (*49*). In two salmonid species, salmon and brown trout (*Salmo trutta*), five different cytosolic GST isotypes

from one class have been purified (*50*), representing the diversity of the gene. The primer sequences used in our real-time PCR assay was designed based on an up-regulated GST sequence that belonged to a π-class sequence from a subtracted salmon cDNA library in our laboratory (*46*). As explained earlier, ARE/EpRE plays a key role in the regulation of GST expression by the monofunctional phenolic antioxidants and/or their metabolite(s). The induction of the "ARE gene battery" appears to be an adaptation to oxidative stress (*51, 52*). Studies have shown BHT-induced oxidative stress in the cardiac muscle of rats (*53*) and increased hydrogen peroxide (H_2O_2) formation in rat liver and lung microsomes and in guinea pig and hamster liver microsomes (*54, 55*). Contrary to the gene expression, specific GST enzyme activity was not significantly altered in our study, compared to the control, and remained unchanged in all treatment groups during the feeding and depuration periods. It has been demonstrated previously that exposure to BHT inhibited GST enzyme activity and depleted levels of glutathione in rat (*56*). In another study, lactating rats receiving a nominal 500 mg BHT/kg body weight/day showed a 40% reduction of glutathione levels compared to control values (*57*). In these studies, GST activities were inhibited by quinone methides (QMs) and attenuated by depleting glutathione (GSH) (*27*). On the basis of the above studies, it is possible that the same inhibitory effect of BHT on both phase I and II enzyme activities may have happened in our study. This is a subject of continued investigation in our laboratory.

Biotransformation Enzyme Genes and Activity during Depuration Period. The production of farmed Atlantic salmon in Norway requires a mandatory 2-week depuration period to ensure elimination of veterinary medicaments and other undesirable components prior to slaughtering and delivery to market. Because of its lipophilicity, BHT is likely to be rapidly and evenly distributed throughout the lipid depot. The major lipid storage sites in fish are variable with species but include mesenteric tissue, muscle, liver, and the often overlooked subdermal fat layer (*58*). The Atlantic salmon stores most its reserve of lipids in the muscle. Farmed salmon of commercial size contain up to 12% lipids in the edible muscle (*59*). Fish muscle lipids exist mainly in three forms, namely neutral lipids (mainly triglyceride) in adipocytes for storage of energy, polar lipids serving as major components of the cell membrane, and intracellular lipid droplets distributed in the cell cytoplasm as a local energy store. During starvation, lipids stored in adipose tissues are utilized before the muscular lipid, hepatic glycogen, and protein sources (*60*). Therefore, the release of parent BHT into the circulating system under starvation could affect the hepatic metabolism as well as biotransformation gene and enzyme expression patterns. BHT may also be transported and retained in other organs, which may provoke a new wave of biotransformation with formation of metabolites that may inhibit biotransformation enzymes and subsequently result in prolonged excretion.

During the 2-week depuration period, mRNA levels of AhR isoforms were not significantly altered in any of the treatment groups and days of exposure. For CYP1A1, CYP3A, GSTπ, and UGT1, the pattern of expression during the depuration period could generally be described as increased expression at depuration day 3, followed by a decrease at day 7 and 14. From the HPLC analysis, we observed a decrease in the concentration of BHT in the salmon liver and muscle during the depuration period. We therefore speculate that high levels of BHT and its metabolites may have been released from the fat depot or other organs during the first period (i.e., 3 days) of the depuration period and activated the transcription of CYP1A1, CYP3A, GSTπ, and UGT1 mRNA levels in a dose-dependent manner. Similar to the feeding period, specific enzyme activities EROD, BROD, GST, and UGT were not significantly affected, compared to controls. This supports our hypothesis that high BHT levels at feeding day 84 (i.e., depuration day 0) and its metabolite due to bioaccumulation, probably inhibited the translation and post-translational modification of phase I and II biotransformation systems.

Food Safety and Fish Health Aspect. At the permitted concentrations used in food, BHT has been part of the human diet for many years without evidence of adverse effects (*61*). Other studies have found BHT to display some evidence of carcinogenicity in animal tests with an uncharacterized mechanism of action (*61*). The BHT doses typically required for the production of toxic effects in animals and in vitro systems are orders of magnitude above the amounts of BHT ingested by humans. The implications of these facts on regulatory decision-making will not be obvious until the pharmacodynamics and relative metabolic rates and pathways in rodents and man are compared. The estimated acceptable daily intake of BHT, as defined by the FAO/WHO, is 0−0.3 mg/kg of body weight per day. This is almost a 1000-fold lower than the dose most often used to produce acute lung injury in mice (*62*). In this study, we showed that the phase I and II pathways in the liver were induced at the transcriptional levels by BHT during the feeding and depuration periods, while the subsequent enzyme activities were inhibited, even with low levels of BHT in the fish feed, that were not measurable in the liver. BHT accumulated in the liver during the whole feeding period, supporting the results that detoxification enzyme activities are inhibited. BHT was also highly retained in the salmon muscle during the depuration period. The highest allowable concentration of BHT in fish feed (150 mg/kg) showed a concentration of 6 μg/kg BHT in the salmon muscle at day 14 of the depuration period (Bohne et al., manuscript in preparation). This is just a part of the total concentration in the salmon, since BHT can accumulate in other organs. The levels of metabolites are unknown and therefore need further investigation. No recommendations have been suggested on the intake of BHT metabolites for fish consumers. Therefore, BHT and its metabolites may have potential for toxicological and adverse health effects for both fish and fish consumers through carry-over processes from the fish feed.

ACKNOWLEDGMENT

Help from Harald Mundheim at Fiskeriforskning (Norway, Bergen) in the formulation and production of the experimental diets is highly appreciated. We are grateful to the technical staff at Matre Research Station for excellent handling of the fish and the technical staff at FettVit laboratory at NIFES for the help during sampling. We thank Anne Mortensen for highly professional technical assistance.

Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Suontama, J.; Karlsen, Ø.; Moren, M.; Hemre, G.-I.; Melle, W.; Langmyhr, E.; Mundheim, H.; Ring, ø, E.; Olsen, R. E. Growth, feed conversion and chemical composition of Atlantic salmon (Salmo salar L.) and Atlantic halibut (Hippoglossus L.) fed diets supplemented with krill or amphipods. *Aquacult. Nutr.* **2007**, *13*, 241–255.

(2) Fritsche, K. L.; Johnston, P. V. Rapid autooxidation of fish oil in diets without added antioxidants. *J. Nutr.* **1988**, *118*, 425–426.
(3) Ministry, Forskrift om fôr varer *Landbruksdepartementet*, 2002, Postboks 8007 Dep 0030 Oslo, Fiskeridepartementet, Postboks 8118 Dep 0032 Oslo.
(4) German, J. B. Antioxidants. In *Food Additives*, 2nd ed.; Branen, A. L.; Davidson, P. M.; Salminen, S.; Thorngate, J. H., III., Eds.; Marcel Dekker, Inc.: New York, 2002; pp 1−19.
(5) Verhagen, H., Adverse effects of food additives. In *Food Safety and Toxicity*, de Vries, J., Ed.; CRC Press: Boca Raton, FL, 1997; pp 1−12.
(6) Marino, A. A.; Mitchell, J. L. Lung damage in mice following intraperitoneal injection of butylated hydroxytoluene. *Proc. Soc. Exp. Biol. Med.* **1972**, *140*, 122–125.
(7) Takahasi, O.; Hiraga, K. Dose response study of hemorrhagic death by dietary butylated hydroxytoluene (BHT) in male rats. *Toxicol. Appl. Pharmacol.* **1978**, *43*, 399–406.
(8) Rostron, C.; Tumour promotion by, B. H. T. *Food Chem. Toxicol.* **1982**, *20*, 329–337.
(9) Nakagawa, Y.; Tayama, K.; Nakao, T.; Hiraga, K. On the mechanism of butylated hydroxytoluene induced hepatic necrosis in rats. *Biochem. Pharmacol.* **1984**, *33*, 2669–2674.
(10) Nakagawa, Y.; Tayama, K. Nephrotoxcity of butylated hydroxytoluene in phenobarbital-pretreated rats. *Arch. Toxicol.* **1988**, *61*, 359–365.
(11) Thompson, D. C.; Thompson, J. A.; Sugumaran, M.; Moldeus, P. Biological and toxicological consequences of quinone methide formation. *Unknown* **1993**, *86*, 129–162.
(12) Pascual, C. Y.; Cresbo, J. F.; Perez, P. G.; Esteban, M. M. Food allergy and intolerance in children and adolescents, an update. *Eur. J. Clin. Nutr.* **2000**, *54*, 75–78.
(13) Bradshaw, T. D.; Trapani, V.; Vasselin, D. A.; Westwell, A. D. The aryl hydrocarbon receptor in anticancer drug discovery: friend or foe. *Curr. Pharm. Des.* **2002**, *8* (27), 2475–90.
(14) Nelson, D. R.; Koymans, L.; Kamataki, T.; Stegeman, J. J.; Feyereisen, R.; Waxman, D. J.; Waterman, M. R.; Gotoh, O.; Coon, M. J.; Estabrook, R. W.; Gunsalus, I. C.; Nebert, D. P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* **1996**, *6*, 1–42.
(15) Gu, Y. Z.; Hogenesch, J. B.; Bradfield, C. A. The PAS superfamily: sensors of environmental and developmental signals. *Annu. Rev. Pharmacol. Toxicol.* **2000**, *40*, 519–61.
(16) Williams, D. E.; Lech, J. J.; Buhler, D. R. Xenobiotics and xenoestrogens in fish: modulation of cytochrome P450 and carcinogenesis. *Mutat. Res.* **1998**, *399* (2), 179–92.
(17) Berdikova Bohne, V. J.; Hamre, K.; Arukwe, A. Hepatic Biotransformation and Metabolite Profile during a 2-Week Depuration Period in Atlantic Salmon Fed Graded Levels of the Synthetic Antioxidant, Ethoxyquin. *Toxicol. Sci.* **2006**, *93* (1), 11–21.
(18) Berdikova Bohne, V. J.; Hamre, K.; Arukwe, A. Hepatic metabolism, phase I and II biotransformation enzymes in Atlantic salmon (Salmo Salar, L) during a 12 week feeding period with graded levels of the synthetic antioxidant, ethoxyquin. *Food Chem. Toxicol.* **2007**, *45* (5), 733–46.
(19) Pesonen, M.; Andersson, T. Subcellar localization and Properties of Cytochrome P-450 and UDP Glucuronsyltransferase in the Rainbow Trout Kidney. *Biochem. Pharmacol.* **1987**, *36*, 823–829.
(20) Burke, M. D.; Mayer, R. T. Ethoxyresorufin: Direct fluorometric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methylcholanthrene. *Drug Metab. Dispos.* **1974**, *2*, 583–588.
(21) Andersson, T.; Pesonen, M.; Johansson, C. Differential induction of cytochrome P-450-dependent monooxygenase, epoxide hydrolase, glutathione transferase and UDP glucuronosyltransferase activities in the liver of the rainbow trout by β-naphthoflavone or Clophen A50. *Biochem. Pharmacol.* **1985**, *34*, 3309–3314.
(22) Habig, W. H.; Pabst, M. J.; Jakoby, W. B. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* **1974**, *249*, 7130–7139.
(23) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. *Anal. Biochem.* **1976**, *72*, 248–254.
(24) Mortensen, A. S.; Arukwe, A. Activation of estrogen receptor signaling by the dioxin-like aryl hydrocarbon receptor agonist, 3,3′,4,4′,5-pentachlorobiphenyl (PCB126) in salmon in vitro system. *Toxicol. Appl. Pharmacol.* **2008**, *227* (2), 313–24.
(25) Arukwe, A. Toxicological housekeeping genes: do they really keep the house. *Environ. Sci. Technol.* **2006**, *40* (24), 7944–9.
(26) Steele, B. K.; Meyers, C.; Ozbun, M. A. Variable expression of some "housekeeping" genes during human keratinocyte differentiation. *Anal. Biochem.* **2002**, *307* (2), 341–347.
(27) Lemercier, J. N.; Meier, B. W.; Gomez, J. D.; Thompson, J. A. Inhibition of glutathione S-transferase P1−1 in mouse lung epithelial cells by the tumor promoter 2,6-di-tert-butyl-4-methylene-2,5-cyclohexadienone (BHT-quinone methide): protein adducts investigated by electrospray mass spectrometry. *Chem. Res. Toxicol.* **2004**, *17* (12), 1675–83.
(28) Hahn, M. E. Aryl hydrocarbon receptors: diversity and evolution. *Chem. Biol. Interact.* **2002**, *141* (1−2), 131–60.
(29) Hansson, M. C.; Wittzell, H.; Persson, K.; von Schantz, T. Unprecedented genomic diversity of AhR1 and AhR2 genes in Atlantic salmon (Salmo salar L.). *Aquat. Toxicol.* **2004**, *68* (3), 219–32.
(30) Abnet, C. C.; Tanguay, R. L.; Hahn, M. E.; Heideman, W.; Peterson, R. E. Two forms of aryl hydrocarbon receptor type 2 in rainbow trout (Oncorhynchus mykiss). Evidence for differential expression and enhancer specificity. *J. Biol. Chem.* **1999**, *274* (21), 15159–66.
(31) Kahl, R. Synthetic antioxidants: Biochemical actions and interference with radiation, toxic compounds, chemical mutagens and chemical carcinogens. *Toxicology* **1984**, *33* (3−4), 185–228.
(32) Van der Oost, R.; Beyer, J.; Vermeulen, N. P. E., Fish bioaccumulation and biomarkers in environmental risk assesment: a review. *Environ. Toxicol. Pharmacol.* **2002**, (13), xxxx.
(33) Eisele, T. A.; Sinnhuber, R. O.; Nixon, J. E. Dietary antioxidant effects on the hepatic mixed-function oxidase system of rainbow trout (Salmo gairdneri). *Food Chem. Toxicol.* **1983**, *21* (3), 273–277.
(34) Price, R. J.; Scott, M. P.; Walters, D. G.; Stierum, R. H.; Groten, J. P.; Meredith, C.; Lake, B. G. Effect of thiabendazole on some rat hepatic xenobiotic metabolising enzymes. *Food Chem. Toxicol.* **2004**, *42* (6), 899–908.
(35) Levine, W. G. Effects of butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) on metabolism of N,N-dimethyl-4-aminoazobenzene (DAB) by rat liver microsomes. *Res. Commun. Chem. Pathol. Pharmacol.* **1984**, *46* (1), 113–20.
(36) Klein, P. J.; Van Vleet, T. R.; Hall, J. O.; Coulombe, R. A., Jr. Effects of dietary butylated hydroxytoluene on aflatoxin B1-relevant metabolic enzymes in turkeys. *Food Chem. Toxicol.* **2002**, *41* (5), 671–678.
(37) Williams, G. M.; Wang, C. X.; Iatropoulos, M. J. Toxicity studies of butylated hydroxyanisole and butylated hydroxytoluene. II. *Food Chem. Toxicol.* **1990**, *28* (12), 799–806.
(38) Williams, G. M.; Iatropoulos, M. J.; Jeffrey, A. M. Anticarcinogenicity of monocyclic phenolic compounds. *Eur. J. Cancer Prev.* **2002**, *11* (Suppl 2), S101−7.
(39) Hayes, J. D.; Chanas, S. A.; Henderson, C. J.; McMahon, M.; Sun, C.; Moffat, G. J.; Wolf, C. R.; Yamamoto, M. The Nrf2 transcription factor contributes both to the basal expression of glutathione S-transferases in mouse liver and to their induction by the chemopreventive synthetic antioxidants, butylated hydroxyanisole and ethoxyquin. *Biochem. Soc. Trans.* **2000**, *28* (2), 33–41.
(40) Buetler, T. M.; Gallagher, E. P.; Wang, C.; Stahl, D. L.; Hayes, J. D.; Eaton, D. L. Induction of phase I and phase II drug-metabolizing enzyme mRNA, protein, and activity by BHA,

ethoxyquin, and oltipraz. *Toxicol. Appl. Pharmacol.* **1995**, *135* (1), 45–57.

(41) Hayes, J. D.; McMahon, M. Molecular basis for the contribution of the antioxidant responsive element to cancer chemoprevention. *Cancer Lett.* **2001**, *174* (2), 103–113.

(42) Buetler, T. M.; Gallagher, E. P.; Wang, C. H.; Stahl, D. L.; Hayes, J. D.; Eaton, D. L. Induction of Phase I and Phase II Drug-Metabolizing Enzyme mRNA, Protein, and Activity by BHA, Ethoxyquin, and Oltipraz. *Toxicol. Appl. Pharmacol.* **1995**, *135* (1), 45–57.

(43) Tephly, T. R.; Burchell, B. UDP-glucuronosyltransferases: a family of detoxifying enzymes. *Trends Pharmacol. Sci.* **1990**, *11* (7), 276–279.

(44) Clarke, D. J.; George, S. G.; Burchell, B. Multiplicity of UDP-glucuronosyltransferases in fish. Purification and characterization of a phenol UDP-glucuronosyltransferase from the liver of a marine teleost, Pleuronectes platessa. *Biochem. J.* **1992**, *284*, 417–23.

(45) George, G. G.; Taylor, B. Molecular evidence for multiple UDP-glucuronosyltransferase gene families in fish. *Mar. Environ. Res.* **2002**, *54*, 253–257.

(46) Mortensen, A. S.; Arukwe, A. Targeted Salmon Gene Array (SalArray): A Toxicogenomic Tool for Gene Expression Profiling of Interactions Between Estrogen and Aryl Hydrocarbon Receptor Signalling Pathways. In 2007.

(47) Parkinson, A. Biotransformation of xenobiotics. In *Cassarett & Doull's: The Basic Science of Poisons*; Klaassen, C. D., Ed.; McGraw-Hill Companies: New York, 2001; pp 133−195.

(48) Kashfi, K.; Yang, E. K.; Chowdhury, J. R.; Chowdhury, N. R.; Dannenberg, A. J. Regulation of Uridine Diphosphate Glucuronosyltransferase Expression by Phenolic Antioxidants. *Cancer Res.* **1994**, *54* (22), 5856–5859.

(49) Willmore, W. G.; Storey, K. B. Storey: Purification and properties of the glutathione S-transferases from the anoxia-tolerant turtle (Trachemys scripta elegans). *FEBS J.* **2005**, *272*, 3602–14.

(50) Novoa-Valinas, M. C.; Perez-Lopez, M.; Melgar, M. J. Comparative study of the purification and characterization of the cytosolic Glutathione S-transferases from two salmonid species: Atlantic salmon (Salmo salar) and brown trout (Salmo trutta). *Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol.* **2002**, *131* (2), 207–213.

(51) Owuor, E. D.; Kong, A. N. T. Antioxidants and oxidants regulated signal transduction pathways. *Biochem. Pharmacol.* **2002**, *64* (5−6), 765–770.

(52) Packer, L.; Cadenas, E., Phenolic Antioxidants. In *Handbook of Synthetic Antioxidants*; Marcel Dekker, Inc.: New York, 1997; pp 188−199.

(53) Faine, L. A.; Rodrigues, H. G.; Galhardi, C. M.; Ebaid, G. M. X.; Diniz, Y. S.; Fernandes, A. A. H.; Novelli, E. L. B. Butyl hydroxytoluene (BHT)-induced oxidative stress: Effects on serum lipids and cardiac energy metabolism in rats. *Exp. Toxicol. Pathol.* **2005**, *57* (3), 221–226.

(54) Rossing, D.; Kahl, R.; Hildebrant, A. G. Effect of synthetic antioxidants on hydrogen peroxide formation, oxyferro cytochrome P-450 concentration and oxygen consumption in liver microsomes. *Toxicology* **1984**, *34* (1), 67–77.

(55) Thompson, D. C.; Trush, M. A. Enhancement of the peroxidase-mediated oxidation of butylated hydroxytoluene to a quinone methide by phenolic and amine compounds. *Chem.−Biol. Interact.* **1989**, *72* (1−2), 157–173.

(56) Kupfer, R.; Dwyer-Nield, L. D.; Malkinson, A. M.; Thompson, J. A. Lung toxicity and tumor promotion by hydroxylated derivatives of 2,6-di-*tert*-butyl-4-methylphenol (BHT) and 2-*tert*-butyl-4-methyl-6-isopropylphenol: correlation with quinone methide reactivity. *Chem. Res. Toxicol.* **2002**, *15* (8), 1106–12.

(57) McFarlane, M.; Price, S. C.; Cottrell, S.; Grasso, P.; Bremmer, J. N.; Bomhard, E. M.; Hinton, R. H. Hepatic and associated response of rats to pregnancy, lactation and simultaneous treatment with butylated hydroxytoluene. *Food Chem. Toxicol.* **1997**, *35* (8), 753–761.

(58) Zhou, S.; Ackman, R. G.; Morrison, C. Storage of lipids in the myosepta of Atlantic salmon (Salmo salar). *Fish Physiol. Biochem.* **1995**, *14* (2), 171–178.

(59) Zhou, S.; Ackman, R. G.; Morrison, C. Adipocytes and lipid distribution in the muscle tissue of Atlantic salmon (Salmo salar). *Fish. Aquat. Sci.* **1996**, *53*, 326–332.

(60) Czesny, S.; Rinchard, J.; Garcia Abiado, M. A.; Dabrowski, K. The effect of fasting, prolonged swimming, and predator prescence on energy utilization and stress in juvenile walleye (Stizostedion vitreum). *Physiol. Behav.* **2003**, *79*, 597–603.

(61) World Health Organization (WHO). Toxicological evaluation of certain food additives and contaminants *WHO Food Additive Series*; Cambridge University Press: Cambridge, 1987; Vol. 21.

(62) Williams, G. M.; Iatropoulos, M. J.; Whysner, J. Safety assessment of butylated hydroxyanisole and butylated hydroxytoluene as antioxidant food additives. *Food Chem. Toxicol.* **1999**, *37* (9−10), 1027–38.

Received for review August 19, 2008. Revised manuscript received October 21, 2008. Accepted October 22, 2008. The Norwegian Research Council (NRC) supported this work under Project 143314/130 SYNTOX.

JF8025524