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Annexe XII. Foan, L., Domercq, M., Bermejo, R., Santamaría, J.M., Simon, V., 2012. Polycyclic aromatic hydrocarbons (PAHs) in remote bulk and throughfall deposition: Seasonal and spatial trends. Environmental Engineering and Management Journal 11 (6), 1101-1110.

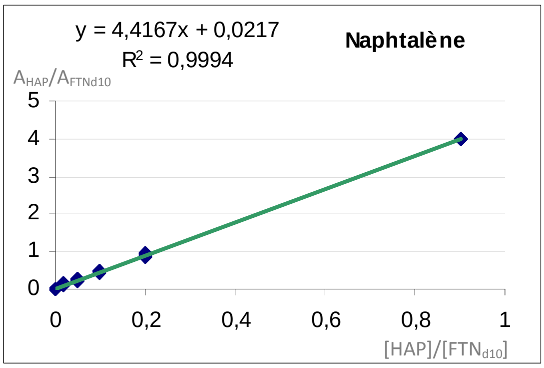
Annexe XIII. Foan, L., Simon, V.. Optimization of pressurized liquid extraction using a multivariate chemometric approach and comparison of solid-phase extraction cleanup steps for the determination of polycyclic aromatic hydrocarbons in mosses, Journal of Chromatography A (sous presse). http://dx.doi.org/10.1016/j.chroma.2012.07.065.

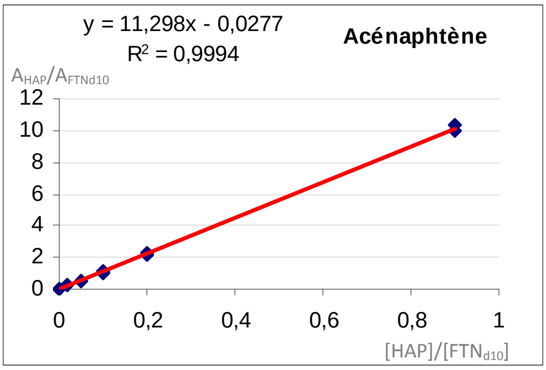
Annexe XIV. Harmens, H., Foan, L., Simon,V., Mills, G., 2010. Mosses as biomonitors of atmospheric POPs pollution: A review.
http://icpvegetation.ceh.ac.uk/publications/documents/FinalPOPsreportDefra21.10.11.pdf

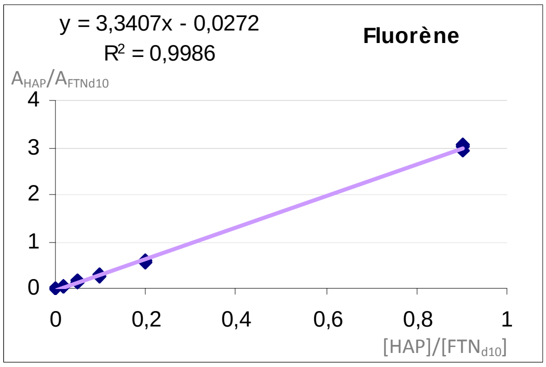
Annexe XV. Harmens *et al.*, 2010. Monitoring of atmospheric deposition of heavy metals, nitrogen and POPs in Europe using bryophytes – Monitoring manual – 2010 survey. http://icpvegetation.ceh.ac.uk/manuals/documents/UNECEHEAVYMETALSMOSSMANUAL2010POPsadaptedfinal_220510_.pdf

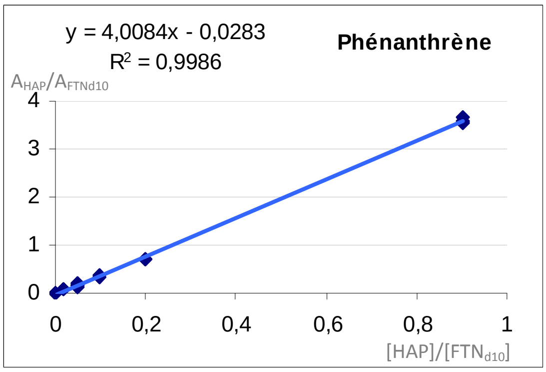
Annexe I.

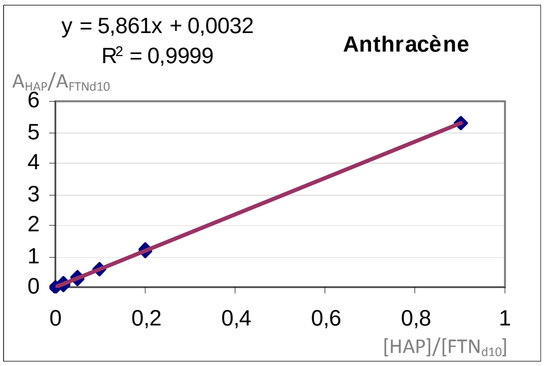
Droites d’étalonnage obtenues par CLHP-FLD pour 15 HAP classés prioritaires par l’USEPA

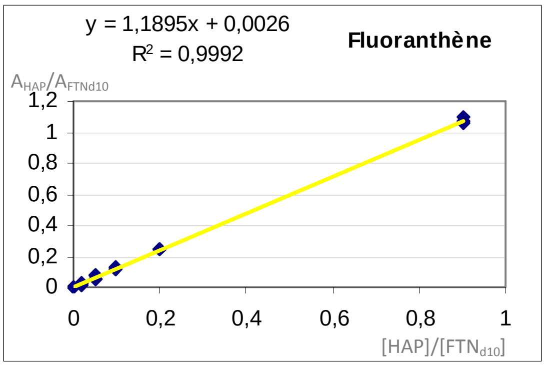


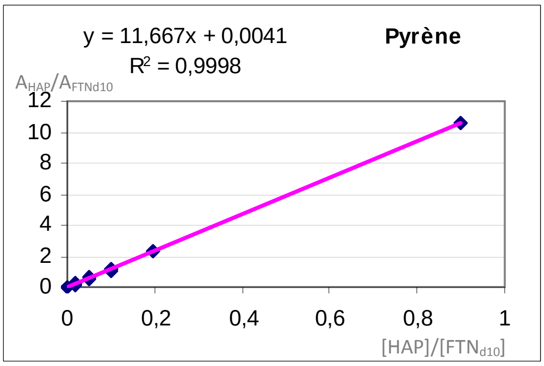


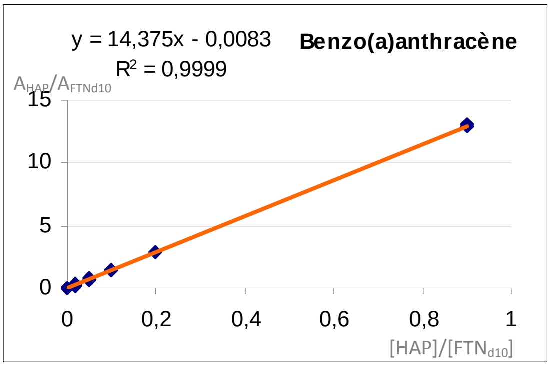


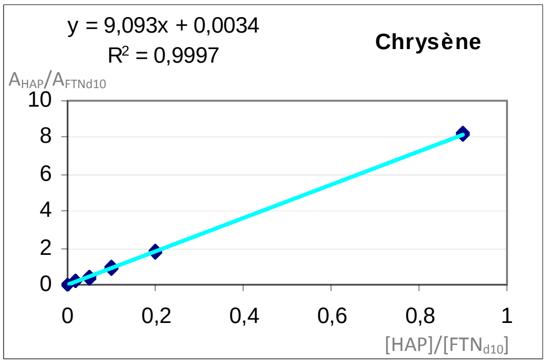








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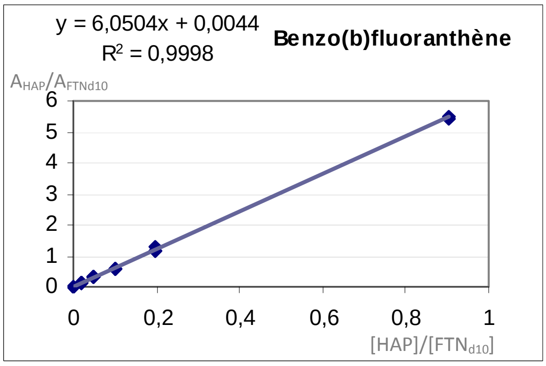
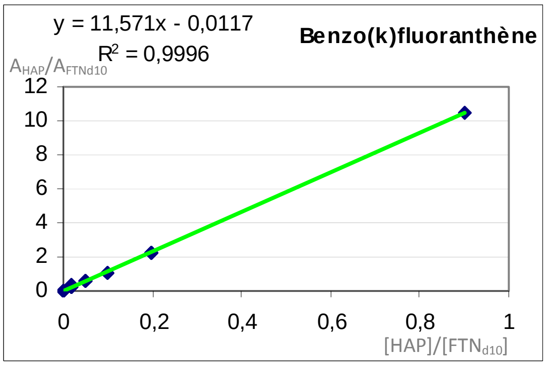
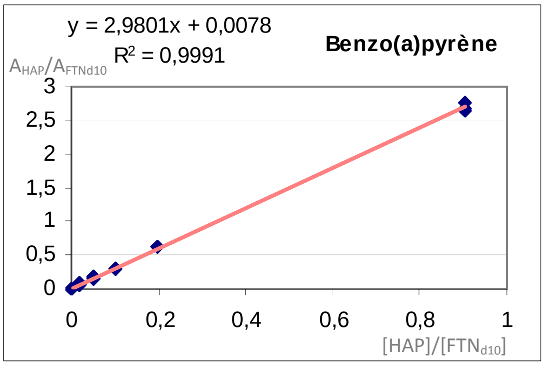
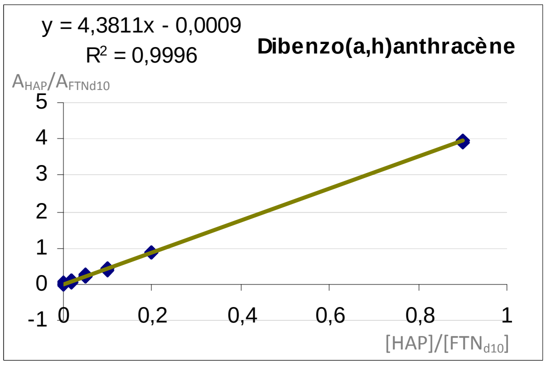
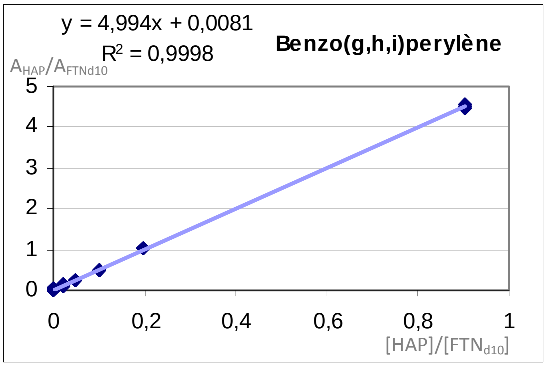
R² = 0,9997
Chrysène
AHAP/AFTNd10
10
8
6
4
2
0
0
0,2
0,4
0,6
0,8
1
[HAP]/[FTNd10]

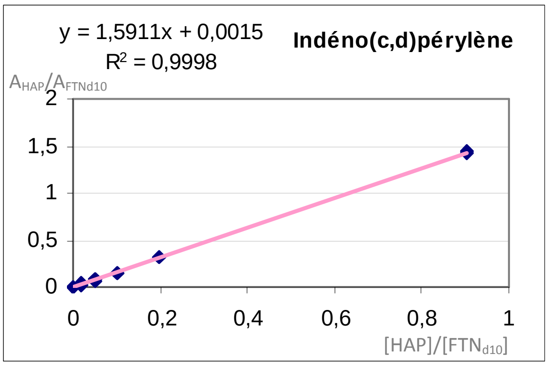

y = 6,0504x + 0,0044
R² = 0,9998
Benzo(b)fluoranthène
AHAP/AFTNd10
6
5
4
3
2
1
0
0
0,2
0,4
0,6
0,8
1
[HAP]/[FTNd10]


y = 11,571x - 0,0117
R² = 0,9996
Benzo(k)fluoranthène
AHAP/AFTNd10
12
10
8
6
4
2
0
0
0,2
0,4
0,6
0,8
1
[HAP]/[FTNd10]


y = 2,9801x + 0,0078
R² = 0,9991
Benzo(a)pyrène
AHAP/AFTNd10
3
2,5
2
1,5
1
0,5
0
0
0,2
0,4
0,6
0,8
1
[HAP]/[FTNd10]


y = 4,3811x - 0,0009
R² = 0,9996
Dibenzo(a,h)anthracène
AHAP/AFTNd10
5
4
3
2
1
0
-1
0
0,2
0,4
0,6
0,8
1
[HAP]/[FTNd10]


y = 4,994x + 0,0081
R² = 0,9998
Benzo(g,h,i)perylène
AHAP/AFTNd10
5
4
3
2
1
0
0
0,2
0,4
0,6
0,8
1
[HAP]/[FTNd10]


y = 1,5911x + 0,0015
R² = 0,9998
Indéno(c,d)pérylène
AHAP/AFTNd10
2
1,5
1
0,5
0
0
0,2
0,4
0,6
0,8
1
[HAP]/[FTNd10]

Annexe II.

Spécimens d'herbier sélectionnés pour l'étude historique des retombées atmosphériques de HAP

Espèce	**Date de prélèvement**	**Lieu d'echantillonage**	**Type de support**
Dicranum scoparium	10/06/1975	Souche d'arbre	Bois
Dicranum scoparium	20/06/1975	Grotte	Pierre
Dicranum scoparium	10/11/1973	Souche d'arbre	Bois
Dicranum scoparium	11/03/2006	Sol	Sol
Dicranum scoparium	12/05/2006	Sol en fôret	Sol
Dicranum scoparium	05/09/2007	Bois mort	Bois
Dicranum scoparium	28/09/2007	Sol	Sol
Dicranum scoparium	19/04/1879	Inconnu	Inconnu
Thamnobryum alopecurum	1/2/1974 & 20/6/1975	Roche de rivière	Pierre
Thamnobryum alopecurum	01/12/2006	Roche humide	Pierre
Thamnobryum alopecurum	09/03/2007	Roche très humide	Pierre
Thamnobryum alopecurum	1879	Inconnu	Inconnu
Thuidium tamariscinum	12/5/2006	Talus	Sol
Thuidium tamariscinum	09/03/2007	Sol au bord d'un ruisseau	Sol
Thuidium tamariscinum	09/03/2007	Roche	Pierre
Thuidium tamariscinum	10/11/1973	Talus	Sol
Thuidium tamariscinum	10/11/1973	Talus	Sol
Thuidium tamariscinum	1881	Inconnu	Inconnu
Hypnum cupressiforme	25/10/2006	Sol	Sol
Hypnum cupressiforme	28/09/2007	Sol	Sol
Hypnum cupressiforme	09/07/2007	Arbre	Bois
Hypnum cupressiforme	05/09/2007	Arbre	Bois
Hypnum cupressiforme	12/05/2006	Talus	Sol
Hypnum cupressiforme	13/10/2006	Sol	Sol
Hypnum cupressiforme	1975	Arbre	Bois
Hypnum cupressiforme	10/11/1973	Talus	Sol
Hypnum cupressiforme	10/11/1973	Talus de route	Sol
Hypnum cupressiforme	1879/1881	Inconnu	Inconnu

Annexe III.

Données de qualité de l'air mesurées à Bertiz entre juillet 2010 et mai 2011 par le LICA (Université de Navarre) : flux moyens journaliers de Cl^-, NO_3^-, SO_4^{2-}, Al, As, Cd, Cr, Cu, Fe, Ni, Mn, Pb et Zn dans les dépôts totaux (en $ng.m^{-2}.d^{-1}$) et teneurs en O_3, NH_3 et NO_2 dans l'air ambiant (en $\mu g.m^{-3}$).

Parcelle	Date de prélèvement	Cl^-	NO_3^-	SO_4^{2-}
Forêt	20/07/2010	3710,5	4043,1	2747,4
Champ	20/07/2010	4507,3	8496,6	3782,6
Forêt	18/08/2010	4025,7	3709,5	3255,0
Champ	18/08/2010	1936,1	3569,9	3500,5
Forêt	15/09/2010	2504,5	3350,6	1964,7
Champ	15/09/2010	1530,7	2414,2	1086,2
Forêt	13/10/2010	8581,8	2435,3	2934,0
Champ	13/10/2010	4310,3	2666,8	2072,2
Forêt	10/11/2010	20826,5	3592,8	4561,6
Champ	10/11/2010	10069,9	4329,6	3411,7
Forêt	09/12/2010	13423,8	5887,1	7885,1
Champ	09/12/2010	29753,8	8324,9	6619,9
Forêt	03/01/2011	1441,9	2641,1	589,2
Champ	03/01/2011	16567,5	6962,7	4091,5
Forêt	02/02/2011	3792,1	2681,2	921,0
Champ	02/02/2011	2580,1	2181,1	946,6
Forêt	01/03/2011	23833,6	4450,8	23228,1
Champ	01/03/2011	16963,6	3072,5	16945,7
Forêt	29/03/2011	6809,2	12547,2	3990,8
Champ	29/03/2011	3306,5	10497,7	3158,5
Forêt	03/05/2011	2715,3	5354,1	2389,0
Champ	03/05/2011	1638,1	4701,8	2361,5

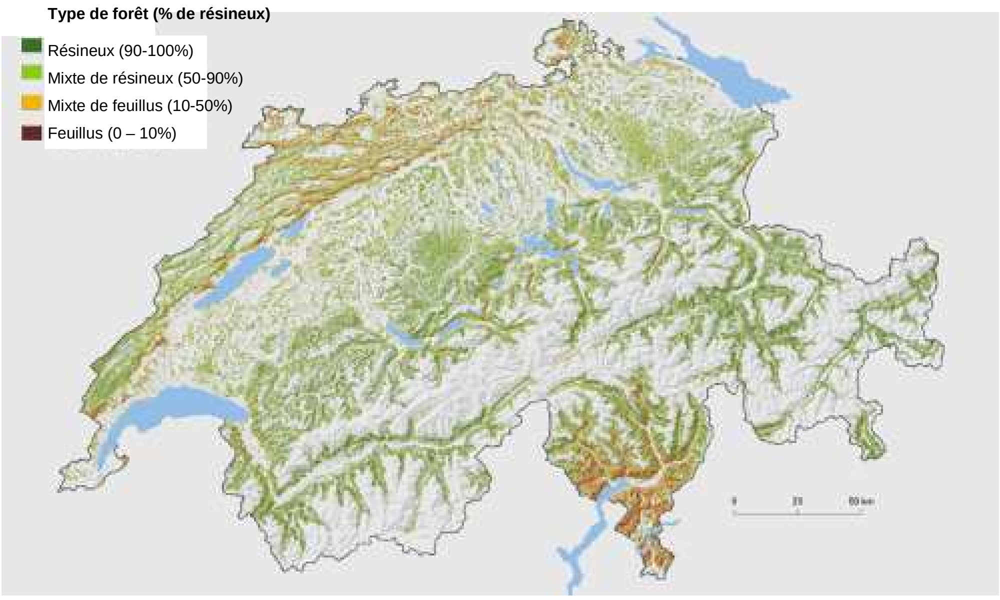
Parcelle	Date de prélèvement	Al	Mn	Fe	As	Cd	Cr	Cu	Ni	Pb	Zn
Forêt	20/07/2010	7,7	55,9	10,8	0,1	0,0	0,2	10,6	0,9	3,4	21,2
Champ	20/07/2010	13,7	4,8	12,3	0,2	0,1	0,5	1,7	1,5	0,6	17,3
Forêt	18/08/2010	16,7	46,2	16,8	0,1	0,1	0,2	2,0	0,7	7,1	4,1
Champ	18/08/2010	6,1	1,2	6,9	0,1	0,0	0,1	1,1	0,4	0,4	4,4
Forêt	15/09/2010	13,5	51,2	13,3	0,1	0,0	0,1	0,2	0,8	0,1	6,3
Champ	15/09/2010	5,9	1,1	2,0	0,1	0,0	0,2	0,2	1,0	0,0	6,6
Forêt	13/10/2010	27,9	74,2	18,8	0,1	0,0	0,3	5,3	1,1	0,3	12,5
Champ	13/10/2010	10,5	2,8	3,6	0,1	0,0	0,3	0,7	0,8	0,1	8,4
Forêt	10/11/2010	17,5	76,6	7,7	0,1	0,1	0,5	0,6	1,9	0,3	9,8
Champ	10/11/2010	14,8	3,4	5,1	0,1	0,3	0,7	0,3	1,3	0,2	11,9
Forêt	09/12/2010	18,6	165,5	6,4	0,1	0,4	0,9	5,2	2,1	7,5	28,7
Champ	09/12/2010	19,8	7,2	6,8	0,1	0,2	0,7	1,3	1,7	1,5	15,9
Forêt	03/01/2011	8,7	16,1	9,9	0,1	0,0	1,3	0,7	1,0	0,2	6,1
Champ	03/01/2011	3,8	0,5	3,6	0,0	0,0	0,1	0,3	0,4	0,1	3,1
Forêt	02/02/2011	2,8	25,8	3,1	0,1	0,0	0,3	0,7	0,3	0,3	3,5
Champ	02/02/2011	3,2	1,3	2,1	0,1	0,0	0,4	0,4	0,5	0,1	5,7
Forêt	01/03/2011	61,6	81,7	179,2	0,3	0,1	3,8	3,1	2,5	1,9	19,0
Champ	01/03/2011	40,7	4,2	138,6	0,2	0,1	2,9	1,0	2,5	1,0	16,6
Forêt	29/03/2011	38,7	72,2	21,3	0,2	0,1	0,4	4,5	1,2	0,4	15,4
Champ	29/03/2011	8,5	8,5	11,7	0,2	0,0	0,3	1,9	0,7	0,1	13,8
Forêt	03/05/2011	11,1	47,4	12,0	0,1	0,0	0,2	1,4	0,8	0,2	6,6
Champ	03/05/2011	5,5	5,5	6,7	2,7	2,7	2,8	3,2	2,9	2,7	5,3

Parcelle	**Date de prélèvement**	**O_3**	**NH_3**	**NO_2**
Forêt	20/07/2010	20,7	0,01	0,2
Champ	20/07/2010	13,3	0,04	1,8
Forêt	18/08/2010	38,0	0,02	0,3
Champ	18/08/2010	27,5	0,04	1,5
Forêt	15/09/2010	34,6	0,04	0,4
Champ	15/09/2010	28,1	0,06	0,7
Forêt	13/10/2010	50,9	0,01	0,4
Champ	13/10/2010	41,4	0,04	1,1
Forêt	10/11/2010	63,6	0,02	0,8
Champ	10/11/2010	39,0	0,04	1,1
Forêt	09/12/2010	60,3	0,08	0,3
Champ	09/12/2010	50,7	0,10	0,2
Forêt	03/01/2011	71,5	0,00	0,4
Champ	03/01/2011	55,6	0,00	0,7
Forêt	02/02/2011	56,8	0,00	0,3
Champ	02/02/2011	42,0	0,00	0,4
Forêt	01/03/2011	27,8	0,07	0,3
Champ	01/03/2011	36,6	0,09	0,1
Forêt	29/03/2011	93,2	0,07	0,5
Champ	29/03/2011	55,8	0,11	0,5
Forêt	03/05/2011	58,9	0,04	0,2
Champ	03/05/2011	51,6	0,07	0,5

Annexe IV.

Occupation du sol par les essences forestières en Suisse (inventaire de 1992 à 1997)

(Source : Office fédéral de la statistique, http://www.bfs.admin.ch/bfs/portal/fr/index.html)

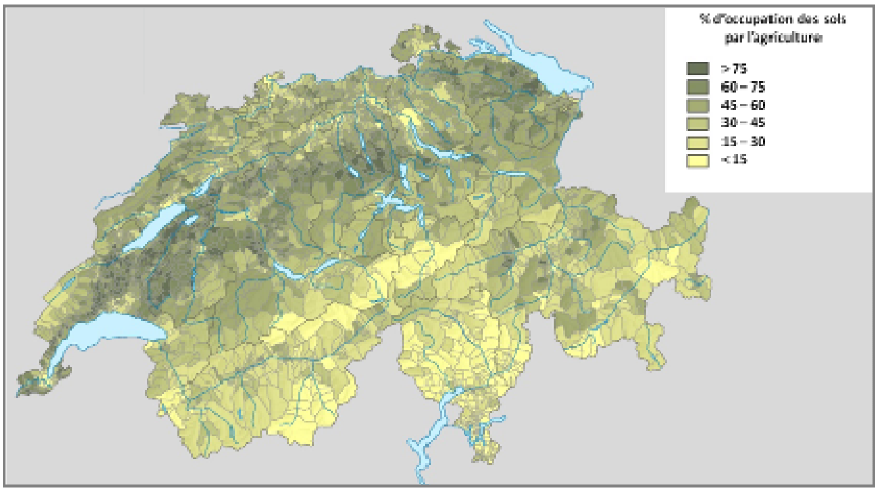


Annexe V.

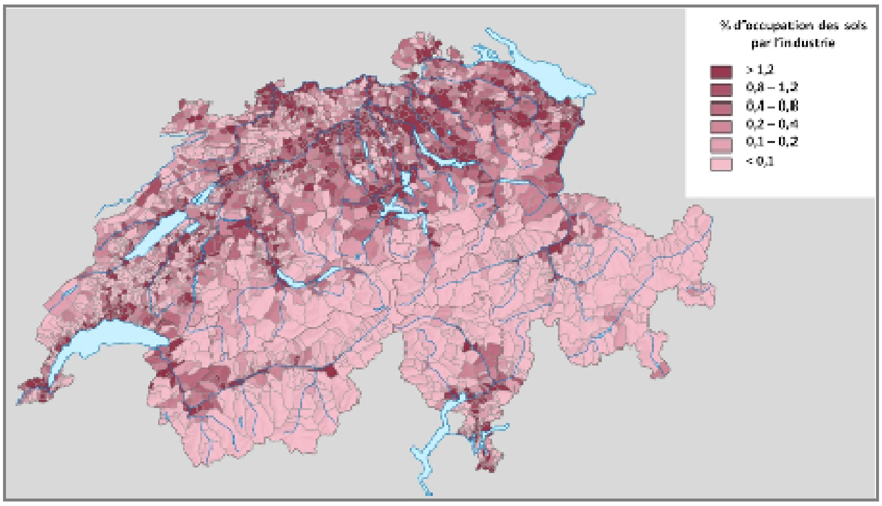
Pourcentage d’occupation des sols suisses par (a) l’agriculture, (b) l’industrie et (c) les zones urbaines (recensement 1992/1997)

(Source : Office fédéral de la statistique, http://www.bfs.admin.ch/bfs/portal/fr/index.html)

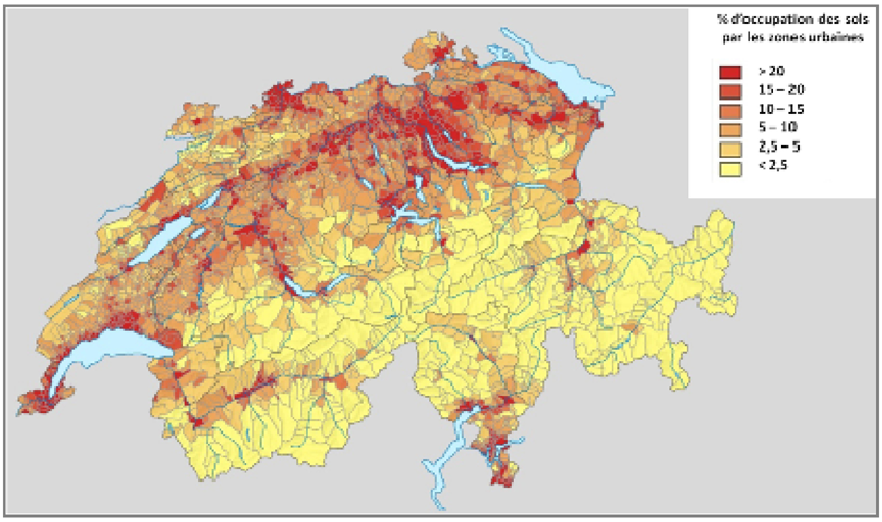
(a)



(b)



(c)



Annexe VI.

Informations concernant l'échantillonnage des bryophytes en Europe destinées à l'analyse des HAP : noms des sites, dates de prélèvement, localisation, coordonnées géographiques et altitude des sites.

Région	Site	Nom	Date	Localisation	Longitude	Latitude	Altitude (m)
Île-de-France	1	F_1	4/10/2010	Forêt Domaniale de Nanteau, 77167 Poligny	48,241389	2,783333	134
	2	F_2	4/10/2010	Forêt Domaniale de Fontainebleau, 77300 Fontainebleau	48,454167	2,700000	84
	3	F_3	4/10/2010	Forêt Domaniale de Jouy, 77160 Chenoise	48,630556	3,183333	152
	4	F_4	4/10/2010	Forêt Domaniale de Malvoisine, 77515 Faremoutiers	48,759167	3,000000	118
	5	F_5	4/10/2010	Forêt Domaniale de Montceaux, 77910 Germigny-l'Evêque	48,967500	2,983333	96
	6	F_6	5/10/2010	Forêt Domaniale de Bois D'Arcy, 78450 Villepreux	48,813611	2,000000	165
	7	F_7	5/10/2010	Forêt des Quatre Piliers, 78610 Saint-Léger-en-Yvelines	48,704167	1,733333	172
	8	F_8	5/10/2010	Forêt Départementale de la Roche Turpin, 91680 Bruyères-le-Châtel	48,620000	2,183333	128
	9	F_9	6/10/2010	95780 La Roche-Guyon	49,090556	1,633333	116
	10	F_10	6/10/2010	Forêt Domaniale de Montmorency, 95570 Attainville	49,032500	2,233333	183
	11	F_11	6/10/2010	95270 Chaumontel	49,143611	2,416667	63
	12	F_12	6/10/2010	Bois de Luzancy, 77410 Villevaudé	48,903333	2,666667	110
	13	F_13	7/10/2010	Forêt Domaniale de Verrières, 91370 Verrières-le-Buisson	48,751667	2,233333	159
	14	F_14	7/10/2010	Forêt Domaniale de Sénart, 91450 Etiolles	48,661667	2,483333	83
	15	F_15	7/10/2010	Forêt Régionale de Gros Bois, 94470 Boissy-Saint-Léger	48,746389	2,533333	92
	16	F_16	7/10/2010	Forêt Régionale de Ferrières, 77090 Collégien	48,811667	2,683333	117
	17	F_17	9/10/2010	Forêt Domaniale de St.Germain-en-Laye, 78100 St.Germain-en-Laye	48,948333	2,066667	43
	18	F_18	10/10/2010	Forêt Domaniale de Meudon, 92370 Chaville	48,799722	2,200000	173

Région		Site	Nom	Date	Localisation	Longitude	Latitude	Altitude (m)
Suisse	Plateau (*Mitteland*)	M2	S_M2	29/08/2010	Forêt de Veytay, Commugny	46,300051	6,153365	427
		M7	S_M7	30/08/2010	Bois du Sépey, Cossonay	46,616544	6,483930	616
		M20	S_M20	30/08/2010	Les Tailles, Payerne	46,796192	6,958987	628
		M24	S_M24	05/09/2010	Altried, Galmwald (Staatswald)	46,920286	7,183772	560
		M32	S_M32	06/09/2010	Könizbergwald, Bern	46,932861	7,405677	620
		M45	S_M45	06/10/2010	Schürhofwald, Aarwangen	47,252494	7,872593	442
		M46	S_M46	05/10/2010	Tannbützen, Ursenbach / Leimiswil (auf Grenze)	47,139070	7,762788	685
		M56	S_M56	06/10/2010	Buech, Staffelbach	47,275074	8,033219	645
		M60	S_M60	07/10/2010	Birch, Egliswil	47,357442	8,191388	565
		M68	S_M68	07/10/2010	Grosshau, Berikon	47,353520	8,404300	650
		M80	S_M80	18/10/2010	Rüti, Zürich	47,366724	8,610417	620
		M88	S_M88	29/09/2010	Brandforen, Aadorf	47,481240	8,887871	606
		M94	S_M94	29/09/2010	Ebni, Hüttlingen	47,568470	8,966941	575
		M98	S_M98	21/09/2010	Frooholz, Birwinken	47,591677	9,207499	560
	Jura (région de Bâle)	J20	S_J20	18/08/2010	Bruederholz, Allme, Therwil	47,505411	7,589683	351
		J24	S_J24	19/08/2010	Chüelenspitz, Möhlin	47,573797	7,865249	325
		P1	S_P1	18/08/2010	Meierhegli, Oberwil	47,525259	7,531317	386
		P2	S_P2	18/08/2010	Zankholz, Kaiseraugst	47,529094	7,754143	378
		P3	S_P3	18/08/2010	Hard, Muttenz	47,544711	7,649567	270
		P4	S_P4	18/08/2010	Metzgerhau, Liestal	47,500133	7,754237	595

Région	Site	Nom	Date	Localisation	Longitude	Latitude	Altitude (m)
Navarre	1	N_1	28/09/2010	Goizueta	43,171769	-1,854140	215
	2	N_2	01/10/2010	Bértiz	43,160572	-1,632878	355
	3	N_3	05/10/2010	Gorramendi	43,225183	-1,454933	625
	4	N_4	05/10/2010	Belate	43,029800	-1,631867	790
	5	N_5	05/10/2010	Egozkue	42,961133	-1,597867	645
	6	N_6	06/10/2010	Roncesvalles	43,000000	-1,332083	1170
	7	N_7	06/10/2010	Lumbier	42,636500	-1,304367	455
	8	N_8	06/10/2010	Nagore	42,850700	-1,385983	620
	9	N_9	07/10/2010	Etxarri - Lizarrusti	42,939583	-2,078633	640
	10	N_10	07/10/2010	Ekai	42,902900	-1,867683	570
	11	N_11	07/10/2010	Ziordia	42,878517	-2,214867	610
	12	N_12	07/10/2010	Gaintza	43,019933	-2,026033	620
	13	N_13	07/10/2010	Beruete	43,040300	-1,842283	905
	14	N_14	14/10/2010	Zúñiga	42,709450	-2,314567	660
	15	N_15	14/10/2010	Monasterio Iranzu	42,770083	-2,053583	840
	16	N_16	15/10/2010	Marañón	42,630917	-2,450600	715
	17	N_17	15/10/2010	Lapoblación	42,606283	-2,465417	950
	18	N_18	15/10/2010	Armañanzas	42,564833	-2,290550	570
	19	N_19	15/10/2010	Sesma	42,513533	-2,149867	495
	20	N_20	16/10/2010	Navascués	42,713367	-1,093433	685
	21	N_21	16/10/2010	Ezkároz	42,636500	-1,304367	940
	22	N_22	16/10/2010	Irati	42,658150	-1,102483	935
	23	N_23	16/10/2010	Zuriza	42,870433	-0,832650	1220

Annexe VII.

Données d’occupation des sols (Source : CORINE Land Cover) – Pourcentage d’occupation par les zones agraires et forestières dans un rayon de 10 km à 50 km autour des sites d’échantillonnage et par les zones urbaines de 10 km à 100 km autour des sites d’échantillonnage en Île-de-France (*sites F*), Suisse (*sites S*) et Navarre (*sites N*).

Site	**AGRAR10**	**AGRAR25**	**AGRAR50**	**FOREST10**	**FOREST25**	**FOREST50**	**URBAN10**	**URBAN25**	**URBAN50**	**URBAN75**	**URBAN100**
F_1	57,426	58,386	63,014	21,782	22,785	18,473	3,960	5,222	5,999	8,064	8,887
F_2	20,792	45,622	57,463	58,086	31,329	17,326	11,221	9,968	13,528	12,330	9,771
F_3	85,149	71,783	63,726	5,941	13,977	16,996	4,290	6,065	7,951	10,478	9,916
F_4	63,696	65,823	57,542	19,142	16,403	16,403	9,241	8,808	15,335	13,432	10,377
F_5	65,347	64,504	57,753	12,871	15,454	17,352	13,531	9,019	14,478	13,854	10,390
F_6	37,954	34,335	48,141	14,851	20,781	15,559	31,023	29,219	23,510	14,838	10,891
F_7	28,383	56,909	59,771	49,505	21,677	13,594	5,941	8,755	15,546	13,690	10,628
F_8	46,535	41,192	50,435	14,851	17,774	16,680	26,073	27,637	22,112	14,018	10,509
F_9	47,195	60,127	56,672	28,053	14,768	17,273	8,911	9,757	11,709	14,018	10,707
F_10	24,422	35,549	45,504	15,512	12,395	16,377	45,875	38,238	24,341	14,838	11,261
F_11	43,894	42,616	49,275	24,752	19,040	16,021	17,162	23,892	21,717	14,604	11,076
F_12	33,993	41,350	47,482	11,551	12,975	16,746	42,574	35,918	24,051	14,627	10,483
F_13	21,782	24,842	45,939	10,231	13,291	17,722	58,416	48,629	24,051	14,744	10,694
F_14	23,432	34,916	47,099	17,162	15,084	18,513	44,884	38,449	23,220	13,924	10,535
F_15	23,432	28,059	44,554	15,842	13,660	19,739	51,485	47,152	24,130	14,135	10,469
F_16	26,403	40,823	47,257	30,693	15,612	18,618	28,713	33,544	23,128	14,276	10,509
F_17	27,723	36,287	44,884	21,452	12,816	17,023	34,983	35,549	24,182	15,143	10,970
F_18	21,122	24,631	45,847	9,241	11,762	16,930	53,135	51,213	24,393	14,838	10,535

Site	AGRAR10	AGRAR25	AGRAR50	FOREST10	FOREST25	FOREST50	URBAN10	URBAN25	URBAN50	URBAN75	URBAN100
S_M2	36,964	30,274	25,870	10,231	32,437	41,021	21,782	11,076	6,355	4,337	3,837
S_M7	70,627	43,249	37,263	28,383	30,802	36,485	3,630	4,483	3,732	3,446	3,468
S_M20	80,528	59,652	44,805	22,112	23,470	29,364	1,980	2,637	3,204	2,696	3,468
S_M24	74,257	60,918	48,813	18,812	25,053	30,986	1,980	4,905	2,782	2,766	3,547
S_M32	58,086	66,139	45,899	32,673	27,057	32,634	11,881	4,641	2,795	3,024	3,850
S_M45	55,776	57,806	49,341	40,594	37,342	37,896	7,591	5,380	7,002	5,602	4,800
S_M46	75,578	61,076	49,565	27,723	37,078	37,816	1,320	3,217	5,076	4,899	4,364
S_M56	57,096	60,074	48,523	40,594	34,441	37,447	6,601	5,802	7,450	5,391	4,918
S_M60	48,515	53,586	48,668	41,584	36,392	37,302	11,221	10,021	7,806	5,579	5,011
S_M68	50,165	53,165	50,211	32,013	30,643	35,575	19,142	13,608	6,949	5,227	5,103
S_M80	33,663	51,846	50,488	27,723	31,276	32,265	32,673	13,608	6,936	4,876	5,037
S_M88	60,396	60,707	48,734	39,934	33,544	29,101	4,290	6,382	6,817	5,860	4,971
S_M94	74,257	56,435	49,117	26,733	29,958	28,600	2,640	6,171	6,870	6,071	5,103
S_M98	63,366	49,156	50,382	16,832	19,831	25,923	7,261	6,593	6,355	5,509	4,918
S_J20	32,013	38,924	43,473	33,993	40,137	39,425	24,752	10,285	7,859	6,564	5,498
S_J24	44,224	35,865	42,840	36,964	46,044	40,730	6,601	9,863	8,347	7,267	5,775
S_P1	37,954	39,926	42,168	22,112	36,709	39,715	28,053	10,759	7,845	6,235	5,591
S_P2	34,323	38,027	43,315	45,875	42,932	40,823	13,861	10,601	7,938	7,243	5,538
S_P3	24,422	39,030	42,774	32,673	39,873	40,322	32,343	10,179	8,004	6,892	5,564
S_P4	36,964	37,869	44,198	47,525	43,882	40,546	13,201	10,812	7,727	7,150	5,512

Site	AGRAR10	AGRAR25	AGRAR50	FOREST10	FOREST25	FOREST50	URBAN10	URBAN25	URBAN50	URBAN75	URBAN100
N_2	16,832	16,350	22,191	43,564	48,998	34,810	0,330	1,002	2,307	1,688	1,661
N_3	13,531	25,264	24,855	35,314	36,603	34,494	0,660	1,424	2,677	1,852	1,912
N_4	17,162	19,040	23,721	70,957	51,319	39,610	0,000	1,582	1,833	1,664	1,648
N_5	25,083	26,055	24,314	62,046	45,833	40,757	0,330	2,215	1,582	1,571	1,714
N_6	10,561	14,346	24,776	64,356	53,586	42,695	0,000	0,369	1,160	1,664	1,925
N_7	49,505	26,530	31,237	18,812	31,435	33,742	0,990	1,319	1,503	1,031	1,437
N_8	15,842	22,890	23,128	50,165	47,363	41,007	0,660	2,268	1,055	1,360	1,899
N_9	14,521	17,932	28,428	62,376	53,903	38,568	0,330	0,475	1,780	1,758	1,582
N_10	24,422	26,793	28,494	45,875	44,673	39,676	0,990	2,004	1,503	1,782	1,543
N_11	12,871	21,361	33,136	66,337	51,108	36,603	0,990	0,633	1,965	1,664	1,477
N_12	12,211	17,194	24,011	61,386	53,956	38,555	0,000	0,527	1,622	1,758	1,622
N_13	17,492	18,829	22,376	59,736	54,219	39,201	0,000	0,475	1,741	1,617	1,675
N_14	28,053	35,338	42,300	53,795	37,922	29,681	0,990	0,949	1,859	1,500	1,345
N_15	19,472	29,378	38,423	46,205	41,297	32,661	0,330	0,844	1,820	1,594	1,437
N_16	41,584	45,306	43,605	32,343	28,745	27,492	0,990	2,268	1,846	1,360	1,319
N_17	50,165	47,890	43,710	26,733	26,477	27,598	0,660	2,215	1,806	1,336	1,358
N_18	48,185	55,063	44,093	11,881	20,570	25,725	1,650	2,426	1,859	1,313	1,305
N_19	70,297	61,023	45,280	5,941	11,392	23,457	1,650	2,479	1,556	1,360	1,319
N_20	3,960	18,196	19,554	53,795	48,681	41,166	0,660	1,319	1,226	0,914	1,767
N_21	49,505	26,530	31,237	18,812	31,435	33,742	0,990	1,319	1,503	1,031	1,437
N_22	7,591	19,937	22,020	47,195	42,880	39,003	0,990	1,319	1,279	0,961	1,701
N_23	2,310	5,907	17,774	60,066	57,911	42,642	0,000	0,105	0,725	1,500	1,846

AGRAR : zones agraires ; FOREST : zones forestières ; URBAN : zones urbaines ; 10 : rayon de 10 km ; 25 : rayon de 25 km ; 50 : rayon de 50 km ; 75 : rayon de 75 km ; 100 : rayon de 100 km (NB : les données n'ont pas été récupérées pour le site N_1).

Annexe VIII.

Pluviométrie annuelle moyenne (en mm) mesurée en Île-de-France (2010), Suisse (2003-2007) et Navarre (2009-2010).

(Sources : MeteoFrance, http://france.meteofrance.com pour les données d'Île-de-France ;
Meteotest, http://www.meteotest.ch pour les données de Suisse ;
Gobierno de Navarra, http://meteo.navarra.es pour la Navarre)

Île-de-France	Precipitations (mm)
F_1	717
F_2	703
F_3	755
F_4	733
F_5	707
F_6	628
F_7	657
F_8	700
F_9	694
F_10	638
F_11	624
F_12	716
F_13	647
F_14	690
F_15	719
F_16	731
F_17	583
F_18	656

Suisse	Precipitations (mm)
S_J20	889
S_J24	990
S_M2	890
S_M7	962
S_M20	890
S_M24	972
S_M32	1011
S_M45	1103
S_M46	1196
S_M56	1075
S_M60	1098
S_M68	1063
S_M80	1214
S_M88	1143
S_M94	951
S_M98	935
S_P1	881
S_P2	914
S_P3	826
S_P4	1005

Navarre	Precipitations (mm)
N_1	1585
N_2	1366
N_3	1843
N_4	1572
N_5	1177
N_6	1545
N_7	733
N_8	793
N_9	1215
N_10	1095
N_11	1199
N_12	1512
N_13	1483
N_14	781
N_15	893
N_16	713
N_17	713
N_18	568
N_19	377
N_20	1053
N_21	1491
N_22	1900
N_23	1362

Annexe IX.

Concentrations en éléments traces métalliques (en µg.g^{-1}, masse sèche) mesurées dans les échantillons d'*Hypnum cupressiforme* Hedw. prélevés en Suisse (*sites S*) et en Navarre (*sites N*) lors de la campagne d'échantillonnage de 2010 du programme PIC-Végétation.

Site	Ag	Al	As	Bi	Cd	Ce	Co	Cr	Cu	Fe	Hg	K	Mg	N	Na	Ni	Pb	S	Se	Ti	V	Zn
S_M2	0,02	287	0,14	0,01	0,16	0,95	0,29	1,20	4,07	243	0,03	2905	1350		51,3	1,68	2,02	699	0,20	11,59	0,94	20,16
S_M20	0,02	301	0,09	0,01	0,06	0,35	0,17	0,97	3,47	286	0,04	7346	1314	9,97	51,4	2,38	1,71	857	0,09	7,18	0,64	12,55
S_M24	0,03	170	0,07	0,01	0,09	0,26	0,25	0,70	3,41	205	0,03	4299	1387		40,2	1,55	1,91	795	0,11	4,60	0,48	17,09
S_M32	0,02	91	0,05	0,01	0,13	0,13	0,13	0,57	3,53	154	0,03	7473	1389		39,4	2,11	1,65	846	0,06	3,30	0,28	19,93
S_M45	0,03	120	0,06	0,02	0,19	0,18	0,15	0,54	3,78	173	0,03	2741	869		52,5	1,11	2,70	578	0,11	3,44	0,46	42,26
S_M46	0,03	119	0,07	0,01	0,20	0,18	0,16	0,46	4,29	143	0,04	2494	805		43,0	1,03	2,05	661	0,11	3,12	0,48	34,87
S_M56	0,02	195	0,10	0,01	0,13	0,31	0,26	0,58	3,90	234	0,03	4206	1064		54,9	1,04	1,96	772	0,13	4,74	0,49	22,19
S_M60	0,03	117	0,06	0,01	0,11	0,16	0,15	0,52	3,23	155	0,03	5171	1387		50,0	1,91	1,80	800	0,09	3,14	0,35	18,19
S_M68	0,03	162	0,07	0,03	0,29	0,23	0,24	0,43	6,65	227	0,03	3729	1134		61,9	1,18	3,17	1005	0,13	4,37	0,50	24,45
S_M7	0,02	287	0,09		0,09	0,28	0,21	0,76	3,52	268	0,03	5726	2030		44,9	5,92	1,91	911	0,09	7,60	0,57	14,99
S_M80	0,02	123	0,07	0,01	0,20	0,20	0,13	0,45	3,73	165	0,02	5755	1441		33,4	1,66	1,70	1007	0,10	3,53	0,34	17,21
S_M88	0,02	135	0,07	0,00	0,12	0,20	0,16	0,34	3,63	169	0,03	5534	1589	12,74	44,2	1,34	1,71	1233	0,08	3,78	0,36	19,23
S_M94	0,02	314	0,14	0,01	0,12	0,40	0,19	0,88	3,59	327	0,03	7582	1866		57,5	5,93	1,56	841	0,11	6,24	0,63	16,41
S_M98	0,04	183	0,11	0,02	0,20	0,28	0,20	0,65	3,33	212	0,03	4711	1483		63,4	1,16	2,17	746	0,13	4,60	0,41	31,33
S_J20	0,06	523	0,20	0,02	0,33	1,17	0,46	1,50	5,01	482	0,03	5282	1106		49,8	3,69	4,13	925	0,21	11,74	1,14	26,78
S_J24	0,07	440	0,22	0,07	0,40	0,71	0,73	1,50	5,91	567	0,03	4878	1713		51,9	2,51	6,53	1104	0,23	13,68	1,07	170,25

Site	Na	K	Mg	Al	Ti	Ca	V	Cr	Mn	Fe	Ni	Cu	Zn	As	Zr	Nb	Mo	Cd	Sb	Pb	Hg
N_1	111,08	3731,40	1223,29	1459,19	23,22	3292,89	2,39	3,31	344,12	1000,91	3,16	6,20	37,85	0,22	0,65	0,10	0,34	0,12	0,09	10,29	0,05
N_2	134,20	3713,32	1360,82	510,99	3,02	5055,52	1,26	1,07	129,92	500,27	1,85	5,02	22,80	0,14	0,17	0,04	0,23	0,08	0,07	3,58	0,05
N_3	289,52	4859,96	1745,47	173,33	2,30	3630,67	0,78	0,43	84,76	170,86	2,97	3,89	20,77	0,10	0,03	0,02	0,18	0,10	0,06	3,30	0,05
N_4	100,66	4087,59	1516,10	556,07	5,83	4812,99	1,57	4,77	253,79	617,16	2,71	4,93	21,47	0,20	0,08	0,02	0,20	0,13	0,09	3,30	0,05
N_5	64,53	3414,55	1220,03	1099,79	5,95	6396,06	2,04	3,51	129,57	1201,71	3,35	4,99	21,86	0,30	0,56	0,04	0,21	0,17	0,05	3,26	0,04
N_6	123,19	3722,55	1093,53	419,08	4,48	4212,00	1,59	1,37	625,04	367,12	1,71	5,99	28,29	0,15	0,01	0,01	0,20	0,17	0,12	7,37	0,06
N_7	80,04	2374,83	906,37	451,30	3,93	7844,37	1,48	1,28	28,03	521,59	1,76	2,87	12,94	0,20	0,15	0,02	0,23	0,19	0,06	1,10	0,04
N_8	67,48	2447,11	892,34	325,55	2,68	5404,85	0,80	0,97	52,18	320,48	1,13	2,64	12,66	0,11	0,07	0,02	0,31	0,10	0,05	1,02	0,02
N_9	95,39	4759,85	1443,89	186,91	2,27	4174,97	0,84	0,56	219,49	202,02	1,41	5,31	29,59	0,11	0,11	0,02	0,25	0,16	0,07	3,62	0,04
N_10	59,78	4330,82	1234,42	331,06	3,68	7235,19	0,91	0,94	30,17	344,60	0,81	4,22	19,53	0,12	0,89	0,09	0,39	0,09	0,07	1,84	0,04
N_11	50,88	4350,89	995,62	588,52	8,17	8880,24	2,21	3,06	45,38	672,63	2,04	5,70	30,22	0,20	0,42	0,04	0,38	0,14	0,08	4,11	0,04
N_12	74,70	4402,08	1464,09	473,32	3,67	6703,31	1,35	1,13	79,51	520,29	1,55	6,43	37,13	0,16	0,25	0,03	0,34	0,15	0,08	5,38	0,06
N_13	57,33	4197,65	1588,48	293,52	3,16	5870,64	1,17	0,73	99,58	267,57	1,52	6,03	28,14	0,13	0,05	0,02	0,24	0,15	0,08	3,85	0,06
N_14	67,57	3124,58	883,56	273,89	2,80	6122,83	0,67	0,59	19,82	235,97	0,61	3,60	18,34	0,10	0,05	0,01	0,22	0,16	0,06	1,82	0,03
N_15	32,93	2753,29	1539,53	1032,58	8,99	7129,47	3,67	2,74	59,74	1121,42	1,87	5,65	29,54	0,37	0,53	0,04	0,36	0,25	0,07	7,39	0,07
N_16	42,40	3472,15	922,58	482,09	4,53	7047,83	0,93	0,94	76,00	445,15	0,87	4,09	18,49	0,16	0,84	0,07	0,26	0,10	0,08	2,61	0,04
N_17	116,04	4722,96	1297,18	732,35	7,75	6546,24	1,71	1,88	83,09	777,43	1,44	7,67	34,37	0,33	0,60	0,05	0,35	0,25	0,08	4,28	0,05
N_18	31,09	3863,38	1399,95	351,17	3,71	6405,47	0,60	1,22	17,49	313,97	0,89	3,86	16,52	0,09	0,30	0,02	0,25	0,09	0,06	1,31	0,02
N_19	38,66	3942,61	1349,57	410,24	4,30	5651,41	0,65	0,73	17,29	343,61	0,58	3,73	18,55	0,13	0,30	0,02	0,37	0,12	0,07	1,14	0,03
N_20	35,36	3245,05	1110,94	377,07	3,55	6544,99	0,73	1,07	32,16	338,44	0,94	2,84	13,56	0,11	0,28	0,01	0,28	0,10	0,05	1,14	0,03
N_21	40,23	3876,64	1268,18	1036,83	4,75	7029,68	1,78	3,82	118,21	1078,08	2,95	5,23	25,79	0,22	0,27	0,02	0,16	0,26	0,04	2,04	0,04
N_22	42,70	4705,26	1186,78	1029,55	3,69	5356,39	1,65	3,00	131,30	826,98	3,94	4,69	17,18	0,19	0,01	0,01	0,13	0,14	0,04	2,74	0,04
N_23	23,47	5030,13	1139,78	740,89	4,27	5198,25	1,32	2,63	73,80	881,36	2,44	5,95	20,55	0,23	0,29	0,02	0,15	0,30	0,04	2,60	0,05

Annexe X.

Teneurs moyennes en NH_3, NO_2 et PM10 dans l'air ambiant en Suisse (en $\mu g.m^{-3}$) : données de 2008-2010.

(*Source : Meteotest, http://www.meteotest.ch/en/*)

Site	**NH₃**			**NO₂**			**PM10**		
	2008	**2009**	**2010**	**2008**	**2009**	**2010**	**2008**	**2009**	**2010**
M 2	0,7	0,8	0,7	17	17	17	16	16	15
M 7	1,5	1,7	1,6	11	11	11	15	14	13
M 20	2,3	2,6	2,4	11	11	11	15	15	14
M 24	1,7	1,9	1,8	11	11	11	15	14	13
M 32	1,6	1,7	1,6	18	18	18	17	17	16
M 45	1,7	1,9	1,8	11	11	11	16	16	15
M 46	3,8	4,1	3,9	10	10	10	15	15	14
M 56	2,5	2,7	2,6	10	10	10	16	16	15
M 60	1,6	1,8	1,7	14	14	14	17	17	16
M 68	1,5	1,7	1,6	12	12	12	17	17	16
M 80	1,0	1,1	1,1	16	16	16	16	16	15
M 88	2,5	2,8	2,6	12	12	12	15	15	14
M 94	2,0	2,2	2,1	12	12	12	15	14	13
M 98	3,2	3,5	3,3	12	12	12	14	13	13
J 20	1,0	1,1	1,1	18	18	18	16	16	15
J 24	1,6	1,8	1,7	14	14	14	15	15	14
P 1	1,2	1,4	1,3	14	14	14	15	15	14
P 2	1,3	1,4	1,3	19	19	19	16	16	15
P 3	1,0	1,1	1,0	21	21	21	18	17	17
P 4	1,7	1,9	1,7	16	16	16	16	16	15

Annexe XI.

Reconstructing historical trends of PAH deposition in a remote area of Spain using moss material from a herbarium

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Reconstructing historical trends of polycyclic aromatic hydrocarbon deposition in a remote area of Spain using herbarium moss material

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ABSTRACT

Herbarium mosses from 1879–1881, 1973–1975 and 2006–2007 were used to investigate the historical changes of atmospheric deposition of polycyclic aromatic hydrocarbons (PAHs) at a remote site in Northern Spain. Natural abundance of nitrogen and carbon isotopes was also measured in order to assess the evolution of emissions from anthropogenic sources. Nitrogen and PAH concentrations as well as $\delta^{13}C$ and $\delta^{15}N$ ratios were significantly higher in 19th century samples compared to present century samples. Moreover, PAH distribution varied over the centuries, with the trend towards enrichment in light PAHs. The carbon, nitrogen and PAH concentrations measured in the mosses tally with the historical evolution of anthropogenic emissions in the area, mainly influenced by changes in economic activities, domestic heating and road traffic density. Mosses provided by herbaria seem to offer the possibility of studying long-term temporal evolution of atmospheric PAH deposition.

1. Introduction

Air quality is generally monitored by physico-chemical detectors giving quantitative data. However, these direct measurements face problems such as low sample representativness, analytical difficulties (trace levels), cost and maintenance of equipment. Biological monitoring is an alternative, consisting of an overall technique that can be used for assessing environmental contamination based on studies of living organisms exposed to pollution episodes. Because of their capacity to act as efficient interceptors and accumulators of chemicals, plants are used widely as passive biomonitors in urban and rural environments (Garrec and Van Haluwyn, 2002), the most common being persistent trees, lichens and mosses (Blasco et al., 2008; Lehndorff and Schwark, 2009a,b; Migaszewski et al., 2009).

Bryophytes in particular have been employed over the past decades as biomonitors for the assessment of airborne pollutant deposition, including heavy metals (Rühling and Tyler, 1969; Tyler, 1990; Harmens et al., 2007), radionuclides (Sumerling, 1984; Sawidis et al., 2009), persistent organic pollutants (Carlberg et al., 1983; Migaszewski et al., 2009) and nitrogen (Solga et al., 2006; Wilson et al., 2008; Poikolainen et al., 2009). The characteristics of mosses make them excellent tools for biomonitoring. Their lack of rooting system plus a well-developed cuticle forces them to obtain most of their nutrient supply from the atmosphere, and their high cationic exchange capacity and surface area to volume ratio favours the accumulation of large amounts of pollutants (Gerdol et al., 2002).

Persistent organic pollutants (POPs) remain in the center of scientific attention due to their slow rate of degradation, their toxicity and their potential for both transport over long distances and bioaccumulation in living organisms (Holoubek et al., 2007). Polycyclic aromatic hydrocarbons (PAHs) are a group of persistent organic pollutants formed by incomplete combustion of organic material by pyrolysis and pyrosynthesis (Kiss et al., 2001) that are emitted into the atmosphere from both anthropogenic and natural sources (Simonich and Hites, 1995). Road traffic, domestic heating and energy production by incineration or natural gas combustion are the main anthropogenic sources of PAH emissions (Mastral et al., 2003). Some of the PAHs are also produced commercially. Acenaphtene, acenaphtylene and anthracene are used in the production of dyes, explosives and fibers, and in the

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pharmaceutical industry (Hawley, 1987). Natural sources are usually volcanic eruptions and biomass burning. Several PAHs are hazardous to living organisms due to their mutagenic, carcinogenic, teratogenic, immunosuppressive and/or neurotoxic properties (Galuszka, 2000) and eight PAHs are classified by US EPA (1997) as possible human carcinogens. Therefore it is important to monitor and regulate the occurrence of PAHs in ambient air.

To further investigate the quantitative importance of different sources of deposition, the use of natural abundance levels of stable isotopes in mosses provides a very effective way of understanding environmental interactions. The isotopic composition of elements such as carbon and nitrogen change in predictable ways as they are cycled through the biosphere, making them ideal tracers of their pathways and origins (Solga et al., 2005; Liu et al., 2008).

Mosses preserved in herbaria should be eminently suitable for determining environmental conditions in the past since they are largely dependent on the atmosphere for their nutrition. It is safe to assume that the concentrations in the herbarium moss samples correlate with atmospheric inputs for the corresponding periods, provided that disruptive factors such as soil particles and leaching processes, sampling contamination and certain preservatives or storage conditions can be excluded (Ross, 1990).

Naturally growing mosses have been extensively employed for studying spatial variations of atmospheric PAHs (Migaszewski et al., 2009), plus temporal variations of these pollutants for periods of up to 10 years (Holoubek et al., 2007). The originality of the present study is to use herbarium moss samples to reconstruct temporal tendencies of PAH deposition over the last 125 years in a Nature Reserve located in Navarra, Northern Spain. Moreover, we propose to investigate the historical changes of natural abundance of nitrogen and carbon isotopes in order to detect the evolution of emissions from anthropogenic sources. The temporal variations were measured using four different mosses in order to study inter-species variability. This study will assess the value of herbarium mosses for reconstructing past PAH deposition trends.

2. Experimental section

2.1. Study area

The “Señorío (Estate) de Bertiz” Nature Reserve is located in the northwest of Navarra (Spain), near the French border. The Park, covering 2040 ha, occupies the eastern side of Bertizarana Valley.

Bertiz has an oceanic climate, with an average annual temperature of 14 °C, not very hot summers, mild winters and little difference between the average monthly maximum and minimum temperatures. This is due to the influence of the Bay of Biscay, only a few kilometres away. Rainfall is one of the highest in Navarra, with an annual average of about 1800 mm, spread throughout the year, but especially between October and May.

The vegetation consists mainly of beech forest (*Fagus sylvatica* L.) with scattered patches of oak trees (*Quercus robur* L.) and pastures.

Due to its ecological richness, the Señorío de Bertiz Park was declared a Nature Reserve in March 1984 (92/43/CEE Directive) and has since become part of the Natura 2000 Network.

2.2. Herbarium specimens

The following species of bryophytes were studied: *Dicranum scoparium* Hedw., *Hypnum cupressiforme* Hedw., *Thamnobryum alopecurum* Hedw. Gangulee and *Thuidium tamariscinum* Hedw. Schimp. The mosses had been conserved in the Herbarium PAMP of the University of Navarra, which houses specimens of bryophytes collected in the Bertiz Nature Reserve since 1879. Samples were separated into three periods: 1879–1881 (Lacoizqueta, 1885), 1973–1975 (Fuertes and Álvarez, 1982) and 2006–2007. A total of 28 samples of the species mentioned above were collected.

Leaves or stem tips were taken from a similar terminal position on each specimen using plastic tweezers. Any extraneous material was removed and the unwashed samples were oven-dried at 30 °C to constant weight (dry weight) and finally ground to a fine powder in a mill.

2.3. Elemental and isotopic determination

Moss samples were analyzed for their carbon and nitrogen content and their respective isotopic signatures ($\delta^{13}C$ and $\delta^{15}N$). Tissue C and N contents (%, dry weight) were determined by an elemental analyzer (EA1108, Carlo Erba, Milano, Italy) with an analytical precision of 0.1%. Calibration was performed using atropine (ThermoQuest Italia, Milan, Italy). Stable C and N isotopic ratios were measured with an isotope ratio mass spectrometer (CONFLO II interface, Thermo Finnigan MAT, Bremen, Germany) operating in continuous flow mode. Calibrations were performed using interspersed international isotopic standards of carbon and nitrogen (IAEA, Vienna, Austria). Isotope data are given as $\delta^{13}C$ and $\delta^{15}N$ values, which represent the relative difference expressed per mil (‰) between the isotopic composition of the sample and that of a standard (Pee Dee Belemnite (PDB) for carbon, and atmospheric N_2 for nitrogen):

$$\delta^{13}C(\text{‰ vs V} - \text{PDB}) = \left(\frac{R_{\text{sample}}}{R_{\text{standard}}} - 1\right) \times 1000$$

$$\delta^{15}N(\text{‰ vs at} - \text{air}) = \left(\frac{R_{\text{sample}}}{R_{\text{standard}}} - 1\right) \times 1000$$

where R_{sample} is the isotope ratio ($^{13}C/^{12}C$) or ($^{15}N/^{14}N$) and R_{standard} the isotope ratio for the standard. The accuracy of the measurements was ±0.15‰ for $\delta^{13}C$ and ±0.3‰ for $\delta^{15}N$.

2.4. Polycyclic aromatic hydrocarbons

2.4.1. Chemicals and standards

A standard mix containing acenaphtene (ACE), fluorene (FLR), phenanthrene (PHE), anthracene (ANT), fluoranthene (FTN), pyrene (PYR), benzo(*a*)anthracene (B(a)A), benzo(*b*)fluoranthene (B(b)F), benzo(*k*)fluoranthene (B(k)F), benzo(*a*)pyrene (B(a)P), dibenzo(*a*,*h*)anthracene (D(ah)A), benzo(*g*,*h*,*i*)perylene (B(ghi)P) and indeno(*c*,*d*)pyrene (IND) at 10 μL mL^{-1} of acetonitrile was used for calibration (PAH Mix 9, Supelco, France). Deuterated PAHs were used as extraction standards (anthracene d_{10} and benzo(*a*)pyrene d_{12} at 300 ng mL^{-1} of acetonitrile) and as an internal standard (fluoranthene d_{10} at 1000 ng mL^{-1} of acetonitrile) (Supelco, France). Anthracene d_{10} was chosen as a reference for the light PAH fraction (ACE to PYR) and benzo(*a*)pyrene d_{12} for the heavy PAH fraction (B(a)A to IND).

All solvents were HPLC grade: *n*-hexane and acetonitrile were provided by VWR Scharlau (Spain), cyclohexane and dichloromethane by VMR Merck (France) and Milli-Q water by VWR Millipore (France).

2.4.2. Extraction and clean-up procedure

In order to determine the PAHs in mosses by High Pressure Liquid Chromatography coupled with fluorescence detection (HPLC-FLD), Soxtec extraction and Solid Phase Extraction (SPE) clean-up were carried out.

The extractions were performed with a Soxtec System HT2 (Tecator, France). Cellulose thimbles were filled with approximately

1 g of spiked moss, anhydrous sodium sulfate and Florisil (U.S. Silica Company, USA). The extract was concentrated with a rotary evaporator at 30 °C (Rotavapor, Büchi, Switzerland), then by nitrogen flow (Alpha 1, Air Liquide, France). A clean-up procedure was carried out with normal phase Florisil SPE cartridges (Supelclean LC-Florisil SPE 1 g, Supelco, France) placed on a Supelco manifold. After solvent exchange, the samples were filtered and an internal standard was added. The extracts were stored at −20 °C.

The overall recoveries of the extraction/purification procedure range from 25% to 71% with an average of 54% for light PAHs (ACE to PYR) and from 27% to 79% with an average of 60% for heavy PAHs (B(a)A to IND).

2.4.3. *HPLC-FLD analysis*

The PAH analyses were performed on a reversed-phase high-performance liquid chromatograph DIONEX RF 2000 (Pump P680 HPLC) (Dionex, USA) equipped with a C18 column Supelcosil™ LC-PAH (250 mm × 4.6 mm × 5 μm) and a precolumn (20 mm × 4.6 mm × 5 μm) (Supelco, France) plus a fluorescence detector (DIONEX RF 2000, Dionex, USA) at a flow rate of 1.5 mL min^{-1} with a water/acetonitrile elution gradient. Analytical parameter details are given in Table 1.

The linearity range for the PAH analysis extends from 0 to 58 ng mL^{-1} with regression coefficients from 0.9992 (B(k)F) to 0.9999 (ANT and B(a)A).

To study the repeatability of the analysis, a standard solution containing low PAH concentrations (2 ng mL^{-1}) was injected 10 times. The standard deviation, determined for each PAH (from 3% for ACE to 14% for PHE), was lower than 20%.

The reproducibility of the method was tested by repeating the entire extraction–purification-analysis procedure 7 times using a recent (collected October 2008) moss sample. The results are shown in Table 2. The standard deviation determined for each PAH was lower than 20% except for ACE, FLR, D(ah)A and B(ghi)P with a maximum of 42% for FLR.

Limits of detection (LOD) and limits of quantification (LOQ) were determined by studying the chromatogram obtained with a 2 ng mL^{-1} standard solution. The LOD was calculated as equal to 3 times the background and the LOQ as 10 times the background (Tranchant, 1995). The limits of quantification are shown in Table 1.

Finally, the accuracy of the analytical procedure was verified by applying the whole extraction–purification-analysis routine 5 times to an organic contamination reference material (IAEA-140OC Fucus (35 g) from ANALAB) containing 9 of the 13 target PAHs. All PAHs measured were included in the reference concentration ranges (95% confidence intervals) except for PYR, overestimated because of interferences with other reference molecules (PCBs or pesticides).

2.5. *Statistical analysis*

One-way analysis of variance (ANOVA) was performed to determine the effects of time and species on the variables. Pearson's correlation coefficients were calculated to examine relationships between the different variables. The statistical analysis was conducted using SPSS 15.0 (SPSS Science, Chicago, USA) and XLSTAT 2008 (Addinsoft, Paris, France).

3. Results

3.1. *Elemental analysis and isotopic determination*

3.1.1. *Nitrogen and carbon concentrations*

Tissue nitrogen concentrations in the herbarium specimens ranged from 0.8% to 1.8%. Nitrogen concentrations were significantly higher ($p < 0.05$) in the 19th century than in the present century (Fig. 1).

The *T. alopecurum* moss had the highest nitrogen concentrations, giving significant differences ($p < 0.05$) with the other species.

For carbon, concentrations ranged from 30.9% to 43.9% and here, values did not show significant differences between species or centuries.

3.1.2. *Nitrogen and carbon isotope signatures*

Mean $\delta^{13}C$ and $\delta^{15}N$ values for the moss species studied ranged from −30.5‰ to −25.1‰ and from −9.3‰ to 0.6‰ respectively. The average $\delta^{13}C$ showed a significant ($p < 0.05$) decrease over the three centuries studied, while in the case of $\delta^{15}N$ only mosses collected in the 19th century gave values significantly higher ($p < 0.05$) than those collected in the 21st century (Fig. 2).

No significant differences were observed in $\delta^{13}C$ values between the four moss species studied. However, in the case of $\delta^{15}N$, significant differences were detected between *D. scoparium* and *T. alopecurum*, which had the lowest and highest $\delta^{15}N$ values respectively.

Table 1

Analytical parameters for PAH analysis by HPLC/FLD. Excitation (Exc. λ) and emission (Em. λ) wavelengths were chosen according to the analyzed compounds. The limits of quantification (LOQ) are expressed as mass of injected compound (pg). As a rough guide, the average equivalent concentrations in moss (ng g^{-1} dry weight) have been calculated (for 1 g of moss sample and using the average PAH recoveries).

Time window	Time (min)	Exc. λ (nm)	Em. λ (nm)	Compounds	Retention time (min)	LOQ (pg)	Moss average LOQ (*ng* g^{-1} DW)
1	8.0	233	320	ACE	9.2	22	2.2
				FLR	9.7	52	5.3
2	10.5	260	380	PHE	11.4	27	2.8
3	12.0	250	375	ANT-d$_{10}$	12.5		
				ANT	13.1	4	0.4
4	13.8	365	462	FTN-d$_{10}$	14.3		
				FTN	14.9	28	2.8
5	15.6	275	380	PYR	16.4	13	1.3
6	18.0	270	300	B(a)A	21.3	15	1.4
7	22.1	302	431	B(b)F	26.5	6	0.6
				B(k)F	28.4	3	0.3
				B(a)P-d$_{12}$	29.0		
				B(a)P	30.0	12	1.1
				D(ah)A	32.3	7	0.6
8	33.4	302	419	B(ghi)P	33.9	6	0.6
9	34.8	300	500	IND	35.8	22	2.1

Table 2
Mean and median concentrations (ng g^{-1} dry weight) and corresponding standard deviations (SD) of polycyclic aromatic hydrocarbons obtained from the same sample ($n = 7$) of *Hypnum cupressiforme* collected at Bertiz in 2008. The results were obtained by repeating the extraction/purification/analysis procedure 7 times on the moss sample.

PAHs	Concentrations (ng g^{-1} DW)		
	Mean	Median	SD
Acenaphtene (ACE)	2.8	3.1	0.9
Fluorene (FLR)	11.1	8.9	4.6
Phenanthrene (PHE)	36.1	35.5	1.9
Anthracene (ANT)	2.2	2.1	0.3
Fluoranthene (FTN)	11.5	11.1	2.0
Pyrene (PYR)	10.8	10.2	1.5
Benzo[a]anthracene (B(a)A)	2.7	2.7	0.5
Benzo[b]fluoranthene (B(b)F)	5.5	5.3	0.6
Benzo[k]fluoranthene (B(k)F)	1.8	1.7	0.3
Benzo[a]pyrene (B(a)P)	2.7	2.7	0.4
Dibenzo[a.h]anthracene (D(ah)A)	1.4	1.1	0.5
Benzo[g.h.i]perylene (B(ghi)P)	8.1	7.5	3.2
Indeno[1.2.3-cd]pyrene (IND)	3.0	3.0	0.3

3.2. PAH concentrations

Except for anthracene and dibenzo(*ah*)anthracene contents which remained low, all PAH concentrations significantly decreased in the mosses between 1879–1881 and 1973–1975 ($p < 0.01$). Only concentrations of the heavy PAHs benzo(*b*)fluoranthene, benzo(*k*)fluoranthene, benzo(*a*)pyrene, benzo(*g*,*h*,*i*)perylene and indeno(*c*,*d*)pyrene significantly decreased between 1973–1975 and 2006–2007 ($p < 0.01$) (Table 3).

As a consequence, total PAH contents decreased significantly between 1879–1881 and 1973–1975 ($p < 0.01$) but not between the 1973–1975 and 2006–2007. The average total of the 13 PAHs was 172 ng g^{-1} (dry weight) for the present century samples compared to 1219 ng g^{-1} (dry weight) for the 130-year-old samples.

Phenanthrene was the highest of the PAH concentrations regardless of moss age. It appears to be the major compound, as its accumulation accounted for 47% of the total PAHs measured in the 2006–2007 samples and this percentage increased over time (24% and 39% respectively in the 1879–1881 and 1973–1975 samples).

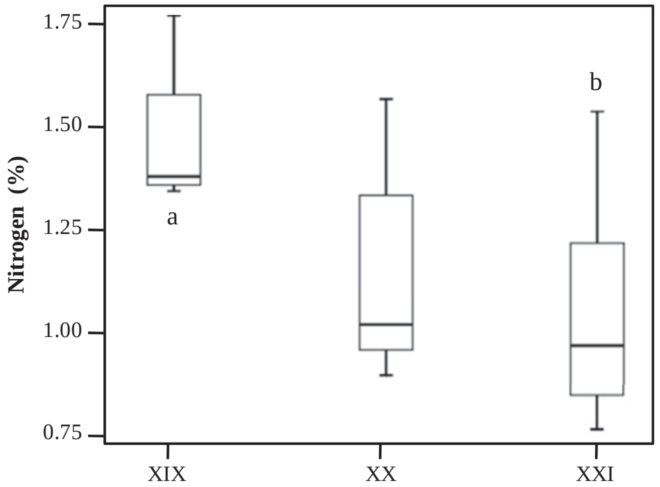

Fig. 1. Temporal variation of nitrogen concentrations (% dry weight) in mosses from Bertiz Nature Reserve. The nitrogen content is shown for the 3 sampling periods: 1879–1881 (XIXth century), 1973–1975 (XXth century) and 2006–2007 (XXIst century). The boundary of the box indicates the 25th and 75th percentile and the line within the box the median. Boxes with different letters are significantly different at the $P < 0.05$ level.

Fluoranthene and pyrene are the other major compounds measured in Bertiz mosses accounting for 33 to 39% of the total PAHs.

Heavy PAHs (benzo(*a*)anthracene to indeno(*c*,*d*)pyrene) accounted for approximately half of the total PAH contents in 19th century mosses, but appeared to be minor compounds in the more recent samples (12%), with individual concentrations of less than 6 ng g^{-1} (dry weight).

Three groups of PAHs appeared to be positively correlated ($p < 0.01$, $R^2 = [0.913–0.978]$)

- acenaphtene and fluorene (2-benzene ring PAHs).
- phenanthrene, fluoranthene and pyrene (3-benzene ring PAHs).
- benzo(*a*)anthracene, benzo(b)fluoranthene, benzo(*k*)fluoranthene, benzo(*a*)pyrene and indeno(*c*,*d*)pyrene (4- and 5-benzene ring PAHs).

The individual and total PAH concentrations measured for the Bertiz mosses did not show significant inter-species variations.

4. Discussion

4.1. Isotopic signatures and atmospheric N, C sources

In contrast with other studies (Peñuelas and Filella, 2001; Solga et al., 2006; Wilson et al., 2008), N concentrations in mosses collected at Bertiz significantly decreased ($P < 0{,}05$) between the 19th and 21st century, and a probable explanation may be found in the 19th century history of the Señorío de Bertiz. At that time, wood burning stoves were the main means of heating houses and the siting of charcoal pits within the forest was common, in order to obtain charcoal to feed the numerous foundries in the surrounding valleys (Erdozáin and Mikelarena, 2002). All these activities emitted high amounts of NOx and consequently, it is very likely that a remote place such as Bertiz was more contaminated in the past than nowadays. As these activities decreased (wood burning) or disappeared (charcoal pits and foundries) at the end of the 19th century, the present lower N concentrations would seem logical.

Moreover, Peñuelas and Filella (2001) suggested that the decrease in foliar N from 1940–1950 to 1960–1970 observed in vascular plants and mosses collected in Spain was the result of increased global CO$_2$ concentrations. Thus, the pattern of N decrease observed in this study could also be explained by a dilution through growth process, as a response to a large increase in CO$_2$ availability.

The highest N concentrations found in *T. alopecurum* could be explained by the different life-form of this bryophyte with respect to the others (Mägdefrau, 1982; Dierssen, 2001). *Thamnobryum* is a hygrophytic moss that grows on rocks by streams and rivers, just above mean water level. Thus, this species is sometimes in contact with surface water, which is much richer in N than rainwater.

The other three moss species are mesophytic, growing on forest soils. Here, the main water source is precipitation, and for *Dicranum scoparium*, the species with the least ability to retain water, this would explain their lower tissue N concentrations. For *T. tamariscinum* and *H. cupressiforme*, both mosses have a weft life-form that allows them to hold considerable quantities of rainwater by capillarity action, thus showing intermediate N concentrations.

Concerning δ^{15}N, isotopic signatures measured in this study were substantially lower than values found by Peñuelas and Filella (2001), but similar to those recorded by Solga et al. (2006) and Liu et al. (2008).

As mentioned above, the highest δ^{15}N values were detected in *T. alopecurum*, while *D. scoparium* showed the most negative

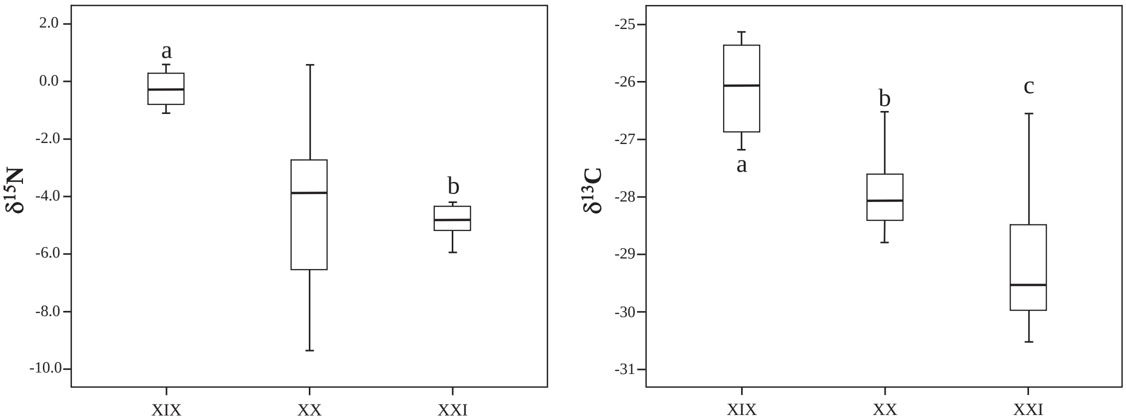

Fig. 2. Temporal variation of $\delta^{13}C$ (‰) and $\delta^{15}N$ (‰) in mosses from Bertiz Nature Reserve. The isotope signatures are shown for the 3 sampling periods: 1879–1881 (XIXth century), 1973–1975 (XXth century) and 2006–2007 (XXIst century). The boundary of the box indicates the 25th and 75th percentile and the line within the box the median. Boxes with different letters are significantly different at the $P < 0.05$ level.

signatures. These findings may also be related to their ecological life-forms. It is known that N absorbed from rain is depleted in ^{15}N, while N taken from surface water is enriched in the heavy isotope (Heaton et al., 1997; Asada et al., 2005). Thus, the different water sources for these species would explain the $\delta^{15}N$ values observed.

A clear trend towards ^{15}N depletion was observed, which agrees with Peñuelas and Estiarte (1997), who observed a $\delta^{15}N$ decline in moss over the past century that was attributed to an increase over time of the uptake of anthropogenic N, which is depleted in ^{15}N. This pattern may be related to the increase of N inputs in the reduced form, which is consistent with the gradual intensification of agriculture in the study area.

Table 3
Means and ranges of PAH concentrations (ng g^{-1} dry weight) in mosses sampled in Bertiz Nature Reserve during the 19th, 20th and 21st centuries. The table indicates the individual PAH concentrations and the total PAH concentration (ΣPAHs) for each sampling period.

Sampling period	1879–1881	1973–1975	2006–2007
Means and observed ranges (ng g^{-1})			
Acenaphtene (ACE)	39.0	2.5	4.1
	12.2–62.4	<1.5–3.3	<1.5–12.7
Fluorene (FLR)	85.5	13.9	15.1
	37.9–143.2	<10.4–18.7	<10.4–21.3
Phenanthrene (PHE)	298.8	127.9	81.1
	118.9–622.0	98.6–174.3	26.9–142.2
Anthracene (ANT)	6.2	4.7	3.2
	2.9–9.4	3.2–9.6	1.2–9.9
Fluoranthene (FTN)	252.2	65.4	38.1
	145.5–466.4	32.7–123.0	10.2–152.7
Pyrene (PYR)	220.9	44.8	18.5
	127.4–436.7	29.1–66.4	6.8–39.0
Benzo(*a*)anthracene (B(a)A)	97.0	8.3	3.1
	66.9–120.1	4.2–11.9	<1.2–7.0
Benzo(*b*)fluoranthene (B(b)F)	86.8	18.8	3.0
	65.6–95.5	9.3–40.9	1.8–5.5
Benzo(*k*)fluoranthene (B(k)F)	40.6	6.6	0.8
	29.6–49.1	3.2–14.3	<0.5–1.8
Benzo(*a*)pyrene (B(a)P)	31.6	9.7	2.4
	28.5–34.8	<1.4–17.8	<1.4–1.7
Dibenzo(*a,h*)anthracene (D(ah)A)	3.6	1.8	4.2
	3.0–4.6	<1.3–3.3	<1.3–7.8
Benzo(*g,h,i*)perylene (B(ghi)P)	31.2	18.5	5.6
	21.7–55.1	10.9–35.3	2.0–16.1
Indeno(*c,d*)pyrene (IND)	25.2	9.9	2.0
	17.5–35.0	5.8–21.0	<2.0–2.5
ΣPAHs	1218.6	328.2	171.8
	782.8–2009.1	206.1–464.6	86.0–372.5

Likewise, Turekian et al. (1998) have shown that the combustion of organic material such as vegetation, produces aerosol particulate material and ashes higher in $\delta^{15}N$ than the source vegetation, material which can be retained by mosses. This could explain the significantly higher values of $\delta^{15}N$ recorded in Bertiz during the 19th century, when burning beech branches (in charcoal pits) was a frequent activity in the forest, endorsing the idea that N fractionation is a good indicator for the isotopic characterization of combustion-derived nitrogenous material.

In spite of the results obtained, it is obvious that the atmospheric concentrations of reactive N have significantly increased all over the world since the Industrial Revolution. In fact, the Ellenberg ecological values calculated from 187 species of mosses collected in Bertiz since 1879, suggest an increase of anthropogenic nitrogen in this area (Ederra and Villaroya, 2009). This means that several factors, such as the local influence of charcoal pits and foundries mentioned above, the increased growth and biomass production of mosses or the seasonal variations of N tissue concentrations, could mask the real increase of anthropogenic N deposition.

With respect to $\delta^{13}C$, values were in agreement with the range of C_3 plants (Liu et al., 2008). The decreasing trend of $\delta^{13}C$ observed over time was consistent with the depletion of ^{13}C detected in the atmosphere over the last centuries due to the increase of anthropogenic emissions, as well as the fractionation by mosses against the heavy isotope of carbon (Fletcher et al., 2006).

4.2. *Temporal variations of PAH concentrations in the mosses*

Bertiz mosses sampled in 2006–2007 gave total PAH concentrations of the same order as other passive biomonitoring studies carried out on moss over the past decade in remote areas of Austria (Zechmeister et al., 2006; Krommer et al., 2007) and the Czech Republic (Holoubek et al., 2007). PAH distribution was also similar, with phenanthrene, fluoranthene and pyrene as major contaminants. Moreover, Holoubek et al. (2000, 2007) observed a significant decrease of total PAH concentrations between mosses from 1988–1994 and 1996–2005, which confirms the temporal trend of PAH depletion in Bertiz mosses.

The significant decrease of individual PAH concentrations since 1879 ($p < 0.01$) can be explained by the evolution of the emissions in the area over the past centuries. During the 19th century, charcoal production in the charcoal pits within the forest, charcoal burning in the local foundries and wood burning in stoves for domestic heating were activities that emitted significant amounts of PAHs as they involved biomass burning under sub-optimum combustion conditions (Freeman and Catell, 1990). Thus, PAH emissions dropped significantly during the 20th century due to the abandoning of charcoal associated activities and the evolution of domestic heating towards systems powered by oil or gas, which emit less PAHs. Indeed, Smith (1987) estimated that PAH emissions from wood combustion in small-space heating stoves exceed those from furnace-oil combustion by a factor of 100 on an energy equivalent basis and Rogge et al. (1993b) showed that home gas appliances have low total fine particulate organic carbon mass emission rates.

The significant decrease of heavy PAHs (4, 5 and 6-benzene rings) between the 1973–1975 and 2006–2007 mosses ($p < 0.01$) should be seen in the context of political measures to limit POP emissions following the signature of the Geneva Convention on Long-range Transboundary Air Pollution in 1979. In particular, the most toxic compounds, benzo(*b*)fluoranthene, benzo(*k*)flouranthene, benzo(*a*)pyrene and indeno(c,d)pyrene were regulated by the Aarhus Protocol (1998). Thus, their emissions were reduced by 56% in France between 1991 and 2007 (CITEPA, 2009) due to the control of industrial emissions, the fitting of catalytic converters to road transport vehicles since 1990, and the recent efficiency improvement of biomass combustion heating systems. Rogge et al. (1993a) showed for example that the total PAH emission rate was 25 times lower for vehicles fitted with catalytic converters. The simultaneous emission limitation of these heavy PAHs may explain why their concentrations are significantly correlated ($p < 0.01$).

Phenanthrene, fluoranthene and pyrene (3-benzene ring PAHs), whose concentrations were significantly correlated ($p < 0.01$), were the major contributors to PAH contamination in Bertiz mosses over the centuries. These compounds are the main PAHs emitted during wood combustion (Khalili et al., 1995; Smith and Harrison, 1998). Thus, the very high concentrations of each of these PAHs (>200 ng g^{-1} dry weight) in the 1879–1881 samples and their significant decrease in the 1973–1975 samples can be explained by the charcoal associated activities and the dominance of domestic heating with wood stoves in the area during the 19th century.

However, in mosses from the 20th and 21st century, phenanthrene, fluoranthene and pyrene remained the major contributors to PAH contamination probably due to the presence of local traffic, in particular the proximity of the main Pamplona-Irun road (N-121A). Indeed, these three compounds are markers of traffic emissions since diesel emission has a high factor loading for phenanthrene, fluoranthene and pyrene and petrol emission for fluoranthene and pyrene (Duval and Friedlander, 1981; Masclet et al., 1986; Khalili et al., 1995; Ho et al., 2002; Omar et al., 2002; Orliński, 2002; Ravindra et al., 2006).

The fact that phenanthrene concentrations in moss increased relatively to fluoranthene and pyrene between the 20th and 21st centuries suggests that diesel-engined traffic is predominant nowadays, since petrol emissions contain relatively low phenanthrene concentrations. Moreover, Marr et al. (1999) showed that light vehicles contribute significantly to 4- and 5-benzene ring PAHs, whereas heavy vehicles, which are diesel powered, are the main source of 3-benzene ring PAHs, i.e., phenanthrene, fluoranthene and pyrene. Thus, the high density of trucks travelling between France and Pamplona (Navarra's main city) appears to be the main PAH contamination source for current Bertiz moss.

4.3. Limits of the method

Passive air quality biomonitoring, i.e., with specimens sampled in-situ, is subject to disruptive factors due to species variability and environmental conditions, particularly meteorological conditions and altitude (Niu et al., 2003; Liu et al., 2005). Moreover, bryophytes can be contaminated by the soil, through wind re-suspension or volatilization of light PAHs, and can lose PAHs through precipitation leaching (Steinnes et al., 1992).

For this study, the number of herbarium samples available was limited and the total quantity of each sample was low. In addition, the only data available on the samples was their age. Hence, no inter-species variability was observed for PAHs. Yet moss morphology and growth conditions appear to have an influence on their contaminant accumulation as was shown for nitrogen stable isotopes in this study and for PAHs in other studies. For example Galuszka (2007) observed higher PAH accumulation in *Hylocomium splendens* than in *Pleurozium schreberi* moss species.

For a more thorough statistical interpretation, more information would be required concerning the exact sampling localisation, dating and meteorological conditions as well as the storage conditions (temperature, preservatives…).

5. Conclusion

This study has assessed the value of herbarium moss samples as biomonitors of atmospheric PAH deposition at a remote Spanish site. The high concentrations of PAHs and N and the high $\delta^{15}N$ values in 1879–1881 mosses of Bertiz Nature Reserve appear to be related to the historical production of charcoal for local foundries in charcoal pits located in the forest and to the exclusive use of wood stoves for domestic heating, before the 20th century. The disappearance of the charcoal pits and foundries at the end of the 19th century, combined with evolution of domestic heating towards less polluting systems during the 20th century, explain the significant PAH, N and ^{15}N depletion in the 1973–1975 mosses. Between the 1973–1975 and 2006–2007 mosses, PAH distribution changed noticeably with a tendency towards 3-benzene ring PAH enrichment, due to political measures limiting 4- and 5-benzene ring PAH emissions (Geneva Convention, 1979; Aarhus Protocol, 1998), and steadily increasing traffic in the area, especially heavy vehicles, confirmed by a significant $\delta^{13}C$ decrease. Thus, in this study, knowledge of nitrogen and carbon signatures in Bertiz mosses helped to identify the evolution of anthropogenic emissions in the area and also explained the evolution of PAH deposition since the 19th century.

Herbarium moss analysis is limited by low sample availability and poor data on sampling and storage. Thus, no evidence of PAH accumulation variability between species could be shown in this study. However, relevant information was obtained on past PAH deposition at Bertiz and it was consistent with the measured carbon and nitrogen signatures. Therefore, herbarium moss samples appear to be an effective tool for reconstructing historical tendencies of atmospheric PAH deposition.

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Annexe XII.

Polycyclic aromatic hydrocarbons (PAHs) in remote bulk and throughfall deposition: Seasonal and spatial trends

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POLYCYCLIC AROMATIC HYDROCARBONS (PAHs) IN REMOTE BULK AND THROUGHFALL DEPOSITION: SEASONAL AND SPATIAL TRENDS

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) were measured in atmospheric deposition in a Nature Park located in the North of Spain over a period of one year (June 2010–May 2011). Total PAH deposition was evaluated monthly by combining samples collected over two-week periods, using 6 throughfall and 2 bulk precipitation collectors distributed over the study area. The latter consisted of glass funnels (with vertical sides) attached to Pyrex glass bottles. PAH determination was performed by liquid-liquid extractions and analysis by high-performance liquid chromatography associated with fluorescence detection. Throughfall deposition revealed a loss under the canopy of 12% of the total atmospheric PAH deposition due to precipitation interception. Spatial variability between the 6 throughfall collectors reached a maximum of 25% during the wet season. Temporal trends showed significant variations of 27 to 54% throughout the year. PAH deposition increased during winter, due to higher emissions from domestic heating, less photoreactivity of the compounds and intense leaching of the atmosphere by wet deposition. Average daily fluxes were estimated for 6 quantified PAHs (PHE, ANT, PYR, B(b)F, B(k)F and B(a)P) at 182 ± 27 ng m^{-2} d^{-1}, which agreed with studies previously carried out in other local rural areas. The major compounds were phenanthrene and pyrene, both markers of traffic emissions.

Key words: annual flux, bulk deposition, polycyclic aromatic hydrocarbon, seasonal trend, throughfall deposition

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1. Introduction

Persistent organic pollutants (POPs) are organic substances that possess toxic characteristics, are persistent, bioaccumulate, are prone to long-range transboundary atmospheric transport and deposition and are likely to cause significant adverse human health or environmental effects near to and removed from their source (UNECE, 1998). They are mainly of anthropogenic origin, show weak degradability and consequently accumulate in the environment worldwide, including in such remote areas as the North and South Poles. The combination of resistance to metabolism and lipophilicity means that POPs will accumulate in foodchains (Jones and de Voogt, 1999). The 1998 Aarhus Protocol on POPs (LRTAP Convention) and the 2001 Stockholm Convention on POPs - a global treaty under the United Nations Environment Programme (UNEP) - aim to eliminate and/or restrict the production and use of selected POPs.

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Polycyclic aromatic hydrocarbons (PAHs) are a family of chemical compounds composed of carbon and hydrogen atoms which form at least two condensed aromatic rings. PAHs are considered as POPs due to their low rates of degradation, toxicity and potential for both long-range transport and bioaccumulation in living organisms (Holoubek et al., 2007). Regulation of PAH emissions and reliable monitoring of PAH concentration in ambient air is thus of paramount importance for public health.

PAHs originate from fossil or non-fossil fuels by pyrolysis or pyrosynthesis. PAHs are emitted into the atmosphere mainly from anthropogenic sources but they also originate from natural ones such as volcanic eruptions and forest fires (Simonich and Hites, 1995). The main sources of PAHs in the environment are aluminium production, coke production from coal, wood preservation and fossil fuel combustion (traffic, domestic heating, electricity production) (Wegener et al., 1992).

PAHs are transported in the atmosphere in two different forms depending on their physical properties: either adsorbed on the particle phase (aerosols) or integrated into the gaseous phase. Light PAHs, which have high saturated vapour pressures, are volatile and occur mainly in the gaseous phase of the atmosphere. Heavy PAHs, tend to adsorb on particulate matter (PM) (Odabasi et al., 1999). The biosphere intercepts these contaminants during atmospheric deposition: wet deposition (e.g. rain, fog, or snow) and dry deposition (gases and aerosols). Each type of deposition contains relatively different PAH levels as a function of their physical properties: volatility, solubility and lipophilicity (Dabestani and Ivanov, 1999). Therefore, to evaluate the impact of total atmospheric deposition of PAHs on ecosystems, total (dry+wet) deposition is studied. Previous studies, performed exclusively on bulk deposition, revealed average daily PAH fluxes ranging from 17 ng m^{-2} d^{-1} in a remote mountainous area (Alps, France) (Fernández et al., 2003) to 19.5 μg m^{-2} d^{-1} in an industrial zone (Bursa, Turkey) (Esen et al., 2008).

The present study was carried out in a Nature Park in the North of Spain to estimate the annual atmospheric PAH deposition and to identify the emission sources. PAH fluxes were evaluated monthly between June 2010 and May 2011. The seasonal trends in a covered and uncovered zone were studied over the whole year and the spatial variability under the canopy for two periods of the year (summer, autumn).

2. Experimental

2.1. Sampling procedure

2.1.1. Study area

The Nature Park of Bertiz (43.14°N, 1.61°W) is located in the Northwest of Navarra (Spain), near the French border (Fig. 1).

Fig. 1. Situation of Bertiz Nature Park

The Park occupies the eastern side of the Bertizarana Valley, covering 2,040 hectares. Bertiz has an oceanic climate, with average annual temperatures of 13.7-15.0ºC, reasonably hot summers, mild winters and low monthly maximum and minimum temperature amplitudes. This is due to the influence of the Bay of Biscay, only a few kilometres away. The rainfall is one of the highest in Navarra, with an approximate annual average of 1530 mm distributed throughout the year, but specially between October and May (Navarra Government, 2011). Vegetation is mainly beech forest (*Fagus sylvatica* L.), although there are some patches of oak woods (*Quercus robur* L.) and a few pastures. Due to its ecological richness, Bertiz was declared a Nature Park in March 1984 (92/43/CEE Directive) and is currently part of the Natura 2000 Network (Navarra Government, 2007).

2.1.2. Sampling site

The devices were set up in two intensive plots within the Bertiz ES02 catchment, which is part of the ICP IM network. This International Cooperative Programme, part of the UNECE Convention on Long Range Transboundary Air Pollution, aims to determine and predict the state and change of terrestrial and freshwater ecosystems over the long-term with regard to the impact of a number of processes, among which air pollution plays a pivotal role. The term ‘intensive plot’ in the framework of the Programme, refers to a site that has been fitted with equipment devoted to simultaneously monitor different compartments of the ecosystem. A total of 8 collectors were distributed over the study area: 6 of them were laid out in the intensive plot situated under the beech forest canopy and the 2 remaining collectors in a pasture area by the forest. The high number of collectors below the forest canopy was because of the variability in throughfall volume and composition, whereas the two parallel gauges were considered to be enough to compensate for that variability in bulk samples (Thimonier, 1998).

2.1.3. Sampling equipment and protocol

Total deposition collectors were specially manufactured for the study. The collection device consisted of glass funnels (I.D. 11.8 cm, with vertical sides of 25 cm) adapted to 2 L glass bottles (Fig. 2a). Teflon tubes (I.D. 5 mm) were inserted in the bottom of the funnels to limit water evaporation during sampling. In order to avoid litter (i.e. leaves, branches, or insects) entering the collection bottles, perforated Teflon® plates attached to stainless steel hooks were placed in the funnels (Fig. 2b). The collectors were laid out on stainless steel supports at a height of 1 m above the ground to avoid contamination from the soil (Fig. 2c). As PAHs are photoreactive, the collection bottles were covered with aluminium foil and enclosed within supporting PVC in order to shield them from sunlight. Furthermore, to keep the samples cool, polystyrene cylinders were inserted between the collection devices and the PVC tubes. Finally, stainless steel rings were fitted around the PVC tubes to stop birds from landing on the collection devices and contaminating the samples with excrement.

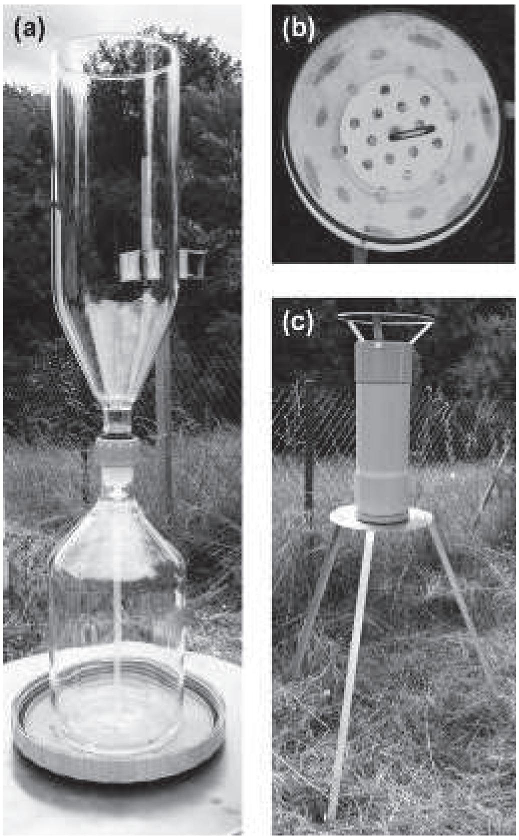

Fig. 2. Total deposition collector manufactured to estimate atmospheric deposition of polycyclic aromatic hydrocarbons: (a) Glass funnel and collection bottle (b) Perforated Teflon® plates for litter recuperation (c) Stainless steel collector support and PVC tube for protection against sunlight

All glass material for field or laboratory use was washed for at least 1 h in an ultrasonic bath with a high pH detergent (Labwash Universal, VWR BDH Prolabo, Radnor, PA, USA) diluted to 5% v/v) in milli-Q water (Millipore, Billerica, MA, USA). Afterwards, the material was rinsed several times with tap water and milli-Q water, dried in a clean oven at 80 °C and then stored in clean cupboards in aluminum foil. Polyethylene bottles (PE) were not cleaned with the detergent as they were new when used. Before use in the laboratory, the material was rinsed with the HPLC grade solvent used during experimentation (*n*-hexane for extraction, acetonitrile for analysis).

Throughfall and bulk deposition samples were recovered every 15 days for a year, from June 2010 to May 2011. Before sample collection, funnels were rinsed with 50 mL of milli-Q water. The glass bottles were then retrieved to the laboratory in cold-boxes. After each sampling, bottles were replaced by clean ones and the collectors were carefully cleaned with 100 mL of acetonitrile/cyclohexane 50:50 (v/v) (HPLC-grade, Scharlau, Sentmenat, Spain) previously prepared in clean PE bottles.

At the laboratory, the sample volume was estimated gravimetrically, then transfered to 1 L PE bottles and subsequently stored at -20 °C until analysis. A maximum of 800 mL of sample was conserved to take into account its expansion on freezing. Therefore the final volume of samples kept for analysis ranged from 50 to 800 mL.

2.1.4. Field blanks and laboratory blanks

To detect contamination due to sampling procedure, field blanks were performed over the entire year. They were prepared in the laboratory in clean 2 L glass bottles (the same as used for collecting deposition). One bottle for each sampling period of 15 days was filled with 1 L of milli-Q water and transported with the sampling bottles to the site. The bottle was opened during the sampling, then reclosed and left for the sampling period on the site close to the collectors and covered with aluminium foil. After 15 days, it was taken to the laboratory along with the samples where, mimicking the sample procedure, 800 mL were transferred to 1 L PE bottles and stored at -20 °C with them.

Moreover, the efficiency of the collector cleaning procedure was verified by wiping the funnels with cotton wool pads after the last sample collection in May 2011. Three pads were used for each collector, then folded, introduced into 100 mL PE bottles, transported to the laboratory in a cold-box and stored at -20 °C until analysis. For control purposes, unused pads were submitted to the same protocol.

To detect any organic contamination due to the use of PE material, laboratory blanks were also made by filling 1 L PE bottles with 800 mL of milli-Q water and storing them at -20 °C until analysis.

2.2. Analytical procedure

2.2.1. Chemicals and standards

A standard PAH mix (Mix 16 HAP, LGC Standards, Teddington, UK) containing naphthalene (NAP), acenaphtene (ACE), fluorene (FLR), phenanthrene (PHE), anthracene (ANT), fluoranthene (FTN), pyrene (PYR), benzo(a)anthracene (B(a)A), chrysene (CHR), benzo(b)fluoranthene (B(b)F), benzo(k)fluoranthene (B(k)F), benzo(a)pyrene (B(a)P), dibenzo(a,h)anthracene (D(ah)A), benzo(g,h,i)perylene (B(ghi)P), indeno(1,2,3-c,d)pyrene (IND) at 10 µg mL^{-1} of acetonitrile was used for calibration and to evaluate the accuracy of the analytical method. Deuterated PAHs were used as surrogate standards (anthracene d_{10} and benzo(a)pyrene d_{12} at 10 µg mL^{-1} of acetonitrile) and as internal standard (fluoranthene d_{10} at 100 µg mL^{-1} of acetonitrile) (LGC Standards, Teddington, UK).

All solvents were HPLC grade: acetonitrile and n-hexane were provided by Scharlau (Sentmenat, Spain) and Milli-Q water by Millipore (Billerica, MA, USA).

2.2.2. Sample and blank preparation

Determination of the PAH concentrations in the samples and the milli-Q water blanks was performed by liquid-liquid extractions with n-hexane, followed by concentration with a TurboVap® apparatus and analysis by high-performance liquid chromatography associated with a fluorimetric detector (HPLC-FLD). The details of the analytical procedure are presented in Fig. 3.

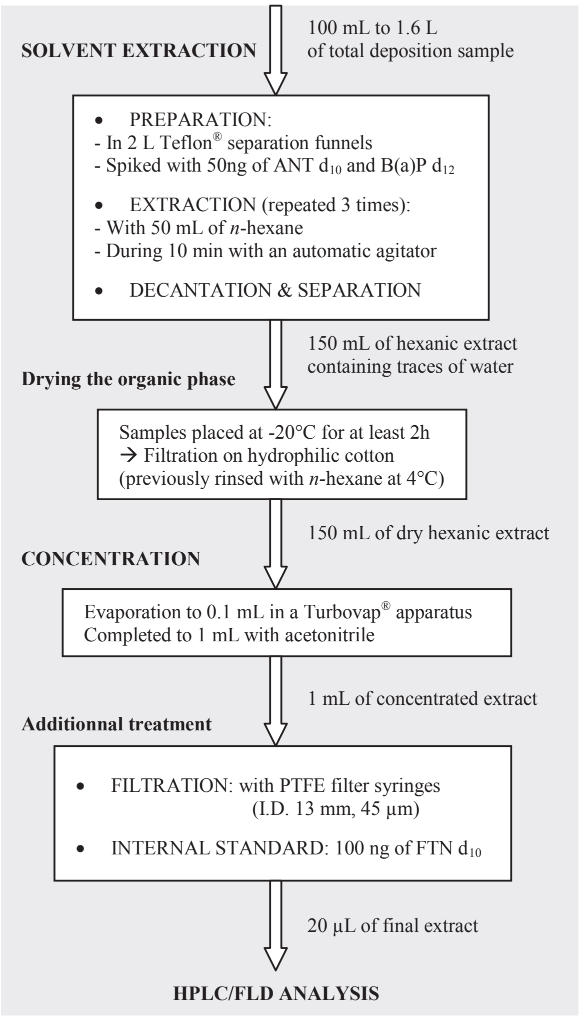

Fig. 3. Diagram illustrating all operations of the analytical procedure to determine PAH concentrations in total deposition samples by solvent extraction

Fortnight samples from each collector were combined to form average monthly samples of 0.1 to 1.6 L. Extractions were carried out in 2 L Teflon® separation funnels using an Agitelec AG automatic agitation device (Grosseron, Saint-Herblain, France). Samples were spiked with 50 ng of deuterated surrogate standards: anthracene d_{10} was chosen as a reference for the light PAH fraction (ACE to PYR) and benzo(a)pyrene d_{12} for the heavy PAH fraction (B(a)A to B(ghi)P). Extractions were carried out with 50 mL of n-hexane for 10 min. After decantation and separation, the lower aqueous phase was recuperated in the same PE as used for sample storage and the organic phase was recuperated in a clean PE bottle. The aqueous phase was then poured back into the funnel and underwent another extraction with 50 mL of fresh n-hexane. This procedure was repeated 3 times to achieve high recoveries. The organic phases were collected in the same bottle and placed in the freezer at -20 °C for at least 2 hours. As n-hexane remains liquid at -20 °C (fusion temperature: -94 °C), the residues of water were eliminated by filtering the frozen organic extracts on cotton wool (previously rinsed with n-hexane at 4 °C).

The extracts were recovered in 200 mL TurboVap® glass tubes and concentrated to 1 mL under a gentle flow of N2 (Alpha 1, Air Liquide, Paris, France) in a TurboVap II apparatus (Caliper Life Sciences, Hopkinton, MA, USA). The tubes were rinsed with 2 mL of n-hexane. The extract was concentrated almost to dryness (0.1-0.2 mL) and finally completed with 1 mL of acetonitrile.The solvent exchange is necessary for chromatographic compatibility. Before analysis, the samples were filtered with PTFE filter syringes (I.D. 13 mm, 45 µm) and 100 ng of internal standard FTN d_{10} was added.

Determination of PAH content on the cotton pads used to wipe the collectors was performed by Pressurized Liquid Extraction (PLE), followed by concentration with a TurboVap® apparatus and analysis by HPLC/FLD. The pads were spiked with 50 ng of surrogate standards, then submitted to PLE with n-hexane during 2 cycles of 5 min at 80 °C. Concentration and preparation for analysis was perfored with the same protocol as the samples.

2.2.3. HPLC/FLD analysis

The PAH analyses were performed with a high-performance liquid chromatography system coupling a 626 pump with a gradient controller 600S (Waters, Milford, MA, USA) and an RF 2000 fluorescence detector (Dionex, Sunnyvale, CA, USA). An automatic injector, Waters 717plus Autosampler, was used. The system was equipped with a 250 mm x 4.6 mm I.D. SupelcosilTM LC-PAH C18 column (particle size 5 µm) and a 20 mm x 4.6 mm I.D. precolumn (particle size 5 µm) (Supelco Analytical, Sigma-Aldrich, St.Louis, MO, USA). Their temperatures were controlled with an Ultimate 3000 column compartment (Dionex, Sunnyvale, CA, USA). Chromeleon 6.80 Chromatography Data System (SR10 Build 2818 (166959)) was used for data acquisition (Dionex, Sunnyvale, CA, USA).

Table 1. Analytical parameters for PAH analysis by HPLC/FLD

Time window	***Time (min)***	***Exc λ [a] (nm)***	***Em λ [a] (nm)***	***Compounds [b]***	***t_R [c] (min)***
1	0.0	206	320	NAP	6.1
2	8.0	233	320	ACE FLR	9.2 9.7
3	10.5	260	380	PHE	11.4
4	12.0	250	375	ANT-d_{10} ANT	12.5 13.1
5	13.8	365	462	FTN-d_{10} FTN	14.3 14.9
6	15.6	275	380	PYR	16.4
7	18.0	270	300	B(a)A CHR	21.3 22.5
8	22.1	302	431	B(b)F B(k)F B(a)P-d_{12} B(a)P D(ah)A	26.5 28.4 29.0 30.0 32.3
9	33.4	302	419	B(ghi)P	33.9
10	34.8	300	500	IND	35.8

[a] *Excitation (Exc λ) and emission (Em λ) wavelengths were chosen as a function of the analyzed compounds*
[b] *Acenaphthylene is not detectable by fluorescence*
[c] *Retention time*

Elution was carried out with a binary solvent gradient of water and acetonitrile (ACN) at a flow rate of 1.5 mL min^{-1}. The gradient elution program was as follows: 60% ACN for 5 min, then a linear ramp to 100% ACN within 25 min and holding at 100% for 10 min. The column temperature was set at 30 °C. Detection was performed at selected fluorescence wavelength programming to obtain better sensitivity and minimal interference. The excitation/emission wavelength pairs (nm) are given in Table 1.

2.2.4. Validation of the analytical procedure

The performance of the analysis by HPLC-FLD was validated with standard PAH solutions prepared by diluting the commercial "Mix 16 HAP" in HPLC-grade acetonitrile. Calibration curves were prepared at six levels (0, 2, 5, 10, 50, 150 ng mL^{-1}) and each calibration level was injected in triplicate. Unweighted least square regression was performed.

To study the repeatability of the analysis, a standard solution containing low PAH concentrations (2 ng mL^{-1}) was injected 10 times. Detection (LOD) and quantification (LOQ) limits were also determined with the chromatograms of that standard solution. The LOD was calculated as equal to 3 times the background (S/N = 3) and the LOQ as 10 times the background (S/N = 10) (Tranchant, 1995).

Before observing the spatial and temporal trends of PAHs in total deposition, it was necessary to validate the entire analytical procedure of liquid-liquid extraction associated with HPLC/FLD analysis. Reproducibility and accuracy were determined using a 4 L average sample obtained by pooling the throughfall deposition collected between 20/12/2010 and 3/01/2011 from 5 forest collectors. 2 L of sample was spiked with a diluted solution of the 16 PAHs standard mixture: 10.25 ng were added per litre.

The analytical procedure was carried out on three 500 mL replicates of spiked samples and non spiked samples. Reproducibility was evaluated with the PAH concentration standard deviations for the non spiked samples. Accuracy was calculated for each PAH using the following expression (Eq. 1):

$$Accuracy = \frac{m_{added} - \left([PAH]_S - [PAH]_{NS}\right) * V_{sample}}{m_{added}} \qquad (1)$$

where:
m_{added} represents the theoretical mass of individual PAH added per litre in the spiked sample, $[PAH]_S$ and $[PAH]_{NS}$ the individual PAH concentrations measured in the spiked and non spiked samples (ng L^{-1}) respectively, V_{sample} the sample volume submitted to the analytical procedure (L).

Finally, analytical blanks were submitted to the analytical protocol to detect eventual contamination during the procedure.

2.2.5. Evaluation of sample storage

PAHs can be adsorbed on the sample containers during storage (Wolska et al., 2005). Storage influence was evaluated by preparing synthetic solutions by addition of standard PAHs to tap water. 12 aliquots of 800 mL were spiked with 40 µL of the standard PAH mix diluted to 200 ng L^{-1} in acetonitrile (10 ng added per litre).

6 aliquots were submitted to the analytical procedure after preparation (t = 0) and the 6 others after storage in PE bottles at -20 °C for 1 month. Non spiked aliquots were also analyzed before and after storage.

2.3. Evaluation of the spatial and temporal trends of atmospheric PAH deposition

Atmospheric daily fluxes were calculated over each sampling period for the individual PAHs from the expression (Eq. 2):

$$\Phi(PAH) = \frac{[PAH]\times V}{S\times T} \qquad (2)$$

where: $\Phi(PAH)$ is the daily PAH flux (ng m^{-2} d^{-1}), $[PAH]$ the PAH concentration measured in total deposition (ng L^{-1}), V the volume of total deposition collected (L), S the surface of the collector (m^2) and T the length of the sampling period (d).

Spatial variability of PAH deposition over the sampling site was studied using average samples for two periods of the year, characteristically different for their climatic conditions and canopy cover: at the start of summer (11/06/2010 to 20/07/2010) and in the middle of autumn (13/10/2010 to 10/11/10). For these sampling periods, the samples of the 2 field and 6 forest collectors were analyzed. Seasonal variability of PAH deposition was evaluated between the 11/06/2010 and 03/05/2011 with average monthly samples from one collector positioned in the field and one positioned in the forest. The same collector was used throughout the year.

3. Results and discussion

3.1. Validation of the analytical procedure

The HPLC-FLD analysis of PAHs showed satisfactory results. The linearity range extended from 0 to 150 ng mL^{-1} with regression coefficients from 0.9996 (ACE) to 0.9999 (PHE, ANT, FTN, B(a)A, CHR, B(b)F, B(k)F, D(ah)A).

Analyses were repeatable as relative standard deviations, determined for each PAH (from 3% for ACE to 14% for PHE), were lower than 20%. Average quantification limits were of the order of a few ng L^{-1} in total deposition (Table 2).

The composite sample used for validating the analytical procedure of liquid-liquid extraction followed by HPLC-FLD analysis contained quantifiable levels of ACE, FLR, PHE, FTN, ANT, PYR, B(a)A, B(b)F, B(k)F, B(a)P and B(ghi)P. NAP, D(ah)A and IND were detected but not quantifiable. Determination of CHR concentrations was impossible due to the presence of an interfering compound co-extracted from the throughfall deposition matrix and co-eluted at the same retention time. Validity of the analytical procedure was evaluated with the 11 quantified compounds. Average recoveries and their corresponding standard deviations (n = 6) were satisfactory: 68 ± 4% for light PAHs (ACE to PYR) and 75% ± 3 for heavy PAHs (B(a)A to B(a)P). The analytical procedure is reproducible for the 11 target compounds as relative standard deviations of PAH concentrations in the non spiked samples were lower than 20% (Table 2). The accuracy of the analytical method was lower than 20% for 9 PAHs (FLR, PHE, ANT, FTN, PYR, B(a)A, B(b)F, B(k)F, B(a)P). ACE and B(ghi)P showed differences between experimental and theoretical concentrations higher than 20%, due to the presence of interfering compounds co-eluted at retention times close to those of the target compounds.

As a result, the analytical procedure assured satisfactory precision for quantification, in the total deposition sample studied, of 5 light PAHs: FLR, PHE, ANT, FTN, PYR; and 4 heavy PAHs: B(a)A, B(b)F, B(k)F, B(a)P. Concentrations determined for these compounds appear in Table 3.

Table 2. Quantification limits, reproducibility and accuracy obtained during validation of the analytical procedure of PAH determination in total deposition

Compounds	*LOQ [a](pg)*	*LOQ in bulk deposition [b] (ng L^{-1})*	*RSD [c](%)*	*Accuracy [d](%)*
NAP	591	86.4	ND [e]	ND
ACE	22	3.2	15	+25
FLR	52	7.6	18	- 5
PHE	27	4.0	6	+3
ANT	4	0.6	4	- 3
FTN	28	4.1	7	- 6
PYR	13	1.9	6	+1
B(a)A	15	2.0	6	+3
CHR	16	2.1	ND	ND
B(b)F	6	0.8	8	+17
B(k)F	3	0.4	5	+15
B(a)P	12	1.6	4	+19
D(ah)A	7	0.9	ND	ND
B(ghi)P	6	0.8	13	+45
IND	22	2.9	ND	ND

[a] *Quantification limits (LOQ), determined with a standard solution of PAHs at 2 ng mL^{-1} in acetonitrile, are expressed as mass of injected compound (pg).*

[b] *As a rough guide, the equivalent concentrations in bulk deposition (ng L^{-1}) have been calculated for 500 mL samples and with global average PAH recoveries of 68% for light PAHs (ACE to PYR) and 75% for heavy PAHs (B(a)A to B(a)P).*

[c] *Relative standard deviations (%) obtained for the evaluation of the reproducibility of the analytical procedure (n=3).*

[d] *Accuracy (%) of the analytical method evaluated by spiking a bulk deposition sample with PAHs (n=3).*

[e] *Non determined.*

Table 3. PAH concentrations (ng L^{-1}) in a composite bulk deposition sample collected in December 2010 under Bertiz Natural Park forest canopy

PAHs	*Concentrations (ng L^{-1})*		
	Mean	*Median*	*SD*
FLR	6.2	5.8	1.1
PHE	11.8	12.2	0.8
ANT	2.3	2.4	0.1
FTN	4.0	4.0	0.3
PYR	4.6	4.5	0.3
B(a)A	2.9	2.9	0.2
B(b)F	2.7	2.6	0.2
B(k)F	2.6	2.6	0.1
B(a)P	2.4	2.4	0.1
Total	***39.6***	***39.4***	***1.8***

The values are close to quantification limits, with individual concentrations from 2.4 ± 0,1 ng L^{-1} for benzo(a)pyrene to 11.8 ± 0.8 ng L^{-1} for phenanthrene. Total concentration of the 9 PAHs quantified is 40 ± 2 ng L^{-1}. Atmospheric fluxes of PAHs were therefore determined in this study for the following compounds: FLR, PHE, FTN, ANT, PYR, B(a)A, B(b)F, B(k)F, B(a)P.

Analytical blanks showed PAH concentrations under the quantification limits for FLR, ANT, FTN, B(a)A and B(b)F. The 4 other target compounds (PHE, PYR, B(k)F, B(a)P) were quantified at levels of the order of the quantification limits. These values were taken into account during the determination of the PAH concentrations in total deposition samples.

3.2. Validation of the sampling protocol

3.2.1. Efficiency of the collectors for sampling bulk deposition

At the Bertiz Nature Park, the average volume of bulk deposition sampled over the two-week periods in the field collectors, ranged from 0 to 2.1 L (Fig. 4). Parallel bulk sampling reproducibility was lower than 10%. Daily precipitations, measured in the intensive plot and reported by the local authorities of Navarra (Navarra Government, 2011), were used to estimate the volume of bulk deposition accumulated in the collectors over each sampling period.

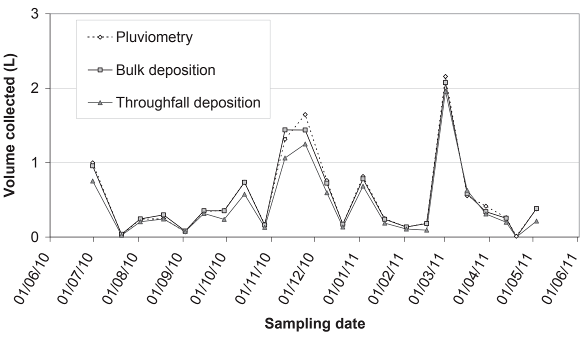

Fig. 4. Temporal variation of the average volume of bulk and throughfall precipitation values and comparison with the theoretical volume calculated from daily pluviometry data (Navarra Government, 2011)

The theoretical values were highly correlated with the average volumes of bulk deposition measured after each sampling period (r = 0.9948).

Moreover, between June 2010 and May 2011, sample loss due to evaporation was 2% of the bulk deposition collected. The collection device therefore appears to be very efficient at conserving bulk deposition.

3.2.2. PAH contamination/loss due to the sampling procedure

Laboratory and field blanks showed the same PAH levels as the analytical blanks used for validation of the analytical procedure. Moreover, PAH content on the cotton wool pads used to wipe the collectors was under quantification limits. The cleaning protocols are therefore efficient and the sampling procedure does not appear to be a source of PAH contamination.

The storage tests revealed an average loss of 67% of the total PAH content, in line with a previous study which found adsorption of up to 70% of the PAHs onto glass container walls (Wolska et al., 2005). As average concentrations measured in the bulk deposition samples collected in the Bertiz Park (0-9 ng L^{-1}) had similar trace levels as the test solutions (spiked with 10 ng per litre), we supposed that the PAH loss in the samples was of the same order as the test solutions. A correction factor was therefore applied during evaluation of PAH fluxes.

3.2.3. Spatial variability of PAH levels under the canopy

In the total deposition samples collected in Bertiz during summer (11/06/2010 → 20/07/2010) and autumn (13/10/2010 → 10/11/10), FLR, FTN and B(a)A showed concentrations under quantification limits. Atmospheric PAH deposition was therefore evaluated with the light PAHs: PHE, ANT, PYR; and heavy PAHs: B(b)F, B(k)F, B(a)P. Average daily fluxes evaluated with the 6 forest collectors and the relative standard deviations are shown in Table 4.

Table 4. Spatial variability under the forest canopy of average daily fluxes of 6 PAHs for two seasons: summer and autumn 2010

	30/06/10 → 1/08/10		***27/10/10 → 23/11/10***	
	Mean [a] ***(ng m^{-2} d^{-1})***	***RSD*** [b] ***(%)***	***Mean*** [a] ***(ng m^{-2} d^{-1})***	***RSD*** [b] ***(%)***
PHE	33.3	13	116.4	25
ANT	8.8	16	18.5	24
PYR	15.8	9	38.5	7
B(b)F	8.5	13	20.3	10
B(k)F	8.8	12	19.4	13
B(a)P	8.5	11	20.6	21

[a] *Mean value of average daily fluxes determined from the 6 forest collectors.*

[b] *Relative standard deviations between the 6 forest collectors.*

During summer 2010, the PAH fluxes measured in the forest presented low spatial variability: between 9% for PYR and 16% for ANT.

During the autumn, PAH fluxes were 2-3 times higher than in the summer and were associated with higher variability between the collectors: 7% for PYR to 25% for PHE. These results validate the choice of using 6 collectors for sampling throughfall deposition.

3.3. Influence of the forest cover

3.3.1. Precipitation interception by the canopy

At the Bertiz Nature Park, the average volume of throughfall deposition collected over the 2 week periods under the forest canopy ranged from 0 to 2.0 L (Fig. 4). These volumes measured were highly correlated (r = 0.9902) with precipitation data measured in the field plot (Navarra Government, 2011). However, between June 2010 and May 2011, throughfall deposition corresponded to only 81% of the bulk deposition. The data obtained with the field collectors showed that only 2% of the precipitation loss is due to sample evaporation from the collection bottles. Therefore, an average value of 17% of the total precipitation amount was intercepted by the canopy and thus the throughfall collectors retained an average of 83% of the total annual incident rainfall.

3.3.2. Difference in PAH fluxes between the forest and the openfield

The PAH fluxes determined in the openfield (Fig. 5a) were generally either of the same order or higher than those registered in the forest throughout the year (Fig. 5b).

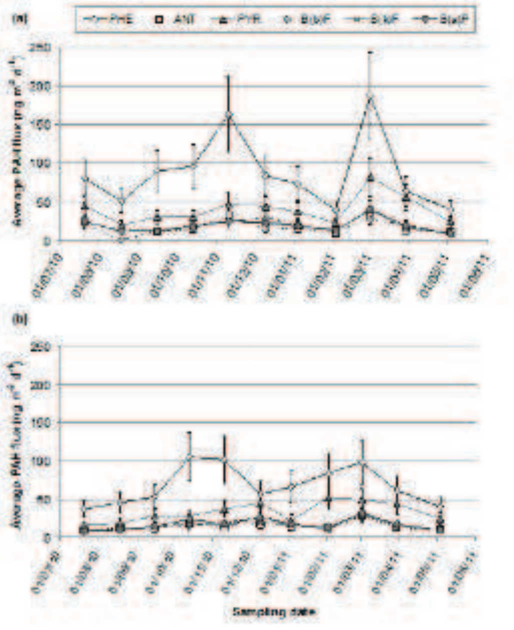

Fig. 5. Temporal variation of the average daily fluxes of 6 PAHs determined from average monthly samples of bulk (a) and throughfall (b) deposition (The error bars represent the standard deviation calculated by studying the spatial variability between the collectors)

In the case of the major target compound PHE, bulk deposition reached a maximum of 169% of the throughfall value during September 2010. This is due to the forest canopy which retains a fraction of the precipitation and as a consequence the forest floor only receives the PAHs contained in throughfall. However, precipitation interception by the forest canopy is variable between sampling periods depending on the precipitation regime (volume, intensity…). During winter months, when the deciduous beech trees had lost their leaves, deposition fluxes were generally similar in the field and under the forest cover. The sample collected during January 2011 revealed fluxes under the canopy 62% higher than those measured in the field, due to exceptionally high concentrations in the forest sample (particularly PYR which was 5 times higher than in the field sample), which reveals a possible local contamination of the forest plot during that particular sampling period. For the whole year, total flux of 6 PAHs in the forest corresponded to 88% of the total flux in the field (Table 5). The 12% loss of PAHs under the forest cover is of the same order as the precipitation loss due to interception by the canopy.

Table 5. Total deposition of 6 PAHs between June 2010 and May 2011 (326 days) in the field and under the forest cover

PAHs	***Total flux (µg m^2)***	
	In the field	***Under the forest canopy***
PHE	26.1	20.6
ANT	5.0	4.7
PYR	11.8	11.6
B(b)F	5.2	5.1
B(k)F	5.7	5.0
B(a)P	5.6	5.4
Total	***59.4***	***52.4***

3.4. Seasonal variability of PAH deposition

PAH fluxes showed important variations throughout the year (Fig. 5). Indeed, relative standard deviations of 27% (B(b)F in the forest) to 54% (PHE in the field) were observed between the 11 average monthly samples. Temporal variations are significantly more important than spatial variations due to sampling, as relative standard deviations between collectors reached a maximum of 25% for PYR in the forest.

Summer and autumn samples, collected under the forest canopy for evaluation of on-site spatial variability, showed that fluxes were 2-3 times higher in autumn than in summer (Table 4). Indeed, we obtained mean values for the major compound PHE of 33 ± 4 ng m^{-2} d^{-1} in the summer against 120 ± 30 ng m^{-2} d^{-1} in the autumn. This is due to variations of PAH emissions and meteorological conditions during the year. First of all, PAH emissions increase during the cold season because of domestic heating using fossil fuels or biomass (Ravindra et al., 2008). A second point concerns the intense photochemical degradation of PAHs in the summer (Dabestani and Ivanov, 1999). Finally, as wet deposition clears the vertical atmospheric column by absorbing soluble gases and/or particles into water droplets, high precipitation levels induce good clearance of the atmosphere, particularly of airborne PAHs (Sicard, 2006). Indeed, PAH fluxes between June 2010 and May 2011 showed temporal trends correlated to the volume of precipitation collected: $r = 0.8459$ in the field and $r = 0.7206$ in the forest ($p < 0.01$).

3.5. Total annual PAH fluxes

The total annual fluxes were determined for 6 PAHs (PHE, ANT, PYR, B(b)F, B(k)F and B(a)P), as these compounds were quantified in all bulk samples analyzed (Table 5). PHE and PYR were the major compounds in bulk deposition, with total annual fluxes in the field and forest of 26.1 ± 3.9 and 20.6 ± 3.1 μg m^{-2} for PHE, 11.8 ± 1.8 and 11.6 ± 1.7 μg m^{-2} for PYR respectively (on the basis of 326 days sampling). PHE contributed to 44% and 39% of the total flux of 6 PAHs in the field and forest respectively, as PYR contributed to 20% and 22% of the total flux in the field and forest respectively. The other compounds presented similar fluxes ranging from 5.0 ± 0.8 μg m^{-2} (ANT) to 5.7 ± 0.9 μg m^{-2} (B(k)F) in the field and from 4.7 ± 0.7 μg m^{-2} (ANT) to 5.4 ± 0.8 μg m^{-2} (B(a)P) under the forest canopy. The contribution of each of these minor compounds (ANT, B(b)F, B(k)F and B(a)P) corresponds to 8-10% of the total atmospheric flux of the 6 compounds. These observations are in accordance with a previous study carried out in the Bertiz Nature Park where airborne deposition of PAHs was monitored biologically with mosses (Foan et al., 2003). The high levels of PHE and PYR are mainly attributable to traffic emissions in the Nature Park surroundings.

Total annual levels for the 6 target PAHs were 59.4 ± 8.9 and 52.4 ± 7.9 μg m^{-2} in the field and forest respectively (on the basis of 326 days sampling). Equivalent daily fluxes are respectively 182 ± 27 and 161 ± 24 ng m^{-2} d^{-1} in the field and forest. The values measured with bulk deposition are higher than previous results obtained in a remote area of the Pyrenees, where average fluxes of 27-76 ng m^{-2} d^{-1} were measured for 25 compounds (Fernández et al., 2003). However, they are significantly lower than in urban areas. For example, Esen et al. (2008) measured in Bursa in Turkey an average flux of 3300 ng m^{-2} d^{-1} between July 2004 and May 2005. Bertiz Natural Park therefore reveals characteristic PAH fluxes of a rural site. The difference in throughfall can be explained by the precipitation interception by the forest canopy.

4. Conclusions

Analysis of total deposition samples between June 2010 and May 2011 showed the existence of differences in PAH deposition between bulk and throughfall collectors placed at the same remote site in Northern Spain. PAH fluxes in the forest are equivalent to approximately 88% of the total PAH deposition received in the field, due to the filtering role of the canopy. Variability of the fluxes under the canopy was also observed, with a maximal relative standard deviation for 6 collectors of 25%.

However, spatial variability on the sampling site was found to be negligible in comparison with the amplitude of temporal trends. Generally, atmospheric deposition of PAHs is greater in winter than in summer, due to more PAH emissions by domestic heating, less photoreactivity of the compounds and intense leaching of the atmosphere by wet deposition.

Annual fluxes were estimated for 6 quantified PAHs (PHE, ANT, PYR, B(b)F, B(k)F and B(a)P). The major compounds were phenanthrene followed by pyrene. Both are markers of traffic emissions. Average daily fluxes of the 6 compounds were estimated at 182 ± 27 g m^{-2} d^{-1} and 161 ± 24 ng m^{-2} d^{-1} in the field and forest respectively, in accordance with studies previously carried out in other local remote areas.

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Annexe XIII.

Optimization of pressurized liquid extraction using a multivariate chemometric approach and comparison of solid-phase extraction cleanup steps for the determination of polycyclic aromatic hydrocarbons in mosses

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Optimization of pressurized liquid extraction using a multivariate chemometric approach and comparison of solid-phase extraction cleanup steps for the determination of polycyclic aromatic hydrocarbons in mosses

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ABSTRACT

A factorial design was used to optimize the extraction of polycyclic aromatic hydrocarbons (PAHs) from mosses, plants used as biomonitors of air pollution. The analytical procedure consists of pressurized liquid extraction (PLE) followed by solid-phase extraction (SPE) cleanup, in association with analysis by high performance liquid chromatography coupled with fluorescence detection (HPLC–FLD). For method development, homogeneous samples were prepared with large quantities of the mosses *Isothecium myosuroides* Brid. and *Hypnum cupressiforme* Hedw., collected from a Spanish Nature Reserve. A factorial design was used to identify the optimal PLE operational conditions: 2 static cycles of 5 min at 80 °C. The analytical procedure performed with PLE showed similar recoveries (~70%) and total PAH concentrations (~200 ng g^{-1}) as found using Soxtec extraction, with the advantage of reducing solvent consumption by 3 (30 mL against 100 mL per sample), and taking a fifth of the time (24 samples extracted automatically in 8 h against 2 samples in 3.5 h). The performance of SPE normal phases (NH$_2$, Florisil®, silica and activated aluminium) generally used for organic matrix cleanup was also compared. Florisil® appeared to be the most selective phase and ensured the highest PAH recoveries. The optimal analytical procedure was validated with a reference material and applied to moss samples from a remote Spanish site in order to determine spatial and inter-species variability.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are products of thermal decomposition, formed during incomplete combustion of organic materials and geochemical formation of fossil fuels. The main anthropogenic sources are power plants, domestic heating, waste incineration, industrial processes and, most importantly, motor vehicle exhaust emissions [1–3]. PAHs are considered to be persistent organic pollutants (POPs) due to their slow rates of degradation, toxicity and potential for both long-range transport and bioaccumulation in living organisms [4]. Carcinogenic, mutagenic and immunotoxic effects of PAHs, detrimental to human health, have frequently been reported [1,5]. Regulation of PAH emissions and reliable monitoring of PAH concentrations in ambient air is thus of paramount importance for public health.

Atmospheric PAH levels are monitored by collecting atmospheric particles and gases with high or low volume samplers, and PAH deposition is evaluated by sampling bulk deposition and/or wet deposition in collectors [4,6,7]. However, the sampling devices have several drawbacks such as low sample representativeness, analytical difficulties (trace levels), cost and maintenance of equipment. Conversely, monitoring using biological samples appears to be a low cost alternative that can be easily developed globally. For this reason, plants are widely used as airborne pollution biomonitors in urban and rural environments [8]. The most common are herbaceous species [9], deciduous tree leaves [10], persistent tree leaves [11], coniferous needles [4,12], tree bark [13], lichens [14,15] and mosses [4,15,16].

Bryophytes in particular have been used over the past decade as biomonitors for the assessment of airborne pollutant deposition: heavy metals [17], radionuclides [18], nitrogen [19], natural abundance of C, N and S isotopes [20,21] and POPs [16,22–24]. As they do not have any root system or cuticle, mosses obtain most of their nutrients from the atmosphere. Moreover, their high cationic exchange capacity and surface to volume ratio favours the accumulation of large amounts of pollutants [25]. The International Cooperative Programme on Effects of Air Pollution on

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Natural Vegetation and Crops (ICP-Vegetation), established under the United Nations Economic Commission for Europe (UNECE) Convention on Long-Range Transboundary Air Pollution (LRTAP), has monitored heavy metal deposition in Europe with mosses since 1990 [17]. A pilot study was initiated by ICP-Vegetation in 2010 to monitor POPs, particularly PAHs, at a European scale. Therefore, development of a fast and efficient analytical procedure for determining PAHs in mosses is necessary.

PAH determination in mosses is currently undertaken by solvent extraction followed by a cleanup step, associated with chromatographic analysis. Traditional extraction procedures using Soxhlet [26–28] or Soxtec apparatus [15,29] do not provide enough energy to release the analytes rapidly and therefore require long extraction times (3.5–24 h) and large amounts of organic solvents (100–200 mL). Thus, sonication extraction [25,30], microwave-assisted solvent extraction [31] and pressurized liquid extraction (PLE) [32–34], have been proposed as alternatives, with extraction taking on average 30 min with 20–30 mL of solvent. However, sonication extraction has the drawback of not being automated and, even though the microwave apparatus extracts 40 samples simultaneously, an extra filtration step is necessary to separate the matrix residues from the solvent. By comparison, PLE can perform 24 sequential extractions, with no further treatment.

With PLE – also called accelerated solvent extraction (ASE) – extractions can be performed at high temperatures, since the solvents remain in a liquid state under the high pressures applied, ensuring high analyte recoveries [35]. Static extraction temperature, time and number of cycles appear to be critical experimental parameters [36–38]. Indeed, temperature has an effect on the solvent viscosity and thereby its ability to wet the matrix and solubilise the target analytes, and certain sample matrices can retain analytes within pores or other structures, thus increasing extraction time allows these compounds to diffuse into the extraction solvent. Finally, the use of static cycles was developed to introduce fresh solvent during the extraction process, which helps maintain favourable extraction equilibrium, without diluting the sample (since the flush volume is the same whatever the number of cycles). The effect of pressure is to maintain the solvents as liquids above their atmospheric boiling point, and the pressures used in PLE are well above the thresholds required to maintain the solvents in the liquid phase. Changing the pressure has very little impact on analyte recovery, so it seems unnecessary to optimize this parameter.

Several optimization procedures for PAH extraction by PLE on plant biomonitors such as mosses [34] and pine needles [39,40] are available in the literature, and the univariate approach was used in these studies: one parameter was optimized while the others were set at a convenient value. However, operational parameters may interact, making it necessary to use a multivariate chemometric approach for developing the analytical method.

Selectivity is a necessary condition for obtaining reliable chromatographic results [41]. Therefore, solid-phase extraction (SPE) cleanup is usually employed to separate co-extracted matrix compounds from the target analytes as they may interfere during final determination [40,42]. Moreover, the isolation of target compounds from plant co-extracts, appears to be highly important when analyzing PAHs by high performance liquid chromatography coupled to fluorescence detection (HPLC–FLD) [39]. To date, no study covering comparison of SPE cleanup steps of moss extracts has been published.

In order to facilitate the use of mosses as bioindicators of organic airborne pollution, this study offers a fast, low solvent consuming and effective analytical procedure for PAH determination in these plants. PLE operating conditions were optimized with a factorial design and PAH recoveries were compared with those obtained by Soxtec extraction, whose efficiency was demonstrated in a previous study [29]. Moss extracts obtained under optimal PLE conditions were subsequently submitted to several normal phase SPE, in order to reveal the most efficient cleanup, i.e. the one offering the highest recoveries and the best selectivity. Finally, after verifying the accuracy of the optimal analytical procedure using reference material, the method was applied to moss samples from a mountainous area in Spain to determine spatial and inter-species variability on the site.

2. Experimental

2.1. Materials and reagents

2.1.1. Moss samples

To develop the analytical procedure, Pleurocarpous mosses *Isothecium myosuroides* Brid. and *Hypnum cupressiforme* Hedw. were collected in the Bertiz Nature Reserve (Navarra, Spain) between 2008 and 2010. The former was used for optimizing the extraction procedure and the latter for finding the most efficient cleanup. Two different species were used, because large amounts of material were needed to develop the analytical procedure, and the Nature Reserve authorities limit the quantity of mosses sampled, for conservation reasons. All analyses were performed on homogeneous material (cf. Section 2.2) prepared with composite samples. Approximately 0.5 m^2 of each species was collected, giving 50 and 25 g (dry weight) of *I. myosuroides* Brid. and *H. cupressiforme* Hedw. respectively.

After validation, the optimal parameters were applied to moss samples collected in June 2010 from a low-alpine site in the Aralar Range (Navarra, Spain). The aim of the study was to validate the use of the moss species *Hylocomium splendens* (Hedw.) Schimp. for monitoring atmospheric deposition of PAHs in a rural environment. Initially, spatial variability in sampling was evaluated by collecting 9 samples from the same site ($\sim$0.02 m^2 per sample), and the PAH levels were compared with those of *H. cupressiforme* Hedw., because the latter had been used in a previous study for monitoring atmospheric deposition of PAHs in Navarra [29]. To this end, samples of *H. cupressiforme* ($\sim$0.04 m^2 per sample) were taken at the same site as *H. splendens*. Mosses were collected under the surveillance of the Navarra authorities, and due to its scarcity in the Aralar Range, a maximum of only 3 samples of *H. cupressiforme* was allowed.

2.1.2. Chemicals and standards

A standard mix containing acenaphthene (ACE), fluorene (FLR), phenanthrene (PHE), anthracene (ANT), fluoranthene (FTN), pyrene (PYR), benz(a)anthracene (B(a)A), chrysene (CHR), benzo(b)fluoranthene (B(b)F), benzo(k)fluoranthene (B(k)F), benzo(a)pyrene (B(a)P), dibenz(a,h)anthracene (D(ah)A), and benzo(ghi)perylene (B(ghi)P) at 10 $\mu g\,mL^{-1}$ of acetonitrile, was used for calibration (Mix 16 HAP, LGC Standards, Teddington, UK). Deuterated PAHs were used as surrogate standards (anthracene d_{10} and benzo(a)pyrene d_{12} at 10 $\mu g\,mL^{-1}$ in acetonitrile) and as internal standard (fluoranthene d_{10} at 100 $\mu g\,mL^{-1}$ in acetonitrile) (LGC Standards, Teddington, UK).

A reference material IAEA-140-OC (35 g) (ANALAB, Bischheim, France), consisting of a common brown seaweed (*Fucus* sp.) and characterized for the mass fractions of certain organic contaminants, was used to evaluate the accuracy of the analytical method. The International Atomic Energy Agency supplies reference values for organochlorine pesticides, PCB congeners, petroleum hydrocarbons and PAHs for this material, based on an international inter-laboratory comparison [43].

All solvents were HPLC grade: acetonitrile, cyclohexane, dichloromethane (DCM) and *n*-hexane were provided by Scharlau

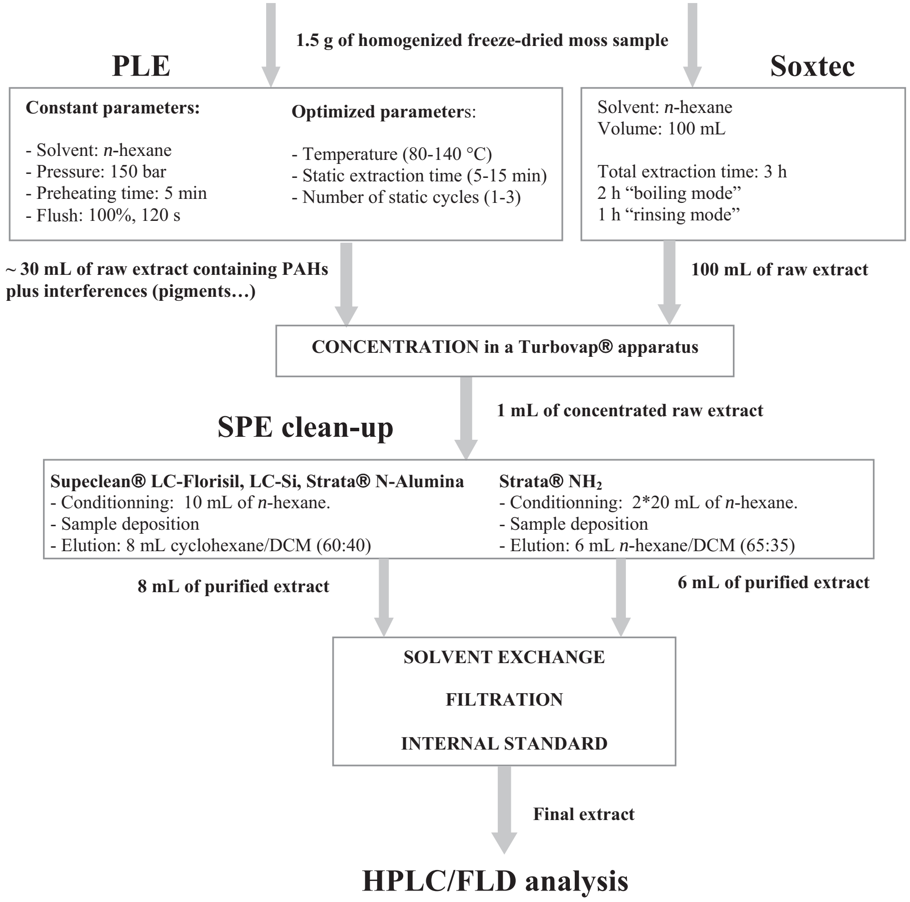

Fig. 1. Diagram illustrating all operations of the analytical procedure to optimize PAH determination in mosses.

(Sentmenat, Spain) and Milli-Q water by Millipore (Billerica, MA, USA).

2.2. Sample preparation

Any litterfall (dead leaves, twigs...) attached to the moss samples was removed with stainless steel tweezers. The green and green-brown shoots from the last three years growth were withdrawn using stainless steel tweezers and scissors. Brown parts were not included in the samples, even if the remaining green parts represented less than three years of growth.

The unwashed samples were freeze-dried – recommended to minimize loss of volatile compounds during preparation [44,45] – with an Alpha 2-4 LD apparatus for 24 h (Martin Christ, Osterode am Harz, Germany). To obtain homogeneous samples, the lyophilized mosses were ground to a fine powder in a stainless steel mill (particle size < 0.5 mm).

2.3. Solvent extraction and SPE cleanup

First, PLE was optimized and compared with previously validated Soxtec extraction [29]. Then, during a second phase, different sorbent phases were compared for the SPE cleanup. The analytical procedure is shown in Fig. 1.

2.3.1. Pressurized liquid extraction

PLE extractions were performed with an ASE 200 apparatus (DIONEX, Sunnyvale, CA, USA). Homogenized moss samples of 1.5 g were extracted in 11 mL stainless steel cells with 0.75 g of anhydrous sodium sulfate and 0.75 g of Florisil® (activated magnesium silicate) from U.S. Silica Company (Berkeley Springs, USA). The sorbents were layered at the outlet of the extraction cells. Anhydrous sodium sulfate was used to ensure total dryness of the cell content to avoid problems of water immiscibility with nonpolar solvents. Florisil® was used to retain co-extracted polar compounds which could interfere with PAHs during analysis. In-line selective removal of interferences avoids drastic post-extraction cleanup steps [46]. Each sample was spiked with 50 ng of surrogate standards ANT d_{10} and B(a)P d_{12}, chosen to estimate the recoveries of the light PAH fraction (ACE to PYR) and heavy PAH fraction (B(a)A to B(ghi)P), respectively.

Extractions were carried out with *n*-hexane at a pressure of 150 bar. The cells were preheated for 5 min to reach thermal equilibrium, then were submitted to one or several static extraction cycles. After each cycle, the cells were flushed with a volume of fresh solvent corresponding to 100% of the cell volume divided by the number of cycles. Finally, at the end of the extraction process, the cells were purged with purified nitrogen for 120 s. The solvent and fixed operational parameters were chosen in the light of a previous study [34] that demonstrated their efficiency for PLE extraction of PAHs from mosses.

Nonpolar/polar solvent mixtures such as DCM/acetone or *n*-hexane/acetone have shown high PAH recoveries in previous PLE studies performed on environmental samples [39,47]. However, high co-extraction of matrix polar compounds such as pigments and lipids was also observed. Hence, with polar solvents, a more drastic cleanup procedure is necessary to ensure interference free chromatographic analysis. Thus the choice of a non-polar solvent, such as *n*-hexane, for PLE extraction seems preferable to obtain lighter extracts containing low matrix residue content. This solvent

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Table 1
Central factorial design used for the PLE optimization of PAH extraction from mosses.

Experiment no.	Temperature, T (°C)	Static time, t_s (min)	Number of cycles, N
1	80	5	1
2	80	5	3
3	80	15	3
4	80	15	1
5	140	5	1
6	140	5	3
7	140	15	1
8	140	15	3
9	80	5	1
10	80	5	3
11	80	15	3
12	80	15	1
13	140	5	1
14	140	5	3
15	140	15	1
16	140	15	3
17	110	10	2
18	110	10	2

also has the advantage of being highly volatile, ensuring a fast concentration step, and is compatible with the normal cleanup phases.

The PLE experimental design was developed to identify the optimal values of static extraction temperature, static time and number of static cycles. Several studies concerning PLE applied to bioindicators for PAH determination use operating temperatures from 80 to 140 °C, static extraction times of 5–15 min and a maximum of 3 cycles [33,34,39]. These limits were assigned to each variable for the experimental design (Table 1).

Before SPE cleanup, the extracts were concentrated to 1 mL with purified N_2 (Alpha 1, Air Liquide, Paris, France) in a Turbovap II apparatus (Caliper Life Sciences, Hopkinton, MA, USA).

2.3.2. Soxtec extraction

The extractions were performed with a Soxtec System HT2 (Tecator, France). 1.5 g moss samples spiked with 50 ng of surrogate standards were extracted in cellulose thimbles with 0.75 g of anhydrous sodium sulfate and 0.75 g of Florisil® (U.S. Silica Company, Berkeley Springs, USA).

Extractions were carried out with 100 mL of *n*-hexane. The thimbles were first immersed in the boiling solvent for 2 h and then raised above the solvent to be rinsed for 1 h by the condensing solvent.

Before SPE cleanup, the extracts were concentrated to 1 mL with purified N_2 (Alpha 1, Air Liquide, Paris, France) in a Turbovap II apparatus (Caliper Life Sciences, Hopkinton, MA, USA).

2.3.3. SPE cleanup procedure

During the PLE optimization and validation, cleanup was performed with Florisil® SPE cartridges (Supelclean LC-Florisil SPE 1 g/6 mL from Supelco Analytical, Sigma–Aldrich, St. Louis, MO, USA). The use of these cartridges and their elution protocol has been validated previously [29].

During the second stage of the study, cleanup using several normal phase SPE cartridges was tested: Supelclean LC-Florisil SPE 1 g/6 mL and Supelclean LC-Si SPE 1 g/6 mL from Supelco Analytical (Sigma–Aldrich, St. Louis, MO, USA); Strata NH_2 1 g/6 mL and Strata Alumina-N 120 μm, 120 Å, 1 g/6 mL from Phenomenex (Le Pecq, France). Cartridges of the same volume were chosen, with equal sorbent mass.

The SPE was performed using a Supelco manifold (Sigma–Aldrich, St. Louis, MO, USA). The LC-Florisil, LC-Si and N-Alumina cartridges were conditioned with 10 mL of *n*-hexane, the NH_2 cartridges with 2× 20 mL of *n*-hexane. After sample deposition, PAH elution was performed with 8 mL of cyclohexane/DCM (60:40, v/v) for the first three cartridges containing polar adsorption media and with 6 mL of *n*-hexane/DCM (65:35, v/v) for the NH_2 bonded cartridges. The elution protocols were based on previous studies of SPE cleanups on pine needles, lichens and moss extracts for PAH analysis [29,40,48], which systematically used a mixture of nonpolar solvent, *n*-hexane or cyclohexane, and a slightly polar solvent, dichloromethane. The polarity of these mixtures ensures efficient PAH elution, as polar co-extracted matrix compounds stay adsorbed on the normal phase sorbents. For example, Blasco et al. carried out lichen extract cleanup with NH_2 bonded cartridges and measured 95% of PAH recovery and 98% of matrix compounds elimination with *n*-hexane/DCM elution [48].

The extracts were concentrated to 0.1 mL under a gentle stream of nitrogen (Alpha 1, Air Liquide, Paris, France), then 1 mL of acetonitrile was added. Finally, extracts were filtered with PTFE syringe filters (I.D. 13 mm, 45 μm) (Xilab, Atlanticlabo-ICS, Bruges, France), and 80 ng of internal standard FTN d_{10} was added. The extracts were stored at −20 °C.

2.4. HPLC–FLD analysis

The PAH analyses were performed with a high-performance liquid chromatography system consisting of a P680 HPLC pump and an RF 2000 fluorescence detector (Dionex, Sunnyvale, CA, USA). An 8125 low dispersion injector (Rheodyne, Rohnert Park, CA, USA) was fitted with a 20 μL loop. The system was equipped with a 250 mm × 4.6 mm I.D. Supelcosil™ LC-PAH C18 column (particle size 5 μm) and a 20 mm × 4.6 mm I.D. precolumn (particle size 5 μm) (Supelco Analytical, Sigma–Aldrich, St. Louis, MO, USA). Their temperatures were controlled with an Ultimate 3000 column compartment (Dionex, Sunnyvale, CA, USA). Chromeleon 6.80 Chromatography Data System (SR10 Build 2818 (166959)) was used for data acquisition (Dionex, Sunnyvale, CA, USA).

Elution was carried out with a binary solvent gradient of water and acetonitrile (ACN) at a flow rate of 1.5 mL min^{-1}. The gradient elution program was as follows: initial conditions with 60% ACN maintained for 5 min, followed by a 25 min linear ramp to 100% ACN and finally a 10 min plateau at 100% ACN. The column temperature was set at 30 °C. Detection was performed with selected fluorescence wavelengths to obtain the best sensitivity and minimal interference. The excitation/emission wavelength pairs (nm) are given in Table 2.

2.5. Quality assurance (QA) and quality control (QC)

Detection (LOD) and quantification (LOQ) limits were determined by studying the chromatogram obtained with a 2 ng mL^{-1} standard solution. The LOD was calculated as equal to three times the background (S/N = 3) and the LOQ as 10 times the background (S/N = 10) [49]. Quantification limits are given in Table 2.

Calibration curves were prepared for six levels (0, 2, 5, 10, 50, 150 ng mL^{-1}) and each calibration level was injected in triplicate. The linearity range for the PAH analysis extended from LOQ to 150 ng mL^{-1} with regression coefficients from 0.9993 (FLR) to 0.9999 (PHE, ANT, FTN, B(a)A, CHR, B(b)F, B(k)F, D(ah)A).

Repeatability was satisfactory since the uncertainty (as defined in (1) [50]) of 10 replicate analyses of the 2 ng mL^{-1} standard solution ranged from 1 to 10%.

$$\text{Uncertainty} = \frac{t \times \text{SD}}{\sqrt{n}} \qquad (1)$$

where t is the Student's t-value, SD is the standard deviation, and n is the number of injections.

The optimal analytical procedure showed average surrogate recoveries of 70% for ANT d_{10} and 68% B(a)P d_{12}. Reproducibility (n = 6) ranged from 1 to 22% (for PYR and ANT respectively) (cf.

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Table 2
Analytical parameters for PAH analysis by HPLC–FLD. Excitation (Exc. λ) and emission (Em. λ) wavelengths were chosen as a function of the compounds analyzed. The limits of quantification (LOQ) are expressed in mass of injected compound (pg). As a rough guide, equivalent concentrations in mosses (ng g^{-1} dry weight) have been calculated (for 1.5 g of moss sample treated, and with satisfactory PAH recoveries of 70%).

Time window	Time (min)	Exc. λ (nm)	Em. λ (nm)	Compounds	Retention time (min)	LOQ (pg)	Equivalent LOQ in mosses (ng g^{-1} DW)
1	8.0	233	320	ACE	9.2	22	1.0
				FLR	9.7	52	2.5
2	10.5	260	380	PHE	11.4	27	1.3
3	12.0	250	375	ANT-d_{10}	12.5		
				ANT	13.1	4	0.2
4	13.8	365	462	FTN-d_{10}	14.3		
				FTN	14.9	28	1.3
5	15.6	275	380	PYR	16.4	13	0.6
6	18.0	270	300	B(a)A	21.3	15	0.7
				CHR	22.5	16	0.8
7	22.1	302	431	B(b)F	26.5	6	0.3
				B(k)F	28.4	3	0.1
				B(a)P-d_{12}	29.0		
				B(a)P	30.0	12	0.6
				D(ah)A	32.3	7	0.3
8	33.4	302	419	B(ghi)P	33.9	6	0.3

Section 3.1). The accuracy was also verified with the reference material (cf. Section 3.4).

2.6. Experimental design approach

To understand the way in which PLE operating variables affect PAH extraction, individual operating variables must be considered along with interaction terms. The operating variables, temperature (T), static extraction time (t_s) and number of cycles (N), were studied using a factorial design (Nemrodw, LPRAI, Marseille, France). Each of the variables was set at 3 coded levels: −1, 0 and +1, requiring 18 experiments in total (Table 1).

The results from the factorial design can be assessed using multilinear regression (Nemrodw, LPRAI, Marseille, France), with an equation of the form:

$$Y = b_0 + b_1T + b_2t_s + b_3N + b_{12}Tt_s + b_{13}TN + b_{23}t_sN$$

where Y is the extraction recovery and b_0 the intercept. This first degree model was validated using a Fischer test. Each regression coefficient was tested using a Student's t test with a corresponding p-value. The factors whose p-values were less than 0.05 were considered as, "statistically significant".

3. Results and discussion

3.1. Optimization of the PLE method

The factorial design revealed recoveries of 56–75% for ANT d_{10} (surrogate for ACE to PYR) and from 58 to 71% for B(a)P d_{10} (surrogate for B(a)A to B(ghi)P) (Fig. 2), in agreement with the results obtained in the literature with n-hexane over the same extraction temperature ranges, static extraction time and number of cycles [33,34].

Variance analysis of the factorial design with a Fischer test validated the choice of a first degree model. Indeed, a variance coefficient of $F = 2$, strictly inferior to the Fischer coefficient $F_{\text{Fischer}} = 6$, showed that the variability of the central point was significantly lower than the variability over the factorial design.

Multilinear regression was applied to the light and heavy PAH recoveries obtained with the central factorial design. Statistical treatment of the results is shown in Table 3. At the 95% confidence level (p-value < 0.05), the PLE operational parameters studied in the factorial design had an insignificant effect on light and heavy PAH recoveries, except for extraction temperature which had a significant effect on heavy PAH recoveries (99% confidence level, p-value < 0.01). This parameter is therefore the key to PLE optimization. The interaction between the terms corresponding to the extraction temperature and the number of static cycles was particularly important as it appeared to have a significant effect on heavy PAH recoveries at the 95% confidence level (p-value < 0.05). The response surface representing heavy PAH fraction recoveries showed that optimal conditions were reached with a minimum temperature of 80 °C and a maximal extraction time of 45 min (3 cycles of 15 min) (Fig. 3). However, at the optimal temperature of

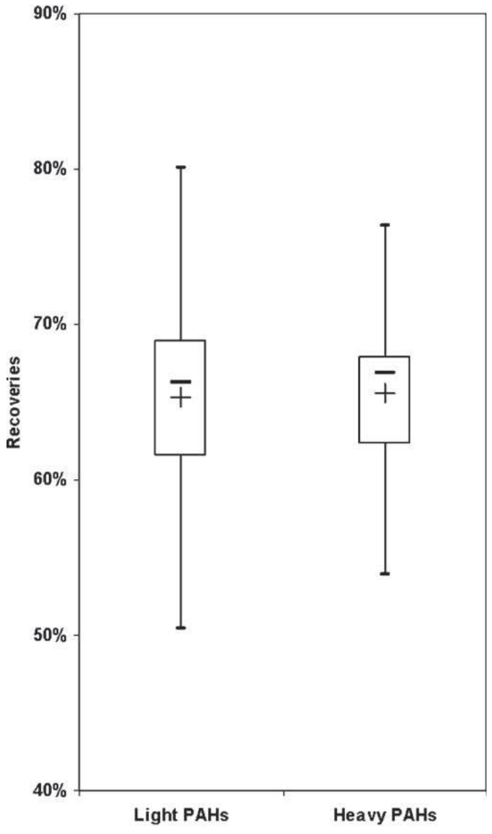

Fig. 2. Box-plots of the recoveries obtained with the PLE factorial design (1–3 static extraction cycles of 5–15 min at 80–140 °C) for light PAHs (ACE → PYR) and heavy PAHs (B(a)A → B(ghi)P). The box boundaries indicate the 25th and 75th percentiles. The horizontal lines and plus signs (+) within the boxes represent the median and mean values respectively.

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Table 3
Multilinear regression coefficients of the first order models of light and heavy PAH recoveries associated with the *p*-values obtained by Student's *t* test and their degree of "statistical significance".

Parameter	Variable	Light PAH recoveries			Heavy PAH recoveries		
		Coefficient	*p* value	Significance	Coefficient	*p* value	Significance
b_0		0.6525	<0.0001	***	0.6600	<0.0001	***
b_1	T	−0.0022	0.8900	–	−0.0281	0.0038	**
b_2	t_s	−0.0041	0.7990	–	0.0128	0.0840	–
b_3	N	−0.0194	0.2540	–	−0.0020	0.7550	–
b_{12}	$T.t_s$	−0.0074	0.6480	–	0.0089	0.2000	–
b_{13}	$T.N$	0.0014	0.9310	–	−0.0206	0.0155	*
b_{23}	$t_s.N$	−0.0286	0.1030	–	−0.0008	0.8990	–

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

80 °C, average recoveries varied insignificantly with total extraction time.

The extracts obtained with *n*-hexane were pale yellow, green or brown, depending on the operational parameters (Fig. 4). At 80 °C (1–4), the colour of the extracts was less intense than at 140 °C (5–8), as low temperatures induced less co-extraction of matrix compounds, as shown previously by Jánská et al. [39]. Moreover, with 80 °C static extraction temperatures, extracts were darker when the PLE was operated with 1 cycle (experiments 1 and 4) than with 3 cycles (experiments 2 and 3). Carrying out several cycles ensures better in-cell cleanup by Florisil®, as less extract passes through the sorbent during each flush. As low interference is necessary during HPLC–FLD analysis, extractions should therefore be performed at 80 °C with at least two static cycles. To develop the shortest analytical procedure possible, and since total extraction time had barely any influence on PAH recoveries, the optimal extraction appeared to be with 2 cycles of 5 min.

After studying the quantitative and qualitative aspects of the PLE method, the optimal parameters chosen were:

- Extraction temperature: 80 °C.
- Static extraction time: 5 min.
- Number of cycles: 2.

These conditions were similar to those found in the literature concerning the optimization of PAH extraction by PLE in environmental samples such as soil, sediments and particulate matter: generally 1–2 cycles of 5 min at 100 °C [36,38].

The optimal conditions were tested using 6 repetitions of the analytical procedure. The extracts obtained were bright yellow and did not contain any visible matrix residues. Moreover, the extracts showed average recoveries of 70% and 68% for light and heavy PAHs respectively. These results were satisfactory as they tallied with the highest quartiles of light and heavy PAH recoveries obtained with the factorial design (shown in Fig. 2). Average total PAH concentrations of 233 ± 6 ng g^{-1} were measured for 13 PAHs in *I. myosuroides* Brid. (Table 4). FLR, PHE, FTN and PYR appeared as the major compounds. These levels were within the ranges of concentrations measured in 2006–2007 in four other pleurocarpous moss species sampled from the same site [29]. These results also validate the reproducibility of the method as the relative standard deviation of the total concentrations was 2% and of the individual concentrations of 1–22% (for PYR and ANT respectively).

3.2. Comparison with Soxtec extraction

Soxtec extraction provided similar yellow extracts to those obtained with optimal PLE, as well as equivalent surrogate recoveries: 77% for light PAHs (ACE to PYR) and 67% for heavy PAHs (B(a)A to B(ghi)P). Moreover, the individual PAH concentrations measured after PLE and Soxtec extraction were within the same range, except for ACE and FLR (Table 4). During analysis, these two-ring aromatic compounds sometimes interfered with matrix compounds, leading to difficult quantification, and as they are volatile, losses may have occurred during Soxtec extraction. These observations confirm the necessity for evaluating the accuracy of the PLE method

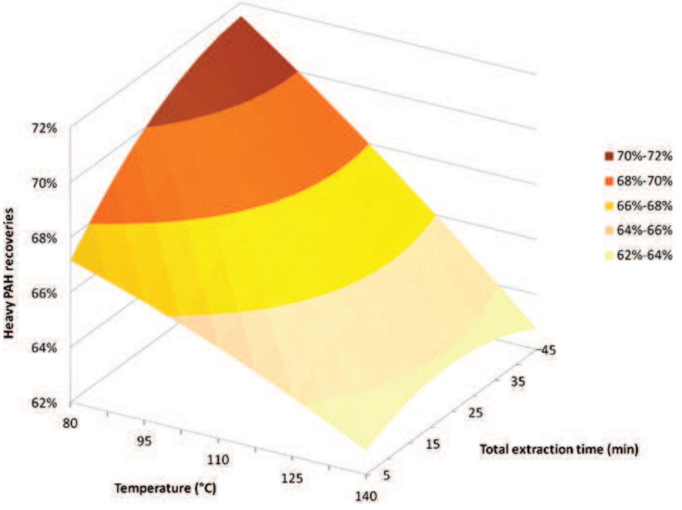

Fig. 3. Response surface of the factorial design representing recoveries of the heavy PAH fraction (B(a)A → B(ghi)P), obtained by plotting extraction temperature against total extraction time.

Table 4
Mean PAH concentrations (ng g^{-1} dry weight) and corresponding standard deviations (SD) measured in *Isothecium myorusoides* Brid. by HPLC–FLD after extraction with the optimal PLE conditions ($n = 6$) and comparison with the levels measured after Soxtec extraction ($n = 3$).

PAHs	Optimal PLE ($n = 6$)		Soxtec ($n = 3$)	
	Mean	SD	Mean	SD
ACE	2.0	0.1	2.6	0.2
FLR	35.6	3.3	14.0	2.2
PHE	29.4	3.1	29.6	3.7
ANT	1.8	0.4	1.7	0.2
FTN	43.4	1.8	42.0	6.8
PYR	96.7	1.3	100.5	18.1
B(a)A	3.4	0.6	4.2	0.4
CHR	9.5	1.1	11.0	1.3
B(b)F	2.3	0.2	2.4	0.1
B(k)F	2.2	0.4	2.1	0.1
B(a)P	4.1	0.5	4.1	0.2
D(ah)A	0.7	0.1	0.9	0.1
B(ghi)P	5.4	0.5	5.3	0.3
Total	233.5	5.5	220.3	31.1

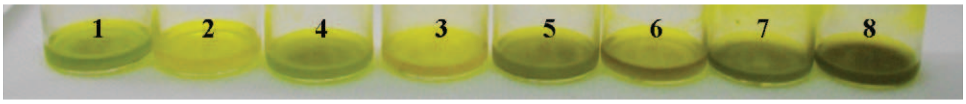

Fig. 4. Concentrated extracts obtained from experiments 1–8 of the factorial design. See Table 1 for details.

with a reference material. Average total concentrations measured with the two methods showed a low relative difference of 6%. Soxtec ensured 2 parallel extractions with 200 mL of solvent in 3.5 h (~0.6 extractions per hour), whereas optimal PLE conditions (2 static cycles of 5 min) led to 3 extractions per hour with 30 mL of solvent per sample. Therefore, optimal PLE conditions were as efficient as Soxtec for PAH extraction from mosses, with the advantage of being five times faster and of consuming three times less solvent.

3.3. Cleanup procedure

Strata NH_2 cartridges were immediately eliminated during preliminary tests because quantification with HPLC/FLD analysis was impossible due to the presence of high quantities of interfering compounds on the chromatograms. Conversely, analysis of the purified extracts obtained with Strata Alumina-N, Supelclean LC-Florisil and Supelclean LC-Si SPE cartridges was possible. Recoveries of light and heavy PAHs were determined for each type of sorbent ($n = 5$) (Fig. 5). Alumina-N recoveries were significantly lower than the 2 other sorbents. For these cartridges,

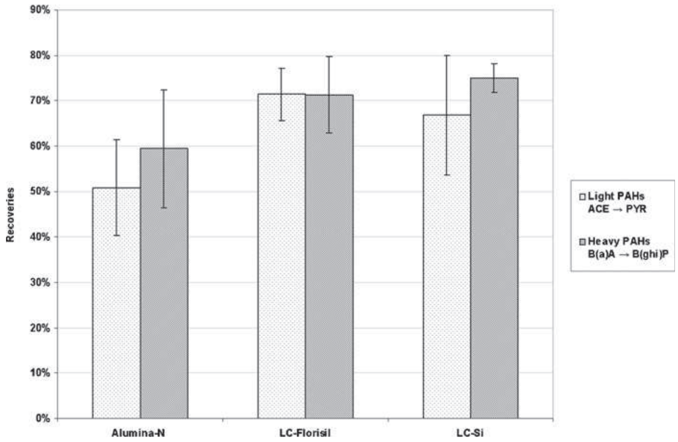

Fig. 5. Light and heavy PAH recoveries obtained under the optimal PLE conditions as a function of the SPE sorbents used for post-extraction cleanup. The error bars represent the standard deviation obtained by repeating the extraction/cleanup/analysis procedure five times with each type of cartridge.

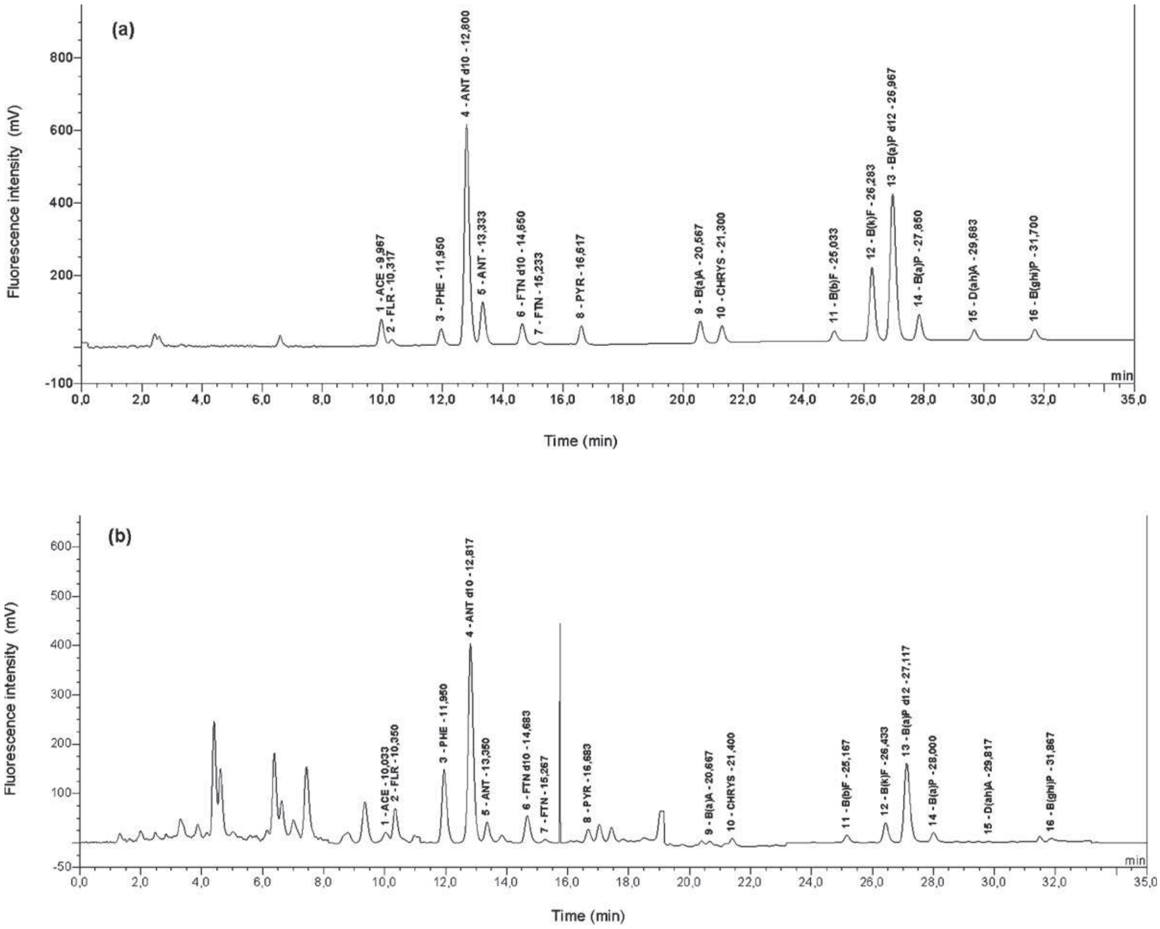

Fig. 6. HPLC chromatograms obtained by fluorescence detection (emission) after injection of: (a) a standard solution of PAHs at 10 ng L^{-1} in HPLC-grade acetonitrile (prepared with Mix 16 HAP, LGC Standards, France). (b) An extract of *Hypnum cupressiforme* moss sampled in 2010 in the Bertiz Nature Reserve, obtained with the optimal PLE method followed by cleanup using Florisil® SPE cartridges. See text and Table 2 for chromatographic experimental conditions.

average recoveries of 51% and 59% were observed for light and heavy PAHs respectively, compared to 71% for both fractions with LC-Florisil and 67% and 75% for light and heavy PAHs respectively with LC-Si cartridges. LC-Florisil and LC-Si cartridges gave equivalent cleanup PAH recoveries. However, light PAH recoveries were much more variable with LC-Si than with LC-Florisil cartridges (RSD of 20% compared to 8%). Therefore, LC-Florisil cartridges appeared to be the most appropriate for moss extract cleanup after PLE extraction, as they were the only type of SPE tested that ensured recoveries higher than 60% for all PAHs. These cartridges also appeared to give the extracts with the least co-extracts.

As cleanup of moss extracts with Florisil® ensured the highest PAH selectivity and recoveries, Supelclean LC-Florisil (1 g/6 mL) cartridges were chosen and used in subsequent experiments. Fig. 6 shows a chromatogram obtained with the optimal analytical procedure combining optimal PLE conditions (80 °C, 2 min × 5 min) and Florisil® cleanup. The chromatogram of a standard solution is also included to show the low interfering compound content in the moss extract chromatogram.

3.4. Validation of the analytical procedure

The accuracy of the optimal analytical procedure was verified by applying PLE extraction three times, under the optimal conditions (2 min × 5 min at 80 °C) followed by cleanup with Supelclean LC-Florisil (1 g/6 mL) cartridges, to a reference material (IAEA-140-OC Fucus (35 g) from ANALAB) containing the 13 target PAHs.

The International Atomic Energy Agency (IAEA) supply reference material with recommended values for 9 target PAHs (ANT, PHE, FTN, PYR, B(a)A, CHR, B(k)F, B(a)P, B(ghi)P), established on the basis of 8–28 statistically valid results submitted during an international laboratory inter-comparison exercise [43]. As shown in Fig. 7, concentrations of 8 target PAHs measured (ANT, PHE, FTN, PYR, CHR, B(k)F, B(a)P, B(ghi)P) were included in the recommended value ranges (95% confidence intervals). Only B(a)A was slightly overestimated, due to the presence of chromatographic interfering peaks, because the reference material contains numerous other organic compounds such as polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs).

The IAEA also supply information values for ACE, FLR, B(b)F and D(ah)A (established with 3–7 accepted laboratory results). The concentrations measured with optimal PLE and SPE conditions were within the information value 95% confidence intervals.

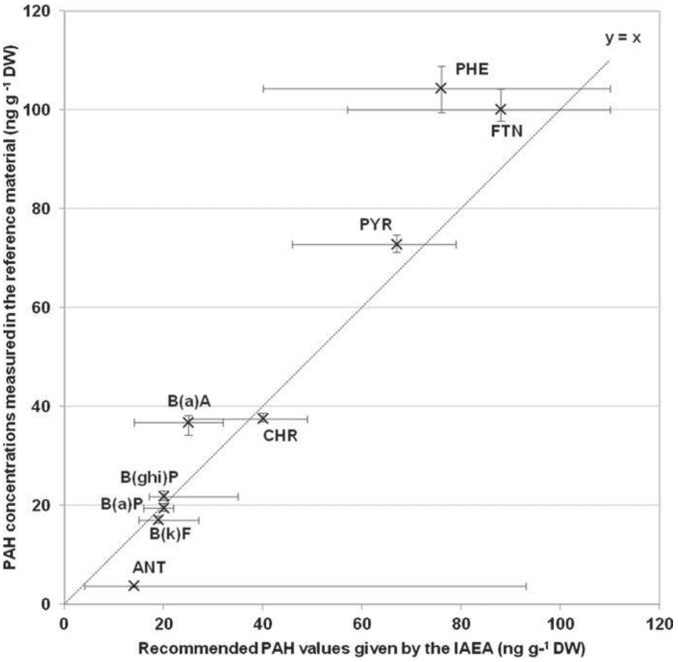

Fig. 7. Concentrations of 9 target PAHs measured in the reference material IAEA-140/OC Fucus (35 g) (ANALAB, France) as a function of the recommended values (ng g^{-1}, based on dry weight DW). The analytical procedure was performed on 3 replicates of reference material. The experimental levels are represented by the mean concentration, and the error bars correspond to the concentration range measured (minimum and maximum). The recommended values are represented by the mean value given by the IAEA, and the error bars correspond to the 95% confidence intervals (based on 8–28 results depending on compound). The dotted line ($y = x$) represents the case where the experimental value is equal to the recommended value.

3.5. Study of spatial and inter-species variability at a rural site

Using the optimal analytical procedure, the 13 target PAHs were quantified in the moss samples collected from the remote site in the Aralar Range located in Navarra, Spain (Table 5). Average total concentrations of 133 ± 5 and 130 ± 5 ng g^{-1} (dry weight) were measured in the *H. splendens* (Hedw.) Schimp. and *H. cupressiforme* Hedw. samples, respectively. The levels were of the same order of magnitude as those measured in the *H. splendens* and *H. cupressiforme* mosses sampled over the past years in rural areas of European countries [4,27,32]. A previous study carried out in 2008

Table 5
Mean and median individual and total concentrations (ng g^{-1} dry weight) and corresponding standard deviations (SD) of 13 PAHs in two species of moss (*Hylocomium splendens* (Hedw.) Schimp. and *Hypnum cupressiforme* Hedw.) sampled at a remote site in the Aralar Range (Navarra, Spain). The results were obtained by applying the optimal extraction/cleanup/analysis procedure to 9 samples of *Hylocomium splendens* and 3 samples of *Hypnum cupressiforme*.

PAHs	Concentrations (ng g^{-1} DW)			Concentrations (ng g^{-1} DW)		
	Hylocomium splendens (Hedw.) Schimp.			*Hypnum cupressiforme* Hedw.		
	Mean	Median	SD	Mean	Median	SD
Acenaphthene (ACE)	3.5	3.1	0.4	2.9	2.9	0.2
Fluorene (FLR)	30.3	30.1	2.8	24.0	24.0	3.3
Phenanthrene (PHE)	19.2	19.8	1.3	19.0	17.9	1.9
Anthracene (ANT)	1.3	1.1	0.2	1.2	1.2	0.1
Fluoranthene (FTN)	15.5	15.5	0.8	15.7	15.5	0.5
Pyrene (PYR)	26.3	25.8	4.0	31.9	32.6	2.3
Benz(a)anthracene (B(a)A)	4.4	4.1	0.7	2.8	2.6	0.3
Chrysene (CHR)	8.2	7.9	0.9	7.6	8.3	1.3
Benzo(b)fluoranthene (B(b)F)	8.5	8.2	0.8	7.2	7.1	0.7
Benzo(k)fluoranthene (B(k)F)	3.5	3.5	0.4	3.0	3.0	0.2
Benzo(a)pyrene (B(a)P)	5.2	5.0	0.5	4.0	4.1	0.3
Dibenz(a,h)anthracene (D(ah)A)	1.6	1.5	0.3	1.2	1.2	0.1
Benzo(g,h,i)perylene (B(ghi)P)	5.8	5.5	0.6	4.4	4.5	0.7
Total	133.3	131.2	4.8	130.0	125.0	4.7

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in the Bertiz Nature Reserve, located 35 km from the Aralar Range sampling site, also showed average total levels for the 13 PAHs of approximately 100 ng g^{-1} (dry weight) in the moss *H. cupressiforme* [29].

Average individual concentrations ranged from 1.3 ± 0.2 to 30 ± 3 ng g^{-1} (dry weight) for ANT and FLR respectively in *H. splendens* and from 1.2 ± 0.1 to 32 ± 3 ng g^{-1} (dry weight) for ANT and PYR respectively in *H. cupressiforme*. The major compounds in both species were FLR, PHE, FTN and PYR, with concentrations over 10 ng g^{-1} (dry weight), as previously found in the Bertiz Nature Reserve [29].

The nine samples of *H. splendens* were sampled from soil at several points over 200 m, at the limit between grassland and forest, where local conditions varied slightly (light intensity, throughfall precipitation...) in function of the canopy cover. The analysis of these samples revealed relative standard deviations lower than 20% for all target PAHs, of the same order as the reproducibility previously determined in this study with a homogenous composite sample. The low variability showed that the local environmental conditions had a negligible influence on bioaccumulation of PAHs by the mosses.

Individual concentrations of FLR, B(a)A, B(b)F, B(k)F, B(a)P, D(ah)A, B(ghi)P were significantly lower in *H. cupressiforme* than in *H. splendens* samples. PYR concentrations were significantly higher in *H. cupressiforme*. The quantity of PAHs bioaccumulated was therefore specific to the moss species, as shown previously by Galuszka [32] who observed higher PAH accumulation in *H. splendens* than in *Pleurozium schreberi* mosses. The variability of bioaccumulation between species can be explained by their different morphologies and/or their growth conditions. *H. splendens* is a weft moss with feathery fronds, whereas *H. cupressiforme* has prostrate, creeping stems which form smooth, dense mats [51]. Moreover, *H. cupressiforme* was growing on tree stumps and dead branches under forest cover, whereas *H. splendens* was collected from soil at the limit between the forest and grassland. Therefore, to biomonitor atmospheric deposition of PAHs with several moss species, preliminary inter-species calibration is necessary.

4. Conclusions

PAH extraction from the moss *I. myosuroides* Brid., using Soxtec with *n*-hexane showed average PAH recoveries of 67–77% and concentrations of approximately 200 ng g^{-1} (dry weight). To increase the extraction turnover and reduce solvent consumption, PLE was tested. The PLE factorial design revealed the optimal extraction conditions: 80 °C for 2 cycles of 5 min, giving maximum recoveries and ensuring good selectivity for moss PAH determination at trace levels. Under optimal PLE conditions, satisfactory average recoveries of 68–70% were obtained and concentrations similar to those for the Soxtec extraction procedure were measured, whereas solvent volume was divided by 3 and total extraction time by 5. Several normal phase SPE cleanup steps were tested. Supelclean LC-Florisil (1 g/6 mL) cartridges appeared to be the most efficient since the purified extracts contained the least interfering compounds and the highest PAH concentrations. The accuracy of the optimal analytical procedure, combining the optimal PLE conditions with cleanup by Florisil® SPE, was verified on a reference material.

Finally, the optimal analytical procedure was applied to samples of *H. splendens* (Hedw.) Schimp. and *H. cupressiforme* Hedw. collected at a remote site in Navarra (Spain) in June 2010. The target PAHs were quantifiable and showed similar levels to those of previous studies carried out in Europe and locally in Navarra. The study revealed negligible on-site spatial variability for the PAH levels in the *H. splendens* samples, but significant differences between the levels in the two species, due to their morphology and growth conditions.

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Annexe XIV.

Mosses as biomonitors of atmospheric POPs pollution: A review

Harmens, H., Foan, L., Simon,V., Mills, G., 2010

http://icpvegetation.ceh.ac.uk/publications/documents/FinalPOPsreportDefra21.10.11.pdf

Mosses as biomonitors of atmospheric POPs pollution:
A review

October, 2011

Defra Contract AQ0816 and CEH project NEC04062

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Executive summary

Background

Worldwide there is concern at the continuing release of persistent organic pollutants (POPs) into the environment. These chemical substances are transported across international boundaries far from their sources and they persist in the environment, bioaccumulate through the food chain, and pose a risk to human health and the environment. International action has therefore been taken in the form of two international agreements to protect human health and the environment from POPs:

- The Protocol on Persistent Organic Pollutants to the Convention on Long-Range Transboundary Air Pollution (treaty for the UNECE region[1]);
- The Stockholm Convention on Persistent Organic Pollutants (a global treaty).

Regulation (EC) No 850/2004 of the European Parliament on persistent organic pollutants and amending Directive 79/117/EEC1 implement both of these agreements for all of EU member states.

In Europe the emission and deposition of POPs are monitored and modelled by the European Monitoring and Evaluation Programme (EMEP). EMEP has established a model for calculating air concentrations, atmospheric transport and deposition fluxes of selected POPs using emission data as input. In 2009, there were 23 EMEP monitoring sites in total in 17 countries measuring selected POPs for model validation. Mosses adsorb/absorb pollutants and nutrients directly from the air as they not have a root system or cuticle. The accumulation of these substances is aided by the high surface to volume ratio of moss tissue. Mosses are successfully being used to monitor spatial patterns and temporal trends of heavy metals and nitrogen at a high spatial resolution in a cost-effective manner. In this report we review whether mosses can also be applied as biomonitors of POPs.

Aims

- To review current knowledge on the application of mosses as biomonitors of POPs;
- To identify knowledge gaps and make recommendations for future research.

Mosses as biomonitors of POPs

The majority of published studies have focussed on the determination of polycyclic aromatic hydrocarbons (PAHs) in mosses and relative few studies have been conducted on other POPs. So far, many studies have focused on spatial trends around pollution sources or the concentration in mosses in remote areas as an indication of long-range transport of POPs. Few studies have determined temporal trends or have directly related the concentrations in mosses with measured atmospheric concentrations and/or deposition fluxes. Examples in the literature show that mosses are suitable organisms to monitor spatial patterns and temporal trends of atmospheric concentrations and deposition of POPs to vegetation. These examples include PAHs, polychlorobiphenyls (PCBs), dioxins and furans, and polybrominated diphenyl ethers (PBDEs).

In the currently ongoing European moss survey of 2010/11 coordinated by the ICP Vegetation, six countries will determine the concentration of selected POPs (PAHs in particular) in mosses in a pilot study to investigate the suitability of mosses as biomonitors of

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[1] United Nations Economic Commission for Europe

POPs at a regional scale. To further establish the suitability of mosses as biomonitors of POPs across Europe it will be paramount to sample mosses at sites where atmospheric POPs concentrations and/or deposition fluxes are determined, for example at EMEP monitoring sites or national POPs monitoring sites. Once a good relationship has been established between POPs concentrations in mosses and measured air concentrations and/or deposition fluxes, data from the European moss monitoring network can be used to complement data from the limited number of EMEP measurement sites to assess the performance of the EMEP model for POPs in a cost-effective manner.

Research recommendations

- To establish the relationship between air concentrations and/or deposition fluxes of POPs and their concentration in mosses, mosses should be sampled near measurement sites for air concentrations and/or deposition fluxes.

- The impacts of factors that might affect such a relationship should be studied in more detail. Such factors might include: different moss species, altitude, amount of precipitation, temperature, seasonality.

- To establish spatial trends in mosses across Europe, bearing in mind the outcome of the above research recommendations, more countries should determine the concentration of various, suitable POPs in mosses as part of the European moss survey.

- To establish temporal trends in mosses across Europe, the POPs survey in mosses should be repeated at regular time intervals, e.g. every five years.

- To assure good quality and comparable data, an inter-laboratory calibration exercise is required to assess the performance of laboratories participating in a European moss survey. In addition, an agreed sampling protocol should be developed.

Contents

1 Introduction

1.1 Aims

- To review current knowledge on the application of mosses as biomonitors of POPs;
- To identify knowledge gaps and make recommendations for future research.

1.2 Background

Worldwide there is concern at the continuing release of persistent organic pollutants (POPs) into the environment. POPs are organic substances that (LRTAP Convention, 1998):

- possess toxic characteristics;
- are persistent;
- bioaccumulate;
- are prone to long-range transboundary atmospheric transport and deposition;
- are likely to cause significant adverse human health or environmental effects near to and distant from their source.

POPs are mainly of anthropogenic origin, show weak degradability and consequently are accumulating in the environment across the globe, including remote areas such as the (Ant)Arctica. The combination of resistance to metabolism and lipophilicity ('fat-loving') means that POPs will accumulate in food chains (Jones and de Voogt, 1999). POPs are readily absorbed in fatty tissue, where concentrations can become magnified by up to 70,000 times the background levels. Fish, predatory birds, mammals, and humans are high up the food chain and so absorb the greatest concentrations. Specific effects of POPs can include cancer, allergies and hypersensitivity, damage to the central and peripheral nervous systems, reproductive disorders, and disruption of the immune system. Some POPs are also considered to be endocrine disrupters, which, by altering the hormonal system, can damage the reproductive and immune systems of exposed individuals as well as their offspring; they can also have developmental effects (Belpomme et al., 2007; Jones and de Voogt, 1999; TFHTAP, 2010; WHO, 2003). Their ecotoxicity has been highlighted in aquatic (Leipe et al., 2005) and terrestrial ecosystems (e.g. Oguntimehin et al., 2008; Smith et al., 2007).

Spatial and some temporal trends in the concentration of selected POPs in fish were reviewed by ICP Waters (2005). Recent results indicated that atmospherically transported POPs can impact the health of fish in remote ecosystems and that they can reach concentrations in fish that exceed health threshold values for consumption by wildlife and humans (TFHTAP, 2010). However, these impacts and exceedances were not observed at Alaskan parks where the contaminant load is due solely to long-range atmospheric transport and not influenced strongly by regional sources. There is currently no strong evidence that Antarctic organisms or ecosystems are being impacted by POPs associated solely with long-range atmospheric transport. There are reasons to believe, however, that Antarctic organisms might be more susceptible to effects of POPs than temperate organisms, though more research is needed in this area (TFHTAP, 2010). Currently there is little information on long-term trends of POPs concentrations in the environment, food and human media

1.3 International agreements to protect the environment from POPs

The 1998 Aarhus Protocol on POPs of the Convention on Long-range Transboundary Air Pollution (LTRAP) and the 2001 Stockholm Convention on POPs, a global treaty under the

United Nations Environment Programme (UNEP), aim to eliminate and/or restrict the production and use of selected POPs.

LRTAP Convention Protocol on Persistent Organic Pollutants

The Executive Body of the LRTAP Convention adopted the Protocol on POPs on 24 June 1998 in Aarhus (Denmark). It focused on a list of 16 substances that have been singled out according to agreed risk criteria; many substances on the list are chlororganic pesticides (Figure 1, Table 1). The ultimate objective is to eliminate any discharges, emissions and losses of POPs. The Protocol bans the production and use of some products outright (aldrin, chlordane, chlordecone, dieldrin, endrin, hexabromobiphenyl, mirex and toxaphene). Others are scheduled for elimination at a later stage (DDT, heptachlor, hexaclorobenzene, PCBs). Finally, the Protocol severely restricts the use of DDT, HCH (including lindane) and PCBs. The Protocol includes provisions for dealing with the wastes of products that will be banned. It also obliges Parties to reduce their emissions of dioxins, furans, PAHs and HCB below their levels in 1990 (or an alternative year between 1985 and 1995). For the incineration of municipal, hazardous and medical waste, it lays down specific limit values.

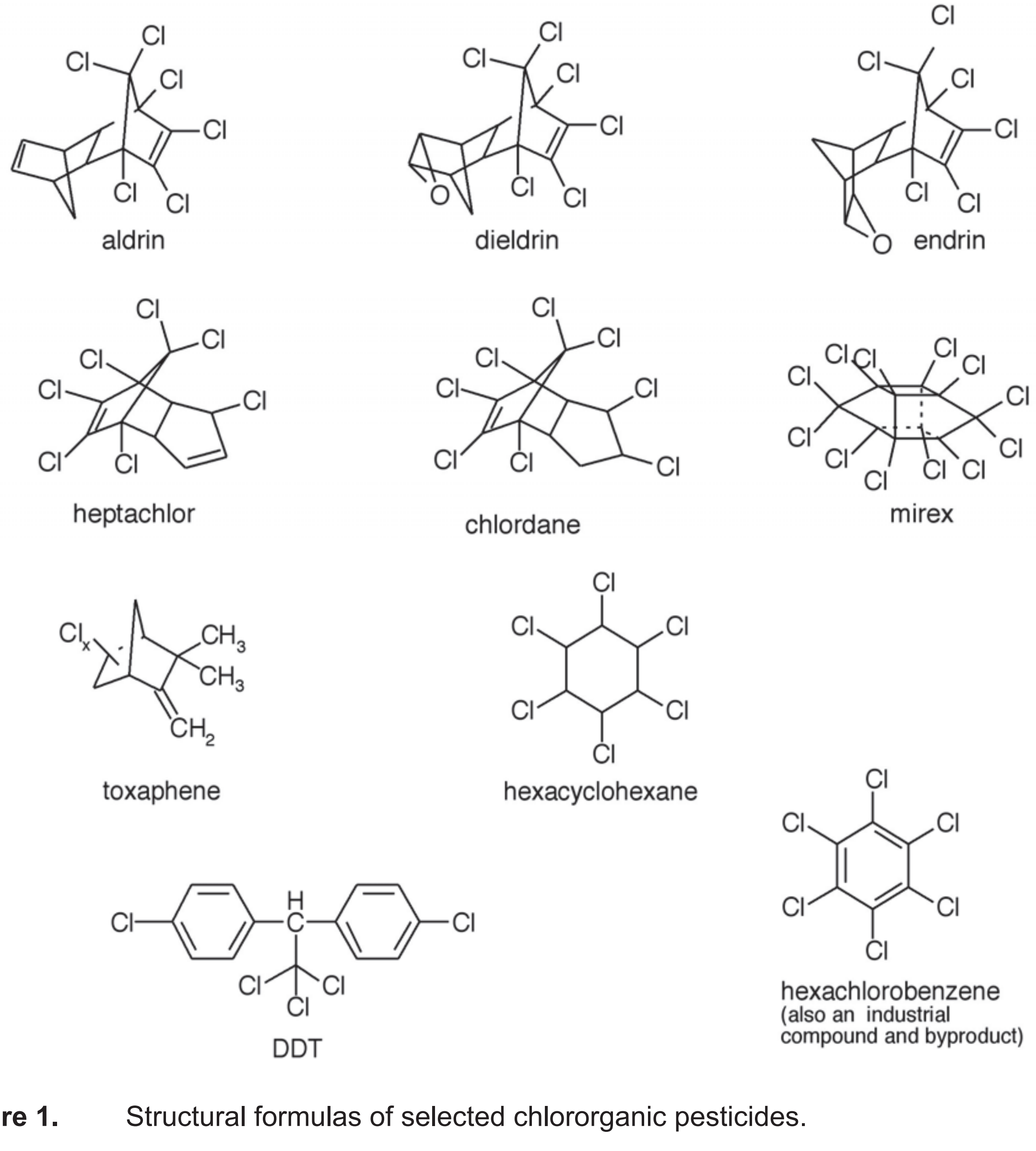

Figure 1. Structural formulas of selected chlororganic pesticides.

The Protocol entered into force on 23 October 2003 and has been signed by 30 Parties to the LRTAP Convention. On 18 December 2009, seven new substances were included in the Protocol (Table 1). Furthermore, the obligations for DDT, heptachlor, hexachlorobenzene and PCBs as well as emission limit values from waste incineration were revised. These amendments have not yet entered into force for the Parties that adopted them.

Stockholm Convention on Persistent Organic Pollutants

The text of the Stockholm Convention on POPs was adopted on 22 May 2001, focusing on 12 substances, and entered into force on 17 May 2004. The Convention text was further amended in 2009 to include nine new substances (Table 1). The Stockholm Convention generally includes the same substances as the POPs Protocol of the LRTAP Convention but not all (e.g. it does not include PAHs and hexachlorobutadiene). Another difference is that the Stockholm Convention is a global treaty whereas the LRTAP Convention Protocol is limited to the UNECE region.

Table 1. POPs included in the Protocol of the LRTAP Convention and the Stockholm Convention. Note: some substances have been grouped.

POP	LRTAP Convention Protocol on POPs	Stockholm Convention	Pesticide	Industrial chemical	By-product
Aldrin	1998	2001	X		
Chlordane	1998	2001	X		
Dichlorodiphenyltrichloroethane (DDT)	1998	2001	X		
Dieldrin	1998	2001	X		
Endrin	1998	2001	X		
Heptachlor	1998	2001	X		
Hexachlorobenzene (HCB)	1998	2001	X	X	X
Mirex	1998	2001	X		
Toxaphene	1998	2001	X		
Polychlorobiphenyls (PCBs)	1998	2001		X	X
Dioxins (PCDDs)	1998	2001			X
Furans (PCDFs)	1998	2001			X
Chlordecone	1998	2009	X		
Hexachlorocyclohexane (HCH) (including lindane)	1998	2009	X		X
Hexabromobiphenyl (HBB)	1998	2009		X	
Polycyclic aromatic hydrocarbons (PAHs)	1998				X
Hexachlorobutadiene	2009				X
Pentachlorobenzene	2009	2009	X	X	X
Polybrominated diphenyl ethers (PBDEs)	2009	2009		X	
Perfluorooctane sulfonic acid, its salts and perfluorooctane sulfonyl fluoride (PFOS)	2009	2009		X	
Polychlorinated naphthalenes	2009			X	X
Short-chain chlorinated paraffins (SCCPs)	2009			X	X

EU regulations

Regulation (EC) No 850/2004 of the European Parliament and the Council of 29 April 2004 on persistent organic pollutants and amending Directive 79/117/EEC[2] implement both of these agreements for all of EU member states. The EU regulation was implemented in the UK in 2007. The EU Regulation REACH on production and use of chemicals in the European Union entered into force in June 2007. Its purpose is to ensure a high level of protection of human health and the environment. In particular, one of the important objectives of the Regulation is to reduce emissions of substances of very high concern (SVHC) by restriction of use and replacement by less dangerous substances or technologies (TFHTAP, 2010). POPs are a subclass of SVHC. REACH implements the precautionary principle and requires the industry to prove that the substances do not adversely affect human health or the environment before they are allowed on the market. According to the Regulation, chemical substances shall not be manufactured or placed on the market in the European Community unless they have been registered in the European Chemical Agency (ECHA). Within the registration process certain information on a substance should be submitted by the industry. In particular, this includes the information on physical-chemical properties of a substance, its environmental fate properties, its toxicological and ecotoxicological properties, possible harmful effects on human health and the environment, emission estimates, and monitoring data for SVHC.

1.4 Measurements and modelling of air concentrations and deposition of POPs

In Europe the emission and deposition of POPs are monitored and modelled by the European Monitoring and Evaluation Programme (EMEP; Gusev et al., 2011). EMEP has established a model for calculating air concentrations, atmospheric transport and deposition fluxes of selected PAHs, dioxins, furans, PCBs and lindane. In 2009, there were 23 EMEP monitoring sites in total in 17 countries measuring POPs for model validation, and 13 of them conducted measurements of POP concentrations in both air and precipitation. POPs measured using passive air samplers included pesticides, PAHs, HCHs, HCB and PCBs (Gusev et al., 2011). Most of the recent additions to EMEP were the sites measuring PAHs which is required by the EU air quality directive. The EMEP model calculated elevated levels of contamination by PAHs for Central and Eastern Europe, Portugal and the western part of Spain in 2009. Benzo[a]pyrene (B[a]P) concentrations at EMEP measurement sites for 2009 are shown in Figure 2. Model simulations of benzo[a]pyrene (B[a]P) pollution within the EMEP region showed that transboundary transport was a significant source of pollution for a lot of the EMEP countries contributing between 30% to 70% to total annual deposition. For 25 countries in 2009 its contribution exceeded 50%. Comparison of modelling results and measurements of B[a]P in air for 2009 showed that for most of the sites differences between the modelled and observed concentrations ranged within 10-30%. According to officially submitted data and expert estimates, emissions of four indicator PAHs within the EMEP domain decreased by 30-40% depending on the compound in the period of 1990-2009. Model evaluation of trends in B[a]P pollution levels showed that levels of its annual mean air concentrations declined in this period by about 30% (Gusev et al., 2011).

High uncertainties exist in the modelling of dioxins and furans (PCDD/F) with modelling values underestimating measurement values by a factor 5 on average. Different level of underestimation for different congeners indicated that there are essential uncertainties in

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[2] Available at http://eur-lex.europa.eu/LexUriServ/site/en/oj/2004/l_229/l_22920040629en00050022.pdf

determination of congener composition of PCDD/F emissions. The reasons for model underestimation of air concentrations and deposition fluxes are both uncertainties of emission totals and uncertainties in evaluation of congener composition of emissions. In addition, uncertainties in measuring PCCD/F were observed with large inter-laboratory differences. In comparison with PAHs and PCDD/Fs, evaluation of HCB pollution levels is complicated by more essential uncertainties in information on current sources of HCB release into the environment and historical emissions. Analysis of modelling results on HCB using available measurements of air concentrations revealed that model predictions underestimated observed pollution levels. This underestimation can be related to the incompleteness of available officially submitted emission data and expert estimates as well as with the underestimation of the role of secondary emission sources. Re-volatilization of HCB from environmental compartments can contribute significantly to the contemporary pollution levels. To evaluate HCB re-emissions elaboration of scenarios of historical HCB emissions is required. The environmental levels of lindane (γ-HCH) have been significantly decreased within the European region during two recent decades due to the reduction of its application. Reduced influence of primary γ-HCH emission sources increase the importance of the contributions of secondary emissions and intercontinental transport to the contamination of the EMEP region (Shatalov et al., 2010).

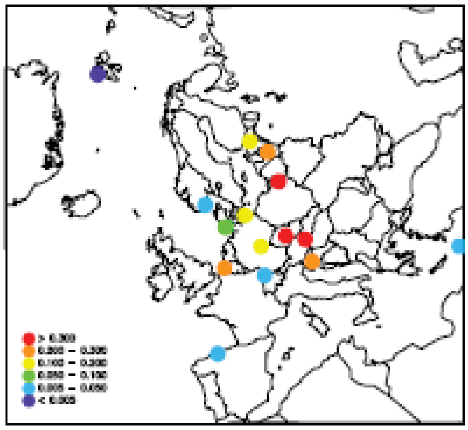

Figure 2. Spatial distribution of the annual average concentrations (ng m^{-3}) of B[a]P in 2009 at EMEP measurement sites (Gusev et al., 2011).

Analysis of sensitivity of POP pollution levels to variation of meteorological and environmental factors showed that factors such as temperature, precipitation amount, wind speed and direction, outflow of air masses through the country boundaries, and vegetation cover, can in most cases explain 90–95% of seasonal variability of chemicals air concentrations for a country. The effect of changes of meteorological and environmental conditions can be different for different POPs due to wide range of variations of their physical-chemical properties. Besides, the sensitivity of POP pollution levels to variations of meteorological and environmental parameters varies within the EMEP region which can lead to varied response to the climatic changes across Europe.

2 Mosses as biomonitors of POPs

2.1 Introduction

As mosses do not have a root system or cuticle, they adsorb/absorb nutrients and pollutants from the air, which often accumulate on or in moss tissue. The accumulation is aided by the high surface to volume ratio of moss tissue. The monitoring of heavy metal and nitrogen concentrations in naturally growing mosses allows determination of spatial patterns and temporal trends of heavy metal and nitrogen pollution and deposition at a high spatial resolution in a cost-effective manner (Harmens et al., 2010, 2011). Detailed statistical analysis of the factors affecting heavy metal concentration in mosses confirmed that EMEP modelled heavy metal deposition was the main predictor for cadmium and lead concentrations in mosses; this was not the case for mercury (Holy et al., 2010; Schröder et al., 2010b). The latter might be related to the specific chemistry of mercury (Harmens et al., 2010). Detailed statistical analysis of the factors affecting nitrogen concentration in mosses also showed that EMEP modelled air concentrations of different nitrogen forms and total nitrogen deposition are the main predictors for the total nitrogen concentrations in mosses (Schröder et al., 2010a), despite the fact that nitrogen is an essential macronutrient and remobilised from senescing tissue (in contrast to the non-essential heavy metals cadmium, lead and mercury). In Switzerland, a high correlation ($r^2 = 0.91$) was found between site-specific measurements of bulk nitrogen deposition and the total nitrogen concentration in mosses, confirming the potential to use mosses as biomonitors of nitrogen deposition at a high spatial resolution (Harmens et al., 2011).

Although mosses have also been used to monitor POPs pollution, the number of studies is limited and most studies have focussed on PAHs. In this chapter we review the application of mosses as monitors of POPs pollution.

2.2 PAHs pollution and biomonitoring with mosses

PAHs are a family of chemical compounds constituted by carbon and hydrogen atoms which form at least two condensed aromatic rings (Figure 3). The majority of PAHs originate from fossil or non-fossil fuels by pyrolysis or pyrosynthesis. PAHs are emitted in the atmosphere mainly from anthropogenic source but they also originate from natural sources such as volcanic eruptions and forest fires (Simonich and Hites, 1995). The main sources of PAHs in the environment are aluminium production, coke production from coal, wood preservation and fossil fuel combustion (traffic, domestic heating, electricity production; Wegener et al., 1992). Eight PAHs have been classified by US Environmental Protection Agency (US EPA, 1997) as potentially carcinogenic: benzo*(a)*anthracene, chrysene, benzo*(a)*pyrene, benzo*(b)*fluoranthene, benzo*(k)*fluoranthene, dibenzo*(a,h)*anthracene, indeno*(1,2,3-c,d)*pyrene and benzo(g,h,i)perylene). Some PAHs also are mutagenic, teratogenic, immunosuppressive and/or neurotoxic (Gałuszka, 2000). They are all listed in the European lists of priority pollutants. However, only benzo*(a)*pyrene is regulated by the Directive 2004/107/EC (EU, 2004) which sets the maximum atmospheric concentration acceptable at 1 ng/m^3 (measurement with PM10, the atmospheric suspended particles of diameter under 10 µm).

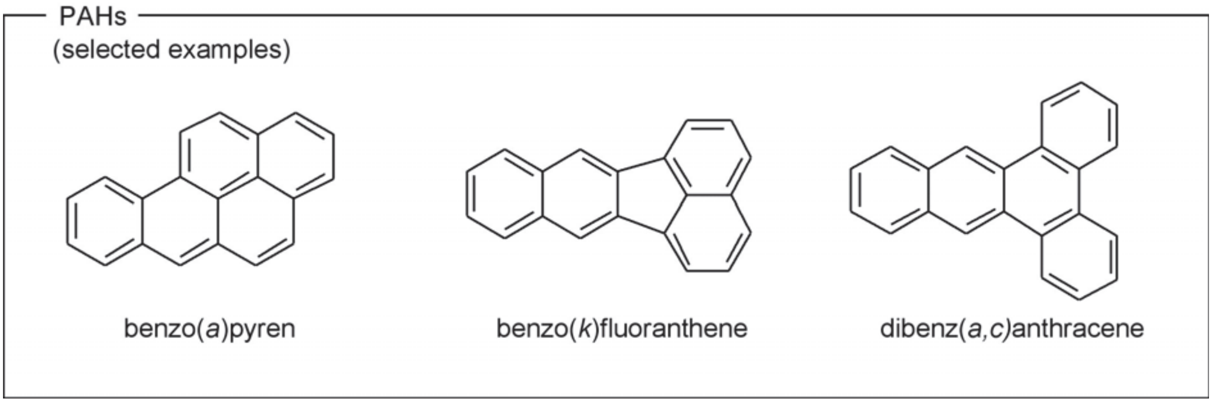

Figure 3. Structural formulas of selected PAHs.

The mechanism of uptake of organic pollutants by vegetation is governed by the chemical and physical properties of the pollutant (such as their molecular weight, aqueous solubility, and vapour pressure), environmental conditions (e.g. atmospheric temperature), and the plant species and structure (Simonich and Hites, 1995). After emission in the atmosphere, the most volatile PAHs remain in gaseous phase whereas the least volatile (5 or 6 rings) are adsorbed on solid atmospheric particles. Deposition to vegetation occurs through uptake of the lipophilic compounds in both vapour and particle phases, but there may also be a removal at for example higher ambient temperatures. PAHs of intermediate volatility (3 or 4 rings) are distributed between gaseous and particulate phases (Viskari et al., 1997). In the winter, however, PAHs are predominantly in the particulate phase due to increased emissions and their low degree of volatilization at low temperatures. PAHs in the gaseous phase are generally transported to areas remote from main pollution sources, whereas particulate absorbed PAHs are generally deposited in higher proportions near emission sources (Thomas, 1986). This might explain why often PAHs in mosses sampled away from local pollution sources are dominated by smaller ring numbers of 3 or 4 (Dołęgowska and Migaszewski, in press; Gałuszka, 2007; Migaszewski et al., 2009; Orliński, 2002). Table 2 provides an overview of the concentrations measured in various mosses sampled in rural environments away from pollution sources. Gerdol et al. (2002) observed that the fraction of low molecular weight volatile PAHs was greater in rural compared to urban sites. On the other hand, the dominance of 3 ring compounds appears to be related to the type of pollution source as are the dominance of individual PAHs (Foan et al., 2010). Phenanthrene, fluoranthene and pyrene have often been reported as the dominant PAHs in mosses sampled away from pollution sources (Foan et al, 2010; Gałuszka, 2007; Krommer et al., 2007; Zechmeister et al., 2006; see Table 2). In Hungary, a good correlation between total PAHs concentrations in *Hypnum cupressiforme* and traffic volume was observed, but not with population density, with 99% of the total PAHs concentration in the moss consisting of low molecular weight (Ötvös et al., 2004).

Ares et al. (2009) showed an exponential decay of PAHs levels in mosses around emission sources. Using moss bags in active biomonitoring of PAHs near a road in Finland, Viskari et al. (1997) found that downwind of the road the concentrations of most PAHs in mosses declined to background levels between 60 – 100 m from the road. Therefore, studies carried out in remote areas, located at a fair distance from emission sources, provide an indication of background levels of atmospheric PAH contamination due to long-range transboundary air pollution.

Table 2. Average and range (within brackets) of PAH concentrations (ng g^{-1} DW) in mosses sampled in rural areas. LOD = limit of detection.

	Holoubek et al., 2000 Czech Republic		**Migazewski et al., 2002 Poland**		**Zechmeister et al., 2006 Austria**	**Krommer et al., 2007 Austria**		**Galuszka, 2007 Poland**		**Foan et al., 2010 Spain**	
Sampling period	1988-1994		2000		2003	2005		2005		2006-2007	
PAHs analyzed (number of rings)	16 (US EPA)		17		16 (US EPA)	17		16		13	
Naphtalene (2)	2.6	(<1 - 640)			6.7	7.3	(1 - 13)				
Acenaphtene (2)	45.3	(<1 - 1183)	<4		1.8	3.1	(2.1 - 5.7)	2	(<1 - 3)	4.1	(<1.5 - 12.7)
Acenaphtylene (2)	7.8	(<0.5 - 163)	5	(4 - 6)	< LOD	0.6	(0.3 - 6.6)	5	(2 - 11)		
Fluorene (2)	68.8	(<1 - 933)	11.5	(10 - 13)	3.9	4.6	(3.8 - 6.6)	13	(8 - 23)	15.1	(<10.4 - 21.3)
Phenanthrene (3)	43.3	(<0.6 - 380)	82.5	(82 - 83)	55	30.1	(24 - 63)	85	(46 - 162)	81.1	(26.9 - 142.2)
Anthracene (3)	68.6	(<0.6 - 2280)	<4		1.4	1.6	(1.2 - 12)	5	(2 - 21)	3.2	(1.2 – 9.9)
Fluoranthene (3)	18.9	(<0.6 - 325)	96	(88 - 104)	14	16.4	(13 - 140)	112	(40 - 420)	38.1	(10.2 - 152.7)
Pyrene (4)	128.5	(<0.9 - 525)	68.5	(65 - 72)	12	12.7	(8.5 - 94)	87	(31 - 356)	18.5	(6.8 – 39.0)
Benzo*(a)*pyrene (5)	13.7	(<0.9 - 311)	22	(18 - 26)	1.5	4.4	(2.9 - 32)	21	(4 - 123)	3.1	(< 1.2 - 7.0)
Chrysene (4)	74.6	(<0.6 - 1190)	69.5	(61 - 78)	4.0	8.4	(5.6 - 27)	44	(15 - 141)		
Benzo*(b)*fluoranthene (4)	5.3	(<0.6 - 84)	71.5	(64 - 79)	4.3	12.9	(8.3 - 46)	41	(19 - 83)	3.0	(1.8 - 5.5)
Benzo*(k)*fluoranthene (4)	6.0	(<0.6 - 120)	33.5	(31 - 36)	2.7	5.3	(3.6 - 18)	11	(<3 - 38)	0.8	(< 0.5 - 1.8)
Benzo*(a)*pyrene (5)	37.9	(<0.3 - 540)	21.5	(12 - 31)	3.5	8.4	(7.3 - 59)	19	(5 - 71)	2.4	(< 1.4 - 1.7)
Benzo*(e)*pyrene (5)			47.5	(43 - 52)				22	(5 - 71)		
Dibenzo*(a,h)*antracene (5)	23	(<0.6 - 460)	< 20		0.8	3	(0.5 - 9)	6	(<5 - 16)	4.2	(< 1.3 - 7.8)
Perylene (5)			<12								
Benzo*(g,h,i)*perylene (6)	14.5	(<0.3 - 290)	39	(37 - 41)	3.8	10.3	(7.1 - 57)	18	(<5 - 63)	5.6	(2.0 - 16.1)
Dibenz[*a*,*h*]anthracene (5)	94.5	(<0.6 - 1087)	42.5	(39 - 46)	2.6	10.8	(8.2 - 27)	21	(<5 - 68)	2.0	(< 2.0 - 2.5)
Coronene (6)					3.6	3.5	(2.2 - 18)				
ΣPAHs	**609.1**	**(<0.3 - 4700)**	**604.5**	**(587 - 622)**	**120**	**137**	**(120 - 730)**	**512**	**(183 - 1629)**	**172**	**(86 - 372)**

Table 3. Moss species used as passive biomonitors of atmospheric PAH deposition.

	Pleurocarpous mosses								**Acrocarpous mosses**	
	Abietinella abietina	*Hylocomium splendens*	*Hypnum cupressiforme*	*Pleurozium schreberi*	*Pseudoscleropodium purum*	*Scleropodium purum*	*Thamnobryum alopecurum*	*Thuidium tamariscinum*	*Dicranum scoparium*	*Tortula muralis*
Knulst et al. (1995)		▲		▲						
Milukaite (1998)		▲		▲						
Gerdol et al. (2002)										▲
Migaszewski et al. (2002)		▲								
Ötvös et al. (2004)			▲							
Gałuszka (2007)		▲		▲						
Holoubek et al. (2000, 2007)			▲							
Krommer et al. (2007)	▲		▲			▲				
Ares et al. (2009)					▲					
Migaszewski et al. (2009)		▲		▲						
Foan et al. (2010)			▲				▲	▲	▲	
Dołęgowska and Migaszewski (in press)		▲		▲						

Many studies on atmospheric pollution (including PAHs) based on moss analysis use pleurocarpous mosses as biomonitors because they generally form broad carpets which almost completely isolate them from the soil. However, pleurocarpous mosses are sensitive to pollution and to dryness, and therefore are rare, sometimes absent, in urban areas. Thus, acrocarpous mosses, which grow on stone or brick walls, are used for studying urban environments (Burton, 1990; Table 3).

One should take care with comparing concentrations between different moss species and different studies. Bioaccumulation of PAHs in mosses might be species-specific as Gałuszka (2007) and Dołęgowska and Migaszewski (in press) observed a higher accumulation of PAHs in *Hylocomium splendens* than *Pleurozium schreberi*. However, Milukaite (1998) reported a similar retention of benzo(a)pyrene in *Hylocomium splendens* and *Pleurozium schreberi*. Migaszewski et al. (2009) found that differences in the accumulation of PAHs between the moss species varied with sampling site and region. Moreover, Ares et al. (2009) noted a seasonal variability due to changes in emissions and climate throughout the year. They also observed spatial variability due to the geomorphology of the study area and the presence of prevailing winds.

Most studies so far have determined the concentration of POPs in mosses as an indication of pollution levels, in particular in remote areas. Few studies have related the concentration in mosses with total atmospheric concentrations or deposition rates. Thomas (1984, 1986) found linear relationships between the accumulation of selected PAHs in *Hypnum cupressiforme* sampled from tree trunks and their concentration in rain water and atmospheric particulate matter, taking into account also the amount of precipitation. The concentration in mosses in the autumn represented mean atmospheric pollution levels in the previous year. He concluded that mosses are most appropriate for measuring environmental chemicals which are deposited in particulate form on the mosses and can be physically retained by them. Milukaite (1998) found that the flux of benzo(a)pyrene from the atmosphere to the ground surface correlated well with its concentration in mosses. However, it should be noted that the accumulation of trace substances in mosses is not only dependent on atmospheric pollution levels but also on enrichment parameters which describe physiological parameters as well as pollutant characteristics (Thomas, 1984). In addition, the presence of water from precipitation might be necessary for PAH accumulation in mosses. Thomas (1986) reported on a marked gradient of the concentration of selected PAHs in mosses in western-northern Europe in agreement with the presence of pollution sources.

Vegetation intercepts 26-62% of the atmospheric PAH deposition (Simonich and Hites, 1994). In addition to mosses, other main bioaccumulators used to date for monitoring atmospheric PAH deposition are lichens (Guidotti et al., 2003; Migaszewski et al., 2002; Blasco et al., 2008), leaves from deciduous trees (Howsam et al., 2000; Jouraeva et al., 2002; Wang et al., 2008; Tian et al., 2008) and conifer needles (Holoubek et al., 2000, 2007b; Migaszewski et al., 2002; Piccardo et al., 2005; Lehndorff and Schwark, 2009a,b; Tian et al., 2008).

Temporal trends of PAHs in mosses

Only a few studies have reported on the temporal trends, generally indicating that the change in concentration and/or composition of PAHs with time reflects the changes in emission sources and levels. Herbarium moss samples appear to be an effective tool for reconstructing historical tendencies of atmospheric PAHs deposition (Foan et al., 2010). The disappearance of the charcoal pits and foundries at the end of the 19th century, combined with the evolution of domestic heating towards less polluting systems during the 20th century, explain the significant decline of PAHs in mosses over that period at a remote site in northern Spain. Between 1973-1975 and 2006-2007, PAH distribution in mosses changed noticeably with a tendency towards 3-benzene ring PAH enrichment, due to the implementation of policies limiting 4- and 5-benzene ring PAH emissions, and a steadily increasing traffic in the area, especially heavy vehicles. Holoubek et al. (2000, 2007) observed a significant decrease in total PAH concentrations in mosses between 1988-1994 and 1996-2005. The small decline in the period 1996-2005 reflected the small decline in PAHs in air (Houlebek et al., 2007).

2.3 **Biomonitoring of POPs other than PAHs**

Mosses have also been sampled to indicate the levels of atmospheric pollution from POPs other than PAHs, although the number of studies for each POP is limited. For these POPs, no attempts have been made so far to relate the concentration in mosses with atmospheric concentrations or deposition fluxes.

Organochlorines (OCs): pentachlorobenzene (PCBz), dichlorodiphenyltrichloroethane (DDT) hexachlorobenzene (HCB), hexachlorocyclo-hexanes (HCHs) and polychlorobiphenyls (PCBs)

Chlorinated hydrocarbons were present in measurable concentrations in mosses in the Antarctica (Bacci et al., 1986). HCB levels from the Antarctic Peninsula were rather similar to those reported for mosses from Sweden and Finland. Although levels of DDT and its derivative were lower in the Antarctica when compared to plant data in Italy and Germany, levels observed in lichens were similar to those observed in Sweden (Bacci et al., 1986). The levels of DDT derivatives appears to originate mainly from DDT deposited in the past. Although levels of PCBs (Figure 4) were near or below the detection limit in the Antarctica in the past (Bacci et al., 1986), recently Borghini et al. (2005) reported PCBs and PCBz being the dominant OCs in mosses from Victoria Land (Antarctica), with all OCs being distributed rather uniformly. The PCBs concentrations from Victoria Land were similar to those reported for mosses in Norway (Lead et al., 1996). In Singapore the concentration of OCs in mosses was also rather uniform, indicating that air masses distributed the pollutants rather evenly over the island; high concentrations of DDT derivates and PCBs were observed compared to those found in mosses in for example the Czech Republic (Lim et al., 2006).

In Norway, the sum of the concentration of the 37 PCB congeners in *Hylocomium splendens* had declined at all locations between 1977 and 1990 (Lead et al., 1996). This decline most likely reflects the reduction in the global use and manufacture of PCBs. While the sum of PCB concentrations have declined, temporal changes in the congener pattern in the samples collected from the same locations were noted. For example, in the south of Norway the relative concentrations of hexa- and heptachlorinated homologue groups decreased to a greater extent than they did in the north. This observation can be interpreted as evidence for differences in congener recycling through the environment according to their volatility, and it was tentatively suggested that this may provide evidence in support of the global fractionation hypothesis (Wania and Mackay, 1993), i.e. compounds will volatilize in warm and temperate areas, will move northward in the Northern Hemisphere (even though atmospheric air flow may not always be in this direction), and will then re-condense when they reach colder circumpolar regions. It has also been hypothesized that differences in the volatility and lability of the individual compounds and in the ambient temperature will lead to a latitudinal fractionation of OCs. In Finland, PCBs concentrations in *Sphagnum* have shown a consistent decline from the 1970s to 1980s. Higher total PCB concentrations were observed in the south compared to the north of Finland (Himberg and Pakarinen, 1994).

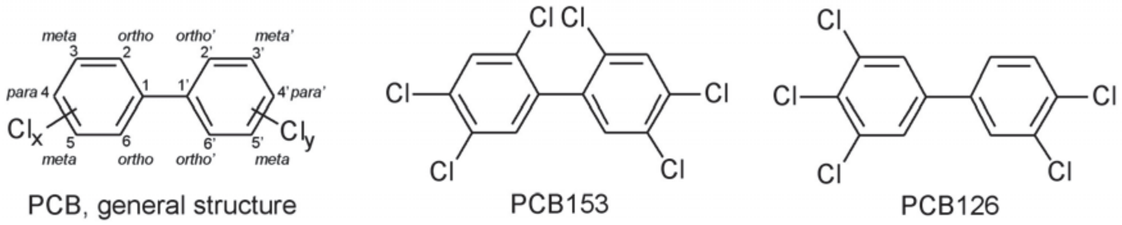

Figure 4. Structural formulas of PCBs.

Dioxins and furans (PCDD/Fs)
Carballeira et al. (2006) concluded that mosses are also good biomonitors for PCDD/Fs (Figure 5). Concentrations of PCDD/F in *Pseudoscleropodium purum* allowed the detection of strong and weak pollution sources. The measurements were sensitive enough to monitor changes in pollution intensity with time, to determine spatial gradients near pollution sources as well as differences in the relative abundance of isomers from different sources.

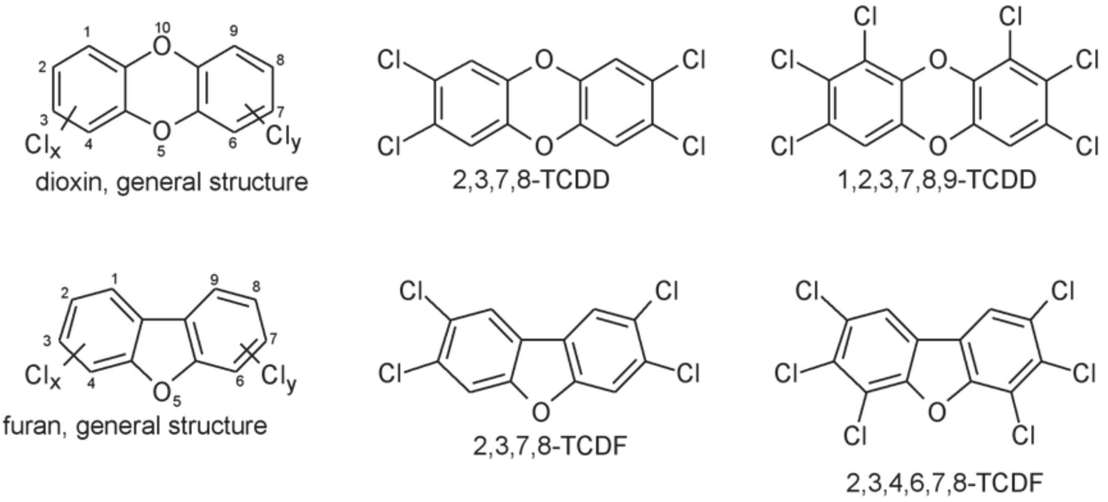

Figure 5. Structural formulas of dioxins and furans (PCDD/Fs).

Polybrominated diphenyl ethers (PBDEs)
In Norway, levels of PBDEs (Figure 6) in mosses showed a general decline towards the northern parts. There was a significant decrease in the concentration of the lower brominated PBDE-congeners in mosses from the south towards the north. This is consistent with the expected atmospheric transport behaviour of these compounds, expected source regions on a European scale (Prevedouros et al., 2004) and results from other investigations. The PBDE levels in Norway were low and are probably of limited toxicological significance (Mariussen et al., 2008). PBDEs were also detected at low levels in mosses sampled on King George Island, Antartica. Concentrations were not statistically different at

sites close to and distant from human facilities, hence long-range atmospheric transport is believed to be the primary source of PBDEs (Yogui and Sericano, 2008).

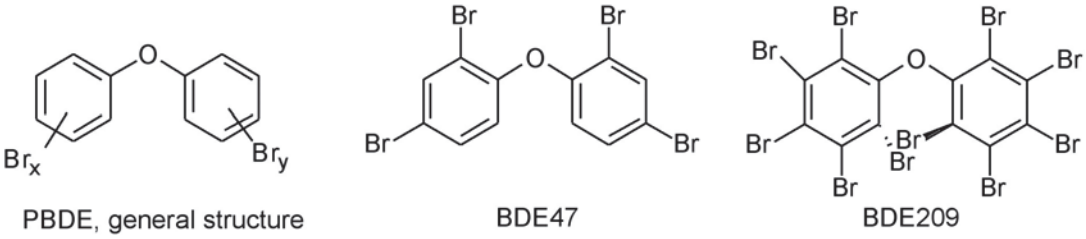

Figure 6. Structural formulas of polybrominated diphenyl ethers (PBDEs).

3 Conclusions and research recommendations

3.1 Conclusions

As for many other air pollutants such as heavy metals and nitrogen (Harmens et al., 2010, 2011), mosses appear to be suitable organisms to monitor spatial patterns and temporal trends of the atmospheric concentrations and deposition of POPs to vegetation. So far, many studies have focused on spatial trends around pollution sources or the concentration in mosses in remote areas as an indication of long-range transport of POPs. Few studies have determined temporal trends or have directly related the concentrations in mosses with measured atmospheric concentrations in rain water or snow (wet deposition) or in particulate matter (dry deposition). The majority of studies have focussed on PAHs and relative few studies have been conducted on other POPs.

In the currently ongoing European moss survey of 2010/11 coordinated by the ICP Vegetation, six countries will determine the concentration of selected POPs (PAHs in particular) in mosses in a pilot study to investigate the suitability of mosses as biomonitors of POPs at a regional scale. To further establish the suitability of mosses as biomonitors of POPs across Europe it will be paramount to sample mosses at sites where atmospheric POPs concentrations and/or deposition fluxes are determined, for example at EMEP monitoring sites (Gusev et al., 2011) or national POPs monitoring sites. Once a good relationship has been established between POPs concentrations in mosses and measured air concentrations and/or deposition fluxes, data from the European moss monitoring network can be used to complement data from the limited number of EMEP measurement sites to assess the performance of the EMEP model for POPs in a cost-effective manner.

3.2 Research recommendations

- To establish the relationship between air concentrations and/or deposition fluxes of POPs and their concentration in mosses, mosses should be sampled near measurement sites for air concentrations and/or deposition fluxes.

- The impacts of factors that might affect such a relationship should be studied in more detail. Such factors might include: different moss species, altitude, amount of precipitation, temperature, seasonality.

- To establish spatial trends in mosses across Europe, bearing in mind the outcome of the above research recommendations, more countries should determine the concentration of various, suitable POPs in mosses as part of the European moss survey.

- To establish temporal trends in mosses across Europe, the POPs survey in mosses should be repeated at regular time intervals, e.g. every five years.

- To assure good quality and comparable data, an inter-laboratory calibration exercise is required to assess the performance of laboratories participating in a European moss survey. In addition, an agreed sampling protocol should be developed.

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Annexe XV.

Monitoring of atmospheric deposition of heavy metals, nitrogen and POPs in Europe using bryophytes – Monitoring manual – 2010 survey

Harmens et les participants du PIC-Végétation, 2010

http://icpvegetation.ceh.ac.uk/manuals/documents/UNECEHEAVYMETALSMOSSMANUAL2010POPsadaptedfinal_220510_.pdf

HEAVY METALS IN EUROPEAN MOSSES: 2010 SURVEY

MONITORING MANUAL

International Cooperative Programme on Effects of Air Pollution on Natural Vegetation and Crops

http://icpvegetation.ceh.ac.uk

wge

Working Group on Effects
of the
Convention on Long-range Transboundary Air Pollution

UNITED NATIONS ECONOMIC COMMISSION FOR EUROPE CONVENTION ON LONG-RANGE TRANSBOUNDARY AIR POLLUTION

MONITORING OF ATMOSPHERIC DEPOSITION OF HEAVY METALS, NITROGEN AND POPs IN EUROPE USING BRYOPHYTES

MONITORING MANUAL

2010 SURVEY

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In collaboration with the participants

May, 2010

1. INTRODUCTION

The UNECE ICP VEGETATION

In the late 1980's, the International Cooperative Programme on the effects of air pollution on natural vegetation and crops (ICP Vegetation, formally ICP Crops) was established to consider the underlying science for quantifying damage to plants by ozone and other pollutants. Scientists from 35 countries currently participate in the ICP Vegetation. The programme is led by the UK and coordinated by the Centre for Ecology and Hydrology at Bangor.

The programme is part of the activities of the Working Group on Effects (WGE) under the Convention on Long-Range Transboundary Air Pollution (LRTAP), which covers the UNECE (United Nations Economic Commission for Europe) region of Europe and North America. The ICP Vegetation is one of several ICPs and Task Forces investigating effects of pollutants on waters, materials, forests, ecosystems, health, and mapping their effects in the ECE region. International cooperation to control pollution is strengthened by the LRTAP Convention. Its Protocols commit countries to reducing pollutant emissions by specific target years. Results from the ICPs are used in both the development of these Protocols and in monitoring their success in reducing the impacts of air pollutants on health and the environment. For further information on the LRTAP Convention, WGE, and other ICPs, please visit the web-pages listed in Annex 1.

Monitoring long-term and large-scale changes in heavy-metal deposition

Increased and excessive accumulation of heavy metals in the soil, ground water and organisms can cause retarded growth of trees and crops and increased levels of heavy metals in the food chain leading to man.

It is apparent that some heavy metals emitted into the air from sources such as industries and power stations are mainly spread locally around the emission source. The affected area might have a diameter of 10-50 km, depending on wind patterns and height of stacks. Examples of this kind of distribution are chromium and nickel. Other metals are transported longer distances due to the formation of a gaseous phase during combustion, leading to a very small and easily transported particles. This appears to be the case with arsenic, cadmium, lead, mercury and zinc. The LRTAP Convention has negotiated the Heavy Metal Protocol in 1998 in Aarhus (Denmark), committing parties to reducing emissions and consequent long-range transport of heavy metals (Working Group on Effects, 2004). However, further information is needed on the concentrations of heavy metals into environment deposition rates and pathways, and effects on human health and the environment. Data from the 2010 moss survey will add to that of previous European surveys in 1990, 1995, 2000 and 2005 (Harmens et al., 2008a), and thus will provide further information on temporal and spatial trends into concentrations of heavy metals in mosses in Europe at a high spatial resolution.

Mosses as biomonitors of atmospheric deposition of heavy metals

Anyone who wants to measure the fallout of heavy metals from the atmosphere has had access to an alternative that is both simple and inexpensive as compared with the rather arduous methods of analysing precipitation with respect to metal concentrations. The dense carpets that ***Hylocomium splendens, Pleurozium schreberi*** and other pleurocarpous mosses form on the ground have turned out to be very effective traps of metals in precipitation and airborne particles. This allowed for a dense biomonitoring network to be established across Europe since 1990.

One of the main benefits to be gained from studying heavy-metal fallout through moss analyses is that metals are accumulated by the moss, leading to concentrations which are much higher than in air, rain and snow. The problems of contamination during sampling and analysis are therefore relatively small, and sampling can be carried out using relatively simple methods.

Mosses as biomonitors of atmospheric deposition of nitrogen

In the 2005 European moss survey, the total nitrogen concentration in mosses was determined for the first time. The spatial distribution of nitrogen concentrations in mosses appears to mirror atmospheric nitrogen deposition across Europe to a high degree and is potentially a valuable tool for identifying areas at risk from high atmospheric nitrogen deposition at a high spatial resolution (Harmens et al., 2008b). Determining the total nitrogen concentration in mosses again in the 2010 survey would allow investigation of temporal trends across Europe.

Mosses as biomonitors of atmospheric deposition of persistent organic pollutants (POPs)

In a pilot study, selected POPs will be determined in mosses for the first time within the framework of the European moss survey. Mosses have been applied in the past as biomonitors of POPs, polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) in particular, at the local or national scale (e.g. Lead et al., 1996; Zechmeister et al., 2003a). In this pilot study we will focus on PAHs, PCBs, polybromodiphenylethers (PBDEs; Mariussen et al., 2008), dioxins (Carballeira et al., 2006) and perfluorooctane sulfonic acid and its salts (PFOS), but other POPs could also be included if there is a national interest.

2. AIMS AND OBJECTIVES

The aims of the 2010 survey are to:

- characterise qualitatively (and quantitatively where possible) the regional atmospheric deposition of heavy metals, nitrogen and POPs in Europe.

- indicate the location of important heavy metal, nitrogen and POPs emission sources and the extent of particularly polluted areas.

- produce maps of the deposition patterns of heavy metals and nitrogen (and possibly for selected POPs) for Europe.

- help to understand the extent of long-range transboundary pollution.

- analyse temporal trends to establish the effectiveness of air pollution abatement policies within Europe.

3. SAMPLING PROGRAMME

Number of sampling sites

Similar to previous surveys each country should aim to collect at least 1.5 moss samples/1000 km^2. If this is not feasible, a sampling density of at least 2 moss samples per EMEP[1] grid (50 km x 50 km) is recommended. It is recommended to make an even and objective distribution of the samples whenever possible, and to have a more dense sampling regime in areas where steep gradients in the deposition of heavy metals can be foreseen. To aid the analysis of temporal trends in the concentration of heavy metals in mosses, it is recommended to collect samples from the same sites as in the previous surveys. Regarding the pilot study for POPs, a lower sampling density is anticipated, depending on national resources available.

Moss species

Only pleurocarpous mosses should be sampled. As in earlier investigations two pleurocarpous moss species are favoured: ***Pleurozium schreberi*** and ***Hylocomium splendens***. However, in some countries it might be necessary to use other pleurocarpous species. In that case, the first choice would be ***Hypnum cupressiforme,*** followed by ***Pseudoscleropodium purum*** (Harmens et al., 2008a). The use of bryophytes other than ***Hylocomium*** or ***Pleurozium*** must be preceded by a comparison and calibration of their uptake of heavy metals relative to the main preferred species. For the correct nomenclature of moss species we refer to Hill et al. (2006). The POPs pilot study should be conducted with ***Pleurozium schreberi*** and ***Hylocomium splendens*** only (and possibly ***Hypnum cupressiforme*** as an alternative).

Field sampling

Sampling in the field should be done according to the following principles:

1. Each sampling point should be situated at least 3 m away from the nearest projected tree canopy: in forests or plantations primarily in small gaps, without pronounced influence from canopy drip from trees, preferably on the ground or on the level surface of decaying stumps.

2. In habitats such as open heathland, grassland or peatland, sampling below a canopy of shrubs or large-leafed herbs should be avoided, as well as areas with running water on slopes.

3. The sampling points should be located at sites representative of non-urban areas of the respective countries. In remote areas the sampling points should be at least 300 m from main roads (highways), villages and industries and at least 100 m away from smaller roads and houses.

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[1] Co-operative programme for monitoring and evaluation of the long-range transmission of air pollutants in Europe. http://www.emep.int/

4. In mountainous areas such as the Alps the sampling points should be below the timberline in order to eliminate confounding influences of altitude on the heavy metal concentration in mosses (Zechmeister, 1995).

5. In order to enable comparison of the data from this survey with previous surveys, it is suggested to collect moss samples from the same (or nearby, i.e. no more than 2 km away but with the same biotope conditions) sampling points as used in previous surveys (at least the same sampling points as used in the 2000 and 2005 survey). In addition, sampling of mosses near (long-term) monitoring stations of atmospheric heavy metal, nitrogen or POPs deposition is recommended in order to directly compare their concentration in mosses with the accumulated atmospheric deposition.

6. It is recommended to make one composite sample from each sampling point, consisting of five to ten (ten for POPs) subsamples, if possible, collected within an area of about 50 x 50 m.

   Mosses: In the composite sample only one moss species should be represented. The sub-samples should be placed side by side or on top of each other in large paper or plastic bags (POPs: polythene bags or glass jars), tightly closed to prevent contamination during transportation. The amount of moss needed is about one litre (or two litres when POPs analysis will be conducted as well). As some POPs are susceptable to volatilization and photochemical breakdown, samples for POPs analysis should be kept cool and in the dark at all times. Note: The latter is less important when analysing only the seven PAHs recommended by the EU (see annex 4).

7. Smoking is forbidden during sampling and further handling of samples, and disposable plastic, non-talcum gloves should be used when picking up the mosses. Do not use vinyl examination gloves if they are powdered with talcum as this will contaminate the samples.

8. Samples should preferably be collected during the period April - October. In arid regions of Europe it is advised to collect the samples during the wet season. Although the heavy metal concentration in ***Hylocomium splendens*** and ***Pleurozium schreberi*** appear not to vary with season (Thöni et al., 1996, Berg and Steinnes, 1997), this might not be true for other moss species (e.g. Couto et al., 2003; Zechmeister et al., 2003b) and all climates in Europe. Therefore, it is suggested to sample the mosses in the shortest time window possible.

9. Each locality must be given co-ordinates, preferably longitude and latitude (Greenwich co-ordinates, 360º system), suitable for common data processing.

10. In order to determine the overall variability associated with the entire procedure (sampling + analysis), multiple moss samples (at least 3 samples per site) must be collected from at least two sites with different levels of overall contamination (one expected to have a high level of contamination and one expected to have a low level of contamination based on the results of the 2005 survey). These multiple moss samples must be collected, processed and analysed individually in order to characterise the overall variability of the data.

4. ANALYTICAL PROGRAMME

Utmost care should be taken in order to avoid contamination from smoke and laboratory tables. The material should therefore be handled on clean laboratory paper, glass shields or clean polythene. Non-talcum, disposable plastic gloves should be worn and no metal tools should be used.

Cleaning and storing of moss samples

If the samples cannot be cleaned straight after sampling, they should be put into paper bags and dried and stored at room temperature (20-25^{o}C) until further treatment. Alternatively, samples can be deep-frozen. For POPs analysis, samples should be stored at 4^{o}C and in the dark (see Field sampling – point 6).

The samples should be carefully cleaned from all dead material and attached litter, so that just the green and green-brown shoots from the last three years growth are included. Brown parts should not be included, even if the green parts only represent the last two to three years of growth. Sampling of ***Hylocomium splendens*** in 2010 would include the fully developed segments from 2007, 2008 and 2009; any segments developed from the 2010 growing season should be discarded, unless sampled at the end of a full growing season in 2010.

If other moss species are collected, shoots corresponding to three years of growth are recommended for the analyses.

Drying of moss samples before determination of heavy metals and nitrogen

The samples should be dried to constant weight at 40ºC, which is used as a reference for the calculations. It is recommended to record the drying loss at 40ºC (compared to room temperature) for future reference. The rest of the dried material not used in analyses should be stored in an environment specimen bank for future investigations.

For mercury, analysis should be conducted on fresh material or material dried at a lower temperature than 40ºC and the determination of drying loss at 40ºC on a separate aliquot is recommended.

Drying of moss samples before determination of POPs

Preparations of the moss samples for the determination of POPs will depend on the compounds analysed and the analytical technique applied in the laboratory. For example, drying of moss samples for the determination of PAHs might be best done by freeze-drying (lyophilisation). However, laboratories need to check for losses of POPs in the various steps leading up to the analysis.

Determination of heavy metals

Digestion

Wet ashing of a homogeneous sub-sample is recommended for the decomposition of organic material. Dry ashing is not acceptable. The preferred method of digestion is microwave digestion. Wet ashing, using nitric acid, has been used in most countries in the past and has proven to give reproducible results. If excess acid is evaporated, samples should not be allowed to become completely dry. **Note:** wet ashing should not be applied when INAA (Instrumental Neutron Activation Analysis) is used as analytical technique; a homogenous, dried sub-sample should be analysed without further pre-treatment.

Analytical technique

The metal determinations can be performed using various analytical techniques. Earlier studies have shown that both AAS (atomic absorption spectroscopy) and ICP-ES/MS (plasma emission spectroscopy) are suitable methods. INAA (instrumental neutron activation analysis) tends to give higher metal concentrations as it determines the total heavy metal concentration (Steinnes et al., 1993). Therefore, it is recommended to compare the results for INAA with other techniques such as ICP-MS using the same moss samples and include standard moss reference material to further compare the performance of these techniques (see below).

An intercalibration of the analytical procedure took place in 1995 and 2005, and will be repeated in 2010. For quality assurance purposes, participants must include the moss standards M2 and M3 that were used in the 1995 and 2005 survey (Steinnes et al., 1997; Harmens et al., 2008a). The moss standards must be analysed at the same time as the collected moss samples. The moss standards will be supplied at a reduced cost by Mr Eero Kubin, Finnish Forest Research Institute, Muhos Research Station, and the distribution of the standards will be coordinated by Juha Piispanen (Juha.Piispanen@metla.fi). Although no recommended values are available for POPs, M2 and M3 should also be included as standards in POPs analyses, to investigate whether recommended values can be established for several POPs. The following certified reference material of organic contamination has to be included for POPs: IAEA-140/OC Fucus (35g) from Analab (seaweed material containing organochlorine compounds (pesticides and PCBs) and petroleum hydrocarbons (aliphatic hydrocarbons and PAHs)).

For quality assurance and cross-border calibration purposes, participants are encouraged to exchange ca. six to ten moss samples (clean and three years growth selected) from selected sites near the border of the country with neighbouring countries.

The following elements with mainly anthropogenic and atmospheric origin should be determined: As, Cd, Cr, Cu, Fe, Hg, Ni, Pb, V and Zn. In addition, Al and Sb should be determined to indicate contamination of the samples by soil/the contribution of wind-blown dust and to indicate anthropogenic origin respectively. Other elements of national concern or local importance (e.g. Bi, Mo, S, Ti, Se) may also be studied. Including as many elements as possible will aid the identification of the sources of heavy metals by applying multivariate analysis.

Determination of nitrogen

The ICP Vegetation encourages participants also to determine the total nitrogen concentration in mosses and hopes to increase the spatial coverage of Europe in comparison to the 2005 survey (Harmens et al., 2008b). To directly compare the nitrogen concentration in mosses with atmospheric nitrogen deposition, it is recommended to include sites near monitoring stations of atmospheric nitrogen deposition. Suggested methods for nitrogen analysis are Kjehldahl (wet digestion) and elemental analysis (Dumas method). For quality assurance purposes the nitrogen concentration in the moss standards M2 and M3 must be determined (in addition to any certified standards for nitrogen) along with the moss samples (see above). Recommended values for M2 and M3 for nitrogen were reported previously (Harmens et al., 2008a).

Determination of POPs

The ICP Vegetation encourages participants also to take part in the pilot study of POPs. Annex 4 provides a list of POPs recommended to be included. No specific analytical techniques are recommended at this stage due to the diverse nature of POPs.

5. FURTHER SITE-SPECIFIC DATA

To determine which site-specific parameters affect the heavy metal and nitrogen concentration in mosses, participants are encouraged to provide further site-specific data via Web MossMet. This will allow detailed geostatistical analysis of factors influencing element concentrations in mosses (Schröder et al., 2008). For further details, please contact Mr Winfried Schröder (wschroeder@iuw.uni-vechta.de) or Mr Roland Pesch (roland.pesch@uni-vechta.de).

6. DATA PROCESSING, REPORTING AND PUBLICATION

The Programme Coordination Centre for the ICP Vegetation (Bangor, UK) will be responsible for common data processing, the construction of maps and the final report. In collaboration with the ICP Vegetation Coordination Centre:

- Data regarding the moss standards will be processed further by Mr Eero Kubin (Finland) and Mr Eiliv Steinnes (Norway).
- Detailed geostatistical analysis of data provided to MossMet will be conducted by Mr Winfried Schröder and colleagues (Germany) in collaboration with the participants.

All data should be sent to Mr Harry Harmens, ICP Vegetation Coordination Centre (see front page for details). Please submit the data by e-mail as an Excel spreadsheet to hh@ceh.ac.uk

THE SPREADSHEET SHOULD CONTAIN THE FOLLOWING INFORMATION (see Annex 2):

Country

Name, address, telephone no., fax no. and e-mail address for all participants

Analytical procedure used for each metal, nitrogen and POP, including sample preparation, digestion method and analytical technique.

Data in rows, with one row for each site sampled. The column headings should read:
Site name
Coordinates
Date sampled
Altitude (m above sea level)
Land cover (according to CORINE classification label level 3; see Annex 3)
Topography (plain or slope)
Any further details regarding the site or climate are optional
Moss species (see Hill et al., 2006)

For each metal, nitrogen and POP, the name and units of concentration. For each metal, nitrogen and POP the quantification limit of the applied analytical technique must be provided.

Data must also include the individual values (metals, N and POPs) for each moss standard, such that the mean value and standard deviations per moss standard can be determined for each participating laboratory. In addition, data for cross-border calibration should be clearly labelled.

A report will be prepared in 2013 that will contain European maps of heavy metal and nitrogen concentrations in mosses and wherever possible, an indication of temporal trends.

7. TIME SCHEDULE

The main sampling period will be April to October 2010 (or 2011, depending on available funding). Data should be submitted to the Coordination Centre as soon as possible, but no later than 1 September 2011 (or 1 April 2012 if survey conducted in 2011). It is envisaged that preliminary maps will be produced by September 2012, and a final report will be prepared early 2013.

8. FUNDING

Sampling and analyses must be paid for by each country separately. Coordination and collating data by the ICP Vegetation Programme Coordination Centre will be funded by the Department for Environment, Food and Rural Affairs (Defra), UK.

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Zechmeister, H.G., Hohenwallner, D., Riss, A., Hanus-Illnar, A. (2003) Variations in heavy metal concentrations in the moss species ***Abietinella abietina*** (Hedw.) Fleisch according to sampling time, within site variability and increase in biomass. Science of the Total Environment 301: 55-65.

Annex 1

WWW LINKS

ICP Vegetation	http://icpvegetation.ceh.ac.uk
UNECE	http://www.unece.org
LRTAP Convention	http://www.unece.org/env/lrtap/welcome.html
Working Group on Effects	http://www.unece.org/env/lrtap/WorkingGroups/wge/welcome.html This web-page contains links to the other ICPs and the Task Force on Health.
EMEP	http://www.emep.int With links to MSC-West and MSC-East.

Stockholm Convention on persistent organic pollutants (POPs) - http://chm.pops.int

Annex 2

Template data sheet

Country																						
For all participants:																						
Name																						
Address																						
Tel.																						
Fax																						
e-mail																						
Full description of analytical procedure for each metal, N and POP, including sample storage and preparation, digestion method and analytical technique																						
Site name	**Longitude**	**Latitude**	**Sample date**	**Altitude (m)**	**Land cover**	**Topography**	**Further details**	**Moss species**	**Al (ug/g)**	**As (ug/g)**	**Cd (ug/g)**	**Cr (ug/g)**	**Cu (ug/g)**	**Fe (ug/g)**	**Hg (ug/g)**	**Ni (ug/g)**	**Pb (ug/g)**	**Sb (ug/g)**	**V (ug/g)**	**Zn (ug/g)**	**N (mg/g)**	**Each POP (unit)**
	xx°xx'xx"	xx°xx'xx"	dd/mm/yr		See annex 3	Plain or slope	Site or climate															
	or in decimals	or in decimals																				
							Quantification limit for each metal															
Also include:																						
Values for all moss standard runs (M2 & M3)																						
Data to determine overall variablility (see Monitoring Manual, field sampling bullet point 10)																						
Also include cross-border calibration data (if done any)																						

Annex 3

Corine Land Cover 2000 classes

Code Level 3	Label Level 1	Label Level 2	Label Level 3
111	Artificial surfaces	Urban fabric	Continuous urban fabric
112	Artificial surfaces	Urban fabric	Discontinuous urban fabric
121	Artificial surfaces	Industrial, commercial and transport units	Industrial or commercial units
122	Artificial surfaces	Industrial, commercial and transport units	Road and rail networks and associated land
123	Artificial surfaces	Industrial, commercial and transport units	Port areas
124	Artificial surfaces	Industrial, commercial and transport units	Airports
131	Artificial surfaces	Mine, dump and construction sites	Mineral extraction sites
132	Artificial surfaces	Mine, dump and construction sites	Dump sites
133	Artificial surfaces	Mine, dump and construction sites	Construction sites
141	Artificial surfaces	Artificial, non-agricultural vegetated areas	Green urban areas
142	Artificial surfaces	Artificial, non-agricultural vegetated areas	Sport and leisure facilities
211	Agricultural areas	Arable land	Non-irrigated arable land
212	Agricultural areas	Arable land	Permanently irrigated land
213	Agricultural areas	Arable land	Rice fields
221	Agricultural areas	Permanent crops	Vineyards
222	Agricultural areas	Permanent crops	Fruit trees and berry plantations
223	Agricultural areas	Permanent crops	Olive groves
231	Agricultural areas	Pastures	Pastures
241	Agricultural areas	Heterogeneous agricultural areas	Annual crops associated with permanent crops
242	Agricultural areas	Heterogeneous agricultural areas	Complex cultivation patterns
243	Agricultural areas	Heterogeneous agricultural areas	Land principally occupied by agriculture, with significant areas of natural vegetation
244	Agricultural areas	Heterogeneous agricultural areas	Agro-forestry areas
311	Forest and semi natural areas	Forests	Broad-leaved forest
312	Forest and semi natural areas	Forests	Coniferous forest
313	Forest and semi natural areas	Forests	Mixed forest
321	Forest and semi natural areas	Scrub and/or herbaceous vegetation associations	Natural grasslands
322	Forest and semi natural areas	Scrub and/or herbaceous vegetation associations	Moors and heathland
323	Forest and semi natural areas	Scrub and/or herbaceous vegetation associations	Sclerophyllous vegetation
324	Forest and semi natural areas	Scrub and/or herbaceous vegetation associations	Transitional woodland-shrub
331	Forest and semi natural areas	Open spaces with little or no vegetation	Beaches, dunes, sands
332	Forest and semi natural areas	Open spaces with little or no vegetation	Bare rocks
333	Forest and semi natural areas	Open spaces with little or no vegetation	Sparsely vegetated areas
334	Forest and semi natural areas	Open spaces with little or no vegetation	Burnt areas
335	Forest and semi natural areas	Open spaces with little or no vegetation	Glaciers and perpetual snow
411	Wetlands	Inland wetlands	Inland marshes
412	Wetlands	Inland wetlands	Peat bogs
421	Wetlands	Maritime wetlands	Salt marshes
422	Wetlands	Maritime wetlands	Salines
423	Wetlands	Maritime wetlands	Intertidal flats
511	Water bodies	Inland waters	Water courses
512	Water bodies	Inland waters	Water bodies
521	Water bodies	Marine waters	Coastal lagoons
522	Water bodies	Marine waters	Estuaries
523	Water bodies	Marine waters	Sea and ocean

Annex 4

Recommended list of persistent organic pollutants (POPs)

Name/Synonym	Group	EMEP modelled	POPs Protocol	Stockholm Convention	Notes		
PCB	Polychlorinated biphenyls		x	x	Dielectric fluids in transformers, capacitors, coolants		
BDE-x	Polybromodiphenylether	BDE-28, 47, 99, 153		2009	Flame retardants		
HBB	Polybrominