Appl Microbiol Biotechnol (1996) 45: 710–718 © Springer-Verlag 1996

ORIGINAL PAPER

F. J. M. Verhagen · H. J. Swarts · T. W. Kuyper
J. B. P. A. Wijnberg · J. A. Field

The ubiquity of natural adsorbable organic halogen production among basidiomycetes

Received: 5 October 1995/Received revision: 28 December 1995/Accepted: 12 February 1996

Abstract Recently, several species of basidiomycetes were shown to produce *de novo* high concentrations of chloroaromatic metabolites. Since these lignocellulose-degrading fungi play a major role in the ecosphere, the purpose of this study was to determine the ubiquity of organohalogen production among basidiomycetes. A total of 191 fungal strains were monitored for adsorbable organic halogen (AOX) production when grown on defined liquid media. Approximately 50% of the strains tested and 55% of the genera tested produced AOX. A low production of 0.1–0.5 mg AOX/l was observed among 25% of the strains, a moderate production of 0.5–5.0 mg AOX/l was observed among 16% of the strains and 9% of the strains produced high levels (5–67 mg AOX/l). The latter group was dominated by species belonging to the genera *Hypholoma*, *Mycena* and *Bjerkandera*, showing specific AOX productions in the range 1074–30 893 mg AOX/kg dry weight of mycelial biomass. Many highly ecologically significant fungal species were identified among the moderate to high producers. These species were also able to produce AOX when cultivated on natural lignocellulosic substrates. *Hypholoma fasciculare* and *Mycena metata* respectively produced up to 132 mg and 193 mg AOX/kg dry weight of forest litter substrate in 6 weeks.

F. J. M. Verhagen (✉) · J. A. Field
Division of Industrial Microbiology, Department of Food Science, Wageningen Agricultural University, P.O. Box 8129, 6700 EV Wageningen, The Netherlands.
Fax: + 31 317 484978
E-mail: Frank.Verhagen@Algemeen.IM.WAU.NL

T. W. Kuyper
Centre for Soil Ecology, Wageningen Agricultural University, Kampsweg 27, 9418 PD Wijster, The Netherlands

H. J. Swarts · J. B. P. A. Wijnberg
Department of Organic Chemistry, Wageningen Agricultural University, Dreijenplein 8, 6703 HB Wageningen, The Netherlands

Introduction

The occurrence of organohalogens in nature has generally been ascribed to anthropogenic activities (Amato 1993). Bulk parameters, like adsorbable organic halogen (AOX), are used to monitor the extent of this type of xenobiotic pollution in the environment. However, the pool of AOX detected in unpolluted environments was recently shown to be at least 300 times greater than that which can be accounted for by anthropogenic sources (Asplund and Grimvall 1991). Forest soil samples collected world-wide contain AOX concentrations between 20 mg and 360 mg/kg dry weight soil.

Since AOX production was shown to take place during the decay of forest litter (Asplund 1995), microorganisms responsible for degradation of lignocellulosic debris are implicated. Basidiomycetes have been recognized as the most ecologically significant group of organisms responsible for lignocellulose decomposition (Swift 1982). Furthermore, several basidiomycetes are known to have the ability to synthesize *de novo* organohalogen metabolites. Both chloromethane (Harper 1985) and several types of chloroaromatic metabolites, such as chlorinated anisyl metabolites (De Jong et al. 1994), chlorinated hydroquinone metabolites (Hsu et al. 1971; Dominguez et al. 1972; Van Eijk 1975; Takahashi et al. 1993) and chlorinated orcinol metabolites (Okamoto et al. 1993), are produced by wood- and litter-degrading basidiomycetes. However, the extent to which basidiomycetes contribute to the natural pool of AOX in the environment is unknown.

In this study, a screening for AOX production by basidiomycetes in laboratory media was carried out to

determine whether the ability to produce organohalogens is widespread among basidiomycetes. Ecologically significant organohalogen-producing species were also tested for AOX production on natural lignocellulosic substrates.

Materials and methods

Microorganisms and culture conditions

Fungal strains were labelled according to their origin. The following labels refer to culture collections: ATCC: American Type Culture Collection, Rockville, Maryland, USA; CBS: Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; CZ: Culture Collection of Basidiomycetes (CCBAS), Prague, Czech Republic; CIMW: Culture Collection of Industrial Microbiology, Wageningen Agricultural University, Wageningen, The Netherlands; MAD: USDA Forest Product Lab., Madison, Wisconsin, USA; EBT: Environmental Biotechnologies Inc., Menlo Park, California, USA; IJFM: Centro de Investigaciones Biologicas, Consejo Superior de Investigaciones Cientificas, Madrid, Spain; ANKE: H. Anke, Department of Biotechnology, University of Kaiserslautern, Kaiserslautern, Germany; INRA: H.-E. Spinnler, Laboratoire de Recherches sur les Aromes, Dijon, France. The following labels refer to fungal strains isolated in the period 1992–1994 in The Netherlands: WIJS, RHEN, ONO, BB, ITAL, BEN, OUDES, PW, ERF, WAG, ARBOR, EPE, HD and DUUR. The following labels refer to fungal strains isolated in 1994 in northern Spain: LAVEC and COFIN; and in Germany: TRIER.

Fungal strains were maintained on two agar media. One of the agar media contained (g/l): glucose $\cdot$ H_2O 20.00 mycological peptone (Oxoid Ltd., Basingstoke, Hampshire, UK) 5.00, yeast extract (Gibco BRL, Life Technology Ltd., Paisley, Scotland, UK) 2.00, KH_2PO_4 1.00, $MgSO_4 \cdot 7H_2O$ 0.50, agar 15.00. The other agar medium contained (g/l): glucose $\cdot$ H_2O 5.00, malt extract (Oxoid) 3.50, agar 15.00. Fungal cultures were stored at 4°C after growth.

AOX screening

Fungal strains were grown in a medium with a high nitrogen content (50 mM N) supplied as peptone, according to Kimura et al. (1990) containing (g/l): glucose $\cdot$ H_2O 20.00, mycological peptone (Oxoid) 5.00, yeast extract (Gibco BRL) 2.00, KH_2PO_4 1.00, $MgSO_4 \cdot 7H_2O$ 0.50, NaCl 0.06. They were also grown in a medium with a low nitrogen content (2.2 mM N) according to Tien and Kirk (1988), containing (g/l): glucose $\cdot$ H_2O 10.00, ammonium tartrate 0.20, 2,2-dimethyl-succinic acid 2.92, KH_2PO_4 2.00, $MgSO_4 \cdot 7H_2O$ 0.50, $CaCl_2$ 0.10, thiamine $\cdot$ HCl 2 mg and trace elements solution 1 ml/l. The composition of the trace elements solution was (g/l): $MgSO_4 \cdot 7H_2O$ 3.00, $MnSO_4 \cdot H_2O$ 0.50, NaCl 1.00, $FeSO_4 \cdot 7H_2O$ 0.10, $CoCl_2 \cdot 6H_2O$ 0.10, $ZnSO_4 \cdot 7H_2O$ 0.10, $CuSO_4 \cdot 5H_2O$ 0.10, H_3BO_3 0.01, $Na_2MoO_4 \cdot 2H_2O$ 0.01, $A1K(SO_4)_2 \cdot 12H_2O$ 0.01, trisodium nitrilotriacetic acid 1.5. The inorganic halide content of the peptone (high N) and mineral medium (low N) were 97.8 ± 0.9 ($n = 6$) and 70.4 ± 0.6 ($n = 6$) mg/l, respectively.

Medium (10 ml liquid volume) in a 100-ml serum bottle was sterilized at 121°C for 30 min. Medium was inoculated with a plug (diameter 5 mm), which was taken from an agar medium covered with fresh mycelium of the fungal strain. Duplicate fungal cultures were incubated in the dark at 25°C under an air atmosphere. A duplicate set of sterile medium containing a sterile agar plug were incubated in parallel to the fungal cultures and were harvested as controls at the time of AOX analysis. When the culture fluid was covered by the mycelium (2–6 weeks), the culture fluid was harvested for organohalogen determination. The measurement of AOX was done according to the method described by the International Organization for Standardization (Anonymous 1989). After centrifugation of the culture fluid at 13 000 g, a subsample (5 ml) was added to a mixture of 50 mg activated carbon and 20 ml demineralized water. The mixture was shaken for 1 h at 250 rpm. The activated carbon, on which the organohalogens were adsorbed, was filtered over a quartz frit and washed five times with 5 ml aqueous $NaNO_3$ (5.4 g/l) supplemented with 0.4 ml nitric acid, and three times with 8 ml demineralized water. The activated carbon on the quartz frit was placed in the combustion room of an AOX analyser (ECS 1600, Euroglas Analytical Instruments, Delft, The Netherlands). The combustion temperature used was 1000°C. Combustion of the activated carbon with the adsorbed organohalogens produced volatile inorganic halides, which were dried and cleaned-up by bubbling through concentrated sulphuric acid. The volatile inorganic halides were then measured in a microcolorimeter, which contained 75% acetic acid as an electrolyte. The precision of the method was checked by determination of AOX in standard solutions of 4-chlorophenol, pentachlorophenol, 2-chloro-1,4-dimethoxybenzene, and 2,4,6-trichlorophenol, ranging in concentrations from 0.8 mg to 25.6 mg organochloride/l. The measured AOX concentrations in the standard solutions were within 10% of the theoretical concentrations. The total halide concentration in a liquid medium was determined by injecting medium (10–100 µl) directly into the microcolorimeter, using a syringe.

Two criteria were used to determine whether a fungal strain was an AOX-producing, positive strain. The first criterion was that the average AOX concentration of duplicate determinations in fungal cultures was at least twofold higher than the average AOX concentration measured in the duplicate sterile controls, which were incubated in parallel with the cultures. The second criterion was that the lowest replicate of the fungal culture was higher than the highest replicate of the controls. Only those strains passing both criteria on at least one of the two media were considered as AOX-positive strains. The minimum net production of AOX required to comply with the criteria was found to be approximately 0.1 mg/l.

Specific AOX production

The specific production of AOX per weight of biomass was determined for 11 ecologically significant fungal strains. Each fungal strain was incubated for 6 weeks at 25°C under an air atmosphere in the two media described above. Fungal cultures, 10 ml liquid volume placed in 100-ml serum bottles, were incubated in quadruplicate in the dark at 25°C under an air atmosphere. A quadruple set of sterile media were incubated parallel to the fungal cultures and were harvested as controls at the time of organohalogen analysis. After 6 weeks, the net AOX concentration as well as the fungal biomass was determined. AOX concentrations were determined as described above, whereas fungal biomass was determined by filtering the biomass over a prewashed and tared GF-50 glass-fibre round filter (Schleicher & Schuell, ’s-Hertogenbosch, The Netherlands), with a pore size of 1 µm, washing the biomass three times with 10 ml demineralized water and drying the filter at 105°C for 24 h.

AOX production on natural substrates

Selected fungal strains were inoculated into media containing different natural substrates. The media contained (g/l): beech (*Fagus sylvatica*) wood 30, pine (*Pinus sylvestris*) wood 30, forest litter [beech leaves 10, beech twigs 5, oak (*Quercus robur*) leaves 10, oak twigs 5], hemp (*Cannabis sativa*) stem wood 30, or wheat (*Triticum*

aestivum) straw 30. All substrates were dried and ground to a particle size of less than 0.5 mm before use. These media were autoclaved at 121°C for 30 min prior to use. The water-soluble inorganic halide contents of the beech wood medium, pine wood medium, forest litter medium, hemp stem wood medium and wheat straw medium after autoclaving were (mg/l) ($n = 3$): 0.45 ± 0.02, 1.28 ± 0.14, 58.7 ± 0.3, 59.2 ± 2.9 and 98.9 ± 1.4 respectively. The total inorganic halide concentrations of the beech and pine wood media after a more extensive extraction (finer milling, below 0.5 mm, and three consecutive 1-h autoclaving treatments at 121°C) were 45.3 ± 2.5 ($n = 3$) and 73.4 ± 9.6 ($n = 3$) mg halide/kg dry substrate, respectively.

Fungal cultures of 10 ml liquid volume placed in 100-ml serum bottles were incubated in quadruplicate in the dark at 25°C under an air atmosphere. A quadruple set of sterile media were incubated parallel to the fungal cultures and were harvested as controls at the time of organohalogen analysis. After 6 weeks, the culture fluid was harvested for AOX determination.

Effect of chloride content on AOX production

The most ecologically widespread AOX-positive fungal strain in The Netherlands, *Hypholoma fasciculare*, was inoculated into media containing ground (less than 0.5 mm) beech wood (30 g/l) to which different amounts of chloride ranging from 0 to 405 mg/l were added. The measured water-soluble inorganic halide concentrations of the media after sterilization (121°C, 30 min) were (mg/l): 0.66, 1.14, 1.42, 2.01, 3.63, 7.10, 15.5, 32.6, 69.2, 135.1 and 406.0, respectively.

Incubations of fungal cultures were done identically to those described under AOX production on natural substrates.

Results

AOX screening

In total, 191 fungal strains belonging to 87 different genera were tested for production of organohalogens in both high-N and low-N media. The AOX-positive strains producing a concentration of 0.2 mg AOX/l or more on at least one of the media are listed in Table 1. Of all strains tested, 95 (49.7%) produced detectable levels of AOX, of which 47 strains (24.6%) produced AOX at concentrations between 0.1 mg/l and 0.5 mg/l (low producers), 30 strains (15.7%) produced AOX at concentrations between 0.5 mg/l and 5.0 mg/l (moderate producers), and 18 strains (9.4%) produced AOX at concentrations between 5.0 mg/l and 66.9 mg/l (high producers). Fungi belonging to the genera *Hypholoma*, *Bjerkandera* and *Mycena* were found to be dominant among the high producers of organohalogens. All *Hypholoma* species tested produced concentrations of AOX between 14 mg/l and 67 mg/l.

Strains with a very low net production between 0.1 mg and 0.2 mg AOX/l were as follows: *Mycena inclinata* ITAL94.11, *Calocybe gambosa* TRIER94.1, *Hebeloma* sp. DUUR94.54, *Psathyrella fulvescens* WIJS94.31, *Psathyrella conopilus* CBS461.86, *Baeospora myosura* ONO93.3, *Armillaria tabescens* ATCC62.537, *Calocybe carnea* WIJS94.15, *Collybia peronata* WIJS94.12, *Pholiota destruens* ARBOR94.1, *Clitocybe marginella* WIJS94.8, *Fomitopsis meliae* EBT259, *Mycena alcalina* CBS489.79, *Phlebia ochraceofulva* CBS142.75, *Hebeloma* sp. DUUR94.43, *Amanita muscaria* WIJS94.20, *Fomes fomentarius* CIMW12.92, *Serpula incrassata* MAD563, *Coprinus atramentarius* ANKE82022, *Panaeolus sphinctrinus* WAG94.2, *Mycena niveipes* ANKE77207, *Gloeophyllum trabeum* MAD617, *Russula cyanoxantha* WIJS94.17, *Marasmius oreades* BB94.5, and *Laccaria laccata* ONO94.4.

Important examples of species that did not produce detectable amounts of AOX in this study include *Phanerochaete chrysosporium*, *Trametes versicolor*, *Ganoderma applanatum*, *Phlebia radiata*, *Ceriporiopsis subvermispora*, *Stereum hirsutum*, *Paxillus involutus*, *Piptoporus betulinus* and *Tyromyces caesius*.

Among the moderate to high AOX producers, 10 species may be regarded as having a high ecological significance in The Netherlands in terms of broad geographical distribution and a high population density within their habitats (Arnolds et al. 1995), as follows: *Hypholoma fasciculare*, *Bjerkandera adusta*, *Collybia dryophila*, *Collybia butyracea*, *Gymnopilus sapineus*, *Mycena galopus*, *Schizopora paradoxa*, *Mycena galericulata*, *Armillaria mellea* and *Megacollybia platyphylla* (see frequency class in Table 1).

Table 1 shows that the nitrogen content of the medium had a great impact on the AOX production. The concentrations of AOX were generally highest in the medium with the high N content. However, many fungi still produced appreciable levels of AOX in the medium with the low N content.

Specific AOX production

The specific AOX productions per weight of dry mycelial biomass for 11 ecologically significant AOX-producing fungi are shown in Table 2. The specific productions in the medium with the high N content were generally higher or equal to those in the medium with the low N content, except for species belonging to the genus *Hypholoma*, which had higher specific productions per dry weight of biomass in the low N medium than in the high N medium. Species belonging to the genus *Hypholoma* showed the highest specific productions. The AOX production in cultures of *Hypholoma elongatum* in the low N medium was equal to about 3% of the weight of the biomass.

AOX production on natural substrates

The AOX concentrations produced by fungal strains inoculated into media containing different natural substrates are given in Table 3. The table illustrates that organohalogens were also produced in considerable

Table 1 Concentrations of adsorbable organic halogens (AOX) produced by basidiomycetes in a medium with a high nitrogen and a low nitrogen content after incubation for 2–6 weeks at 25°C. For every species the FC (frequency class) in The Netherlands is indicated, based on identification in 5-km^2-grid blocks: 0, not known from the Netherlands; 1, < 0.2%; 2, 0.2%–0.6%; 3, 0.6%–2%; 4, 2%–5%; 5, 5%–10%; 6, 10%–25%; 7, 25%–45%; 8, 45%–75%; 9, 75%–100% of grid blocks investigated, after Arnolds et al. (1995). – no FC given because species name is unknown. The net AOX production is the value obtained after correction for levels in sterile controls incubated in parallel which gave approximately 0.07–0.15 mg AOX/l. Values are means of duplicate determinations. ND not detectable (detection limit was approx. 0.1 mg/l); NM not measured. Abbreviations of strains and locations of the culture collections are given in Materials and methods under Microorganisms and culture conditions

Species	Strain	FC	AOX concentration (mg/l	
			High-N medium	Low-N medium
High producers				
Hypholoma elongatum	WIJS94.28	6	66.95	32.35
Hypholoma elongatum	CBS776.87	6	58.05	18.34
Mycena metata	RHEN93.1	7	54.13	1.91
Hypholoma capnoides	ONO93.7	7	28.53	22.12
Bjerkandera adusta BEUK47	WAG2.91	9	27.45	9.21
Hypholoma fasciculare	RHEN93.5	9	16.46	10.36
Mycena epipterygia	ITAL94.3	7	15.36	2.51
Hypholoma sublateritium	WIJS94.27	8	14.49	5.05
Hypholoma elongatum	COFIN94.2	6	13.72	6.04
Phylloporia ribis	IJFM(B7)	4	12.91	ND
Bjerkandera sp. BOS55	CIMW1.91	–	12.30	4.79
Peniophora pseudopini	CBS162.65	0	10.87	4.14
Collybia sp.	BB94.4	–	10.22	0.78
Stropharia aeruginosa	CBS839.87	7	7.09	2.55
Mycena inclinata	ITAL94.11	6	6.40	2.66
Pholiota squarrosa	ONO93.6	7	6.07	0.89
Phellinus torulosus	IJFM(B8)	1	5.83	0.71
Bjerkandera fumosa	CBS152.79	8	5.22	3.08
Moderate producers				
Phellinus fastuosus	ATCC26.125	0	4.36	0.14
Pholiota adiposa	BEN94.1	6	3.58	0.28
Ischnoderma benzoium	CBS250.30	6	3.19	ND
Collybia dryophila	WIJS94.3	8	2.78	1.22
Clitocybe odora	CBS512.87	6	2.64	1.03
Collybia butyracea	OUDES93.1	8	2.60	0.96
Gymnopilus sapineus	WIJS94.10	8	1.98	0.47
Coprinus comatus	PW94.9	8	1.91	ND
Mycena galopus	ITAL94.4	8	1.86	ND
Schizopora paradoxa	CBS319.53	8	1.83	0.81
Stropharia aeruginosa	ERF94.1	7	1.78	0.73
Pholiota aurivella	ANKE80256	6	0.95	1.43
Agaricus bisporus	AH94.1	5	1.42	0.41
Bjerkandera adusta	RHEN93.4	9	0.96	1.33
Mycena galericulata	PW93.1	9	1.29	ND
Mycena galericulata	WIJS94.23	9	1.16	ND
Armillaria mellea	INRA57	6	1.15	0.80
Hericium erinaceus	WAG93.2	4	1.11	ND
Mycena sp.	WIJS94.29	–	ND	1.07
Psilocybe semilanceata	ITAL94.8	6	NM	1.05
Agaricus arvensis	WAG93.1	7	NM	0.96
Trametes sp.	LAVEC94.3	–	0.95	ND
Clitocybe agrestis	WIJS94.24	6	0.87	ND
Gymnopilus junonius	PW93.3	7	0.61	0.83
Mycena galopus var. candida	ITAL94.5	6	0.79	0.26
Macrocystidia cucumis	BB94.3	5	0.78	0.29
Hericium erinaceus	CBS302.89	4	0.73	0.24
Collybia sp.	BB94.6	–	0.66	ND
Gymnopilus sapineus	ONO93.4	8	0.65	0.12
Pleurotus cornucopiae	CBS383.80	3	0.61	ND
Xerula radicata	WIJS94.22	7	0.53	ND
Megacollybia platyphylla	WIJS94.11	8	0.50	ND
Low producers				
Phellinus ferruginosus	ANKE80027	5	0.49	ND
Pleurotus ostreatus	CBS358.88	7	0.33	0.47
Agaricus xanthoderma	CBS200.48	5	0.44	ND
Clitocybe phyllophila	ARBOR94.2	6	0.41	ND
Amanita rubescens	WIJS94.19	8	0.40	0.18
Armillaria lutea	CBS851.85	7	0.37	0.19
Phellinus pini	CBS170.79	2	0.45	ND
Trametes hirsuta	CBS282.73	6	0.43	0.32
Flammulina velutipes	PW93.5	8	0.43	ND
Galerina marginata	CBS339.88	5	0.40	0.17

Table 1 (Continued)

Species	Strain	FC	AOX concentration (mg/l	
			High-N medium	Low-N medium
Heterobasidion annosum	EPE93.1	7	NM	0.39
Oudemansiella mucida	HD94.1	6	0.39	ND
Psilocybe phyllogena	ITAL94.2	3	0.38	ND
Coniophora puteana	CBS230.87	6	0.34	0.20
Mycena megaspora	ANKE77173	4	0.32	0.15
Marasmius androsaceus	ITAL94.1	7	0.32	ND
Meripilus giganteus	WIJS94.21	7	0.28	ND
Pholiota gummosa	PW94.6	6	0.27	ND
Kuehneromyces mutabilis	CIMW6.92	8	0.27	ND
Agrocybe cilindracea	LAVEC94.1	5	NM	0.25
Macrotyphula fistulosa	WIJS94.13	6	0.25	ND
Phellinus robiniae	ATCC62.802	0	0.12	0.25
Phellinus robiniae	CBS254.51	0	0.14	0.22
Lepista nuda	BB93.1	8	0.22	ND
Xerocomus badius	ITAL94.9	8	NM	0.22

Table 2 Biomass production and specific AOX production of some ecologically significant fungi in 10 ml medium with a high nitrogen and a low nitrogen content after incubation for 6 weeks at 25°C. Specific AOX production is based on the net AOX production after correction for levels in sterile controls incubated in parallel. Determinations were done in quadruplicate

Species	Strain	Biomass production (mg dry weight fungus)		Specific AOX production (mg/kg dry weight fungus)	
		High N	Low N	High N	Low N
Hypholoma elongatum	WIJS94.28	27.9 ± 6.2	12.9 ± 0.9	25930 ± 5031	30893 ± 4473
Hypholoma capnoides	ONO93.7	28.3 ± 2.4	7.0 ± 1.0	10066 ± 1304	18881 ± 756
Hypholoma fasciculare	RHEN93.5	21.3 ± 1.6	11.7 ± 0.6	8987 ± 1384	18173 ± 2117
Mycena metata	RHEN93.1	64.7 ± 5.3	8.7 ± 1.4	14626 ± 1117	4794 ± 1358
Collybia butyracea	OUDES93.1	53.7 ± 1.5	29.5 ± 3.2	1112 ± 27	501 ± 32
Bjerkandera sp.	BOS55	48.9 ± 6.5	18.0 ± 1.5	1074 ± 164	472 ± 79
Gymnopilus sapineus	WIJS94.10	35.8 ± 1.8	34.1 ± 1.7	878 ± 88	252 ± 53
Collybia dryophila	WIJS94.3	92.3 ± 8.0	37.5 ± 2.0	579 ± 104	212 ± 41
Mycena galopus	ITAL94.4	67.9 ± 7.1	18.7 ± 1.7	533 ± 83	361 ± 44
Mycena galericulata	WIJS94.23	81.5 ± 3.8	35.2 ± 3.3	188 ± 65	43 ± 8
Megacollybia platyphylla	WIJS94.11	49.1 ± 6.3	4.5 ± 1.3	100 ± 34	110 ± 29

amounts when growth was on natural substrates. Concentrations of AOX were higher in the medium containing forest litter or annual fibre crops (hemp stems and wheat straw) than in those containing pine wood or beech wood. Comparison of the AOX concentrations produced by *H. fasciculare* when cultivated on all five natural substrates showed that the production was strongly influenced by the substrate used, since the AOX concentrations measured after 6 weeks of incubation varied between 40 mg and 132 mg AOX/kg dry beech wood and forest litter, respectively. The highest concentration of AOX after 6 weeks of incubation was measured in the culture fluid of *Mycena metata*, cultivated in a medium containing forest litter, and amounted to 193 mg AOX/kg dry weight substrate. In some cases, such as *H. fasciculare* on pine wood and *Hypholoma* spp. on beech wood, the AOX production was apparently 20–25 mg/kg higher than that which could be accounted for by the water-soluble inorganic halide concentration in the original medium. This anomaly is due to the fact that not all of the halides in wood were completely solubilized in the bulk liquid of the medium, as was shown by a more extensive aqueous extraction of the woody substrates (see Materials and methods).

Effect of chloride content on AOX production

H. fasciculare was cultured in medium containing beech wood supplemented with different amounts of chloride. The measured water-soluble inorganic halide concentrations at the time of inoculation ranged from 23 mg to 13 533 mg chloride/kg dry substrate. It appeared that the extra addition of chloride to the media had no noteworthy effect on the concentrations of AOX after incubation. Measured concentrations of AOX varied between 27 mg and 47 mg AOX/kg dry

Table 3 Concentrations of AOX produced by selected fungi in 10 ml medium containing 300 mg dry weight of natural substrate after incubation for 6 weeks at 25°C. Net AOX concentration after correction for levels in sterile controls incubated in parallel. Control AOX values (mg/kg dry weight): pine wood 2.5, beech wood 1.9, forest litter 6.2, hemp stem wood 7.7, wheat straw 7.2. Calculations are based on the dry weight of substrate at the time of inoculation. Determinations were done in quadruplicate

Substrate	Species	Strain	AOX concentration (mg/kg substrate)
Pine wood	*Hypholoma fasciculare*	RHEN93.5	60.7 ± 3.3
	Hypholoma capnoides	ONO93.7	20.2 ± 3.9
	Peniophora pseudopini	CBS162.65	13.5 ± 1.7
	Mycena epipterygia	ITAL94.3	12.7 ± 2.2
	Gymnopilus sapineus	WIJS94.10	10.0 ± 2.3
	Stropharia aeruginosa	CBS839.87	2.9 ± 2.3
Beech wood	*Hypholoma sublateritium*	WIJS94.27	41.3 ± 2.7
	Hypholoma fasciculare	RHEN93.5	40.8 ± 3.5
	Mycena galericulata	WIJS94.23	5.6 ± 0.9
	Megacollybia platyphylla	WIJS94.11	4.6 ± 1.1
	Gymnopilus sapineus	WIJS94.10	3.9 ± 1.0
Forest litter	*Mycena metata*	RHEN93.1	192.9 ± 17.2
	Hypholoma fasciculare	RHEN93.5	132.4 ± 3.3
	Mycena galopus	ITAL94.4	28.4 ± 5.5
	Collybia butyracea	OUDES93.1	21.9 ± 4.6
	Collybia dryophila	WIJS94.3	17.0 ± 2.0
	Megacollybia platyphylla	WIJS94.11	13.2 ± 3.2
Hemp stem wood	*Hypholoma fasciculare*	RHEN93.5	75.9 ± 8.5
Wheat straw	*Hypholoma fasciculare*	RHEN93.5	115.2 ± 8.6

substrate, irrespective of the chloride concentration of the medium.

Discussion

Ubiquity of AOX formation by basidiomycetes

Approximately 50% of the fungal strains tested and 55% of the genera tested produced detectable levels of AOX, indicating that organohalogen production is a ubiquitous capacity among commonly occurring basidiomycetous fungi. Many of the high- and moderate-AOX-producing species are highly ecologically significant fungi. Considering that many AOX-producing basidiomycetes play a major role in the decomposition of lignocellulosic debris and that most of the terrestrial biomass is present in the form of lignocellulose, basidiomycetes probably are a major source of natural organohalogens in forest ecosystems.

Correspondence to known organohalogen-producing genera

The correspondence between genera known from the literature for *de novo* production of specific chloroaromatic metabolites with the AOX-positive genera found in this study was high (Field et al. 1995). All of the 19 known chloroaromatic-producing genera of basidiomycetes tested in this study were found to be AOX-positive. Aside from these 19 genera, 3 other AOX-positive genera tested in this study have been reported in the literature to produce halomethanes (Field et al. 1995). However, production of organohalogens was detected for the first time in 27 additional genera, of which 3 were among the high producers: *Phylloporia*, *Peniophora* and *Collybia*; 8 of these genera were among the moderate producers: *Ischnoderma*, *Clitocybe*, *Gymnopilus*, *Schizopora*, *Psilocybe*, *Macrocystidia*, *Xerula* and *Megacollybia*; and 16 of these genera were among the low producers: *Flammulina*, *Galerina*, *Coniophora*, *Meripilus*, *Agrocybe*, *Macrotyphula*, *Xerocomus*, *Calocybe*, *Hebeloma*, *Baeospora*, *Phlebia*, *Fomes*, *Serpula*, *Panaeolus*, *Laccaria* and *Gloeophyllum.*

AOX production on lignocellulosic substrates

Ecologically significant strains of basidiomycetes were found to produce AOX when grown on natural lignocellulosic substrates. This observation indicates that AOX production by basidiomycetes probably also occurs in the environment as was confirmed by the measurements of chlorinated anisyl metabolites in wood and leaf litter colonized by commonly occurring basidiomycetes (De Jong et al. 1994).

Effect of environmental factors

Several environmental factors may influence the amount of AOX produced by fungal strains. Firstly nitrogen was found to influence AOX production. Concentrations of AOX measured in the medium with the high N content were generally higher than those in

the medium with the low N content (Table 1), which can be explained by the greater biomass in the high N medium. However, it is not only the amount of biomass that plays a role in the AOX production, because it was shown in Table 2 that a higher AOX production per weight of biomass has been found in the high N medium for most fungi, except for the species belonging to the genus *Hypholoma*. As woody plant tissues usually contain only 0.03%–0.1% nitrogen (Cowling and Merrill 1966), the production of AOX by fungi that grow on wood under natural conditions should be compared with our measurements in the low N medium. On the other hand, beech and oak leaves have nitrogen contents ranging from 1.3% to 2.7% (Janel et al. 1979; Laishram and Yadava 1988). Consequently, AOX productions by fungal species growing under natural conditions on leaf litter should be compared with our measurements in the high N medium. Secondly, the effect of the chloride concentration in the environment may influence AOX production. It is clear from Table 3 that the chloride content of the medium played a minor role in the production of AOX. The minor role of the chloride content of the growth medium has already been reported by De Jong et al. (1994), who found that the production of chlorinated anisyl metabolites by *Bjerkandera* sp. strain BOS55 was not significantly influenced by a variable chloride concentration ranging from 0.4 mM to 20 mM, indicating a high affinity of the halogenating enzymes for chloride. This observation is confirmed by the experiment in which *H. fasciculare* was grown in beech wood medium supplemented with different amounts of chloride. Thirdly, the type of lignocellulosic substrate may influence the AOX production. The AOX concentrations measured after 6 weeks of incubation in cultures of *H. fasciculare* that were cultivated on all five natural substrates varied between 41 mg/kg and 132 mg/kg dry substrate (Table 3). The lowest concentrations of AOX were found in the media containing beech wood and pine wood, whereas higher AOX concentrations were found in the medium containing hemp stem wood and the highest concentrations were observed in the wheat straw- and forest litter-containing media. As in the media used for the screening, nitrogen may play a role in this observation. The nitrogen content of the woody substrates is less than 0.1% (Cowling and Merrill 1966), whereas annual hemp stem wood, wheat straw and beech/oak leaf litter contain about 0.5% (F. de Vries, personal communication), 0.6% (Committee on Animal Nutrition and National Committee on Animal Nutrition, Canada 1969) and 1.3%–2.7% nitrogen (Janel et al. 1979; Laishram and Yadava 1988), respectively. Moreover, there are differences in porosity between the woody and non-woody substrates. Valmaseda et al. (1990) studied the weight losses of wheat straw and beech wood during decay by a number of ligninolytic fungi and found that, for almost all fungi, the losses of weight were greater when the fungi were cultivated on wheat straw (50%–53% maximum weight loss) than on beech wood (25%–33% maximum weight loss).

AOX production in natural environments

In order to estimate the production of AOX in a forest, the specific biomass and the total biomass of basidiomycetes have to be determined. Frankland (1982) estimated the total (i.e. living and dead) standing crop of basidiomycete biomass in the organic litter and soil horizons of a temperate deciduous woodland with mull humus (Meathop Wood, Cumbria, UK) as slightly over 40 kg dry weight/ha with an additional 217 kg dry weight/ha in the woody debris. Frankland also noted about 1630 kg dry weight/ha mycelial biomass in dead roots, but it is not clear whether these data pertain to ectomycorrhizal fungi or to saprotrophic basidiomycetes. Calculations by Swift (1982), based on annual litter fall data per hectare per year in Meathop Wood, Cumbria, UK (5000 kg/ha), the degree of colonization by basidiomycetes after 1 year (50%) and yield efficiency of the mycelium (0.5 g mycelium/g leaf litter), provided an estimate of the production of basidiomycetous mycelium and its organic secretions of 938 kg dry weight ha^{-1} $year^{-1}$.

Since the ratio between high- and low-AOX-producing fungal species may vary greatly between habitats, estimations of the total AOX production in specific habitats were made by adding up the AOX productions of the separate species present, on the basis of estimated biomass values and specific AOX productions (Table 2). For certain forest associations, where quantitative data on yield of sporocarps are available, a rough estimate of biomass could be made assuming a ratio of mycelial biomass to sporocarp of 5 (Frankland 1982). The estimated biomass values in three different habitats and the calculated total AOX productions in grams ha^{-1} $year^{-1}$ are given in Table 4. For *Bjerkandera adusta* and for *Hypholoma* spp. (except *H. elongatum*), the specific productions measured in the low-nitrogen medium were used, whereas for the other species (including *H. elongatum*) the specific productions in the high-nitrogen medium were used. Total AOX productions in the three different forest ecosystems ranged from 105 g to 190 g AOX ha^{-1} $year^{-1}$. However, all the estimates are obviously underestimations. First, the calculations do not take into account all AOX-producing species. Second, mycelium may exist for several years without producing sporocarps. Third, the calculations do not consider the enhanced production of chlorinated aromatics by antagonistic interactions between competing fungal species, as was described by Sonnenbichler et al. (1994). In a peat bog dominated by

Table 4 Estimated annual biomass production (kg dry weight ha^{-1} year^{-1}) and calculated total AOX production of selected saprotrophic fungal species in forests dominated by three different tree species in The Netherlands

Species	Estimated fungal biomass (kg dry weight ha^{-1} year^{-1})		
	Pinus sylvestris[a]	*Quercus robur*[b]	*Fagus sylvatica*[c]
Hypholoma spp.[d]	5.0	7.0	10.0
Hypholoma elongatum	0.2	0.0	0.0
Megacollybia platyphylla	0.1	7.0	10.0
Mycena galericulata	0.1	3.0	1.0
Gymnopilus sapineus	2.0	1.0	0.5
Collybia butyracea	1.2	0.5	1.5
Collybia dryophila	0.5	1.0	0.7
Mycena spp.[e]	0.7	0.2	0.7
Bjerkandera adusta	0.0	0.0	1.0
Total calculated AOX production (g AOX ha^{-1} year^{-1})	105	130	190

[a] After B.W.L. de Vries et al. (unpublished data)
[b] After E. Arnolds et al. (unpublished data)
[c] After Arnolds et al. (1994)
[d] Includes mainly *H. fasciculare*, partly also *H. capnoides* and *H. sublateritium*
[e] Includes mainly *M. epipterygia*, *M. galopus* and *M. metata*

the moss *Sphagnum cuspidatum*, investigated by J.J. Barkman (unpublished observations), the mycelial biomass of *H. elongatum* amounted to 80 kg ha^{-1} year^{-1}. Using a specific AOX production of 25 930 mg AOX/kg dry weight of this fungus (Table 2) would translate into 2074 g AOX ha^{-1} year^{-1} for that fungal species alone.

Acknowledgements This project was financially supported by the Technology Foundation, Utrecht, The Netherlands, under project number WLM33.3127, entitled: Fungal chlorinated aromatic metabolites: natural priority pollutants and dioxin precursors in the environment. The work was funded in part by the Royal Netherlands Academy of Arts and Sciences (J.A.F.). The authors acknowledge Euroglas Analytical Instruments, Delft, The Netherlands for technical assistance with the ECS 1600 AOX analyser.

References

Amato I (1993) The crusade against chlorine. Science 261:152–154
Anonymous (1989) Water quality – Determination of adsorbable organic halogens (AOX). ISO 9562, p. 1–8. International Organization for Standardization, Geneva, Switzerland
Arnolds E, Opdam A, van Steenis W, de Vries BWL (1994) Mycocoenology of stands of *Fagus sylvatica* L. in The Netherlands. Phytocoenologia 24:507–530
Arnolds E, Kuyper TW, Noordeloos ME (1996) Revised Checklist of Dutch macromycetes. Dutch Mycological Association, Wijster, The Netherlands (in press)
Asplund G (1995) Origin and occurrence of halogenated organic matter in soil. In: Grimvall A, Leer EWB de (eds) Naturally-produced organohalogens. Kluwer, Dordrecht, pp 35–48
Asplund G, Grimvall A (1991) Organohalogens in nature, more widespread than previously assumed. Environ Sci Technol 25:1346–1350
Committee on Animal Nutrition and National Committee on Animal Nutrition, Canada (1969) U.S.-Canadian tables of feed compositions. Publication 1684. National Academy of Sciences – National Research Council, Washington DC
Cowling EB, Merrill W (1966) Nitrogen in wood and its role in wood deterioration. Can J Bot 44:1539–1554
De Jong E, Field JA, Spinnler HE, Wijnberg JBPA, Bont JAM de (1994) Significant biogenesis of chlorinated aromatics by fungi in natural environments. Appl Environ Microbiol 60:264–270
Dominguez XA, Butruille SD, Gomez Parra F, Reyes C (1972) Aislamiento del 1,4-dimethoxy-2,3,5,6-tetrachlorobenceno y una lactona del *Fomes robiniae*. Anal Quim 68:917–920
Field JA, Verhagen FJM, De Jong E (1995) Natural organohalogen production by basidiomycetes. Trends Biotechnol 13:451–456
Frankland JC (1982) Biomass and nutrient cycling by decomposer basidiomycetes, In: Frankland JC, Hedger JN, Swift MJ (eds) Decomposer basidiomycetes: their biology and ecology. Cambridge University Press, Cambridge, pp 241–261
Harper DB (1985) Halomethane from halide ion – a highly efficient fungal conversion of environmental significance. Nature 315:55–57
Hsu CS, Suzuji M, Yamada Y (1971) Chemical constituents of fungi. I. 1,4-Dimethoxy-2,3,5,6-tetrachlorobenzene (*O*-methyldrosophilin A) from *Phellinus yucatensis*. Chem Abstr 75:115864a
Janel P, Jocteur Monrozier L, Toutain F (1979) Caracterisation de l'azote des litieres et des sols par hydrolyse acide. Soil Biol Biochem 11:141–146
Kimura Y, Asada Y, Kuwahara M (1990) Screening of basidiomycetes for lignin peroxidase genes using a DNA probe. Appl Microbiol Biotechnol 32:436–442
Laishram ID, Yadava PS (1988) Lignin and nitrogen in the decomposition of leaf litter in a sub-tropical forest ecosystem at Shiroy hills in north-eastern India. Plant Soil 106:59–64
Okamoto K, Shimada A, Shirai R, Sakamoto H, Yoshida S, Ojima F, Ishiguro Y, Sakai T, Kawagishi H (1993) Antimicrobial chlorinated orcinol derivatives from mycelia of *Hericium erinaceum*. Phytochemistry 34:1445–1446
Sonnenbichler J, Dietrich J, Peipp H (1994) Secondary fungal metabolites and their biological activities. V. Investigations concerning the induction of the biosynthesis of toxic secondary metabolites in basidiomycetes. Biol Chem Hoppe-Seyler 375:71–79

Swift MJ (1982) Basidiomycetes as components of forest ecosystems. In: Frankland JC, Hedger JN, Swift MJ (eds) Decomposer basidiomycetes: their biology and ecology. Cambridge University Press, Cambridge, pp 307–337

Takahashi A, Agatsuma T, Ohta T, Nunozawa T, Endo T, Nozoe S (1993) Russuphelins B, C, D, E and F, new cytotoxic substances from the mushroom *Russula subnigricans* HONGO. Chem Pharm Bull 41:1726–1729

Tien M, Kirk TK (1988) Lignin peroxidase of *Phanerochaete chrysosporium.* Methods Enzymol 161B: 238–248

Valmaseda M, Almendros G, Martinez AT (1990) Substrate-dependent degradation patterns in the decay of wheat straw and beech wood by ligninolytic fungi. Appl Microbiol Biotechnol 33:481–484

Van Eijk GW (1975) Drosophilin, a methyl ether from *Mycena megaspora.* Phytochemistry 14:2506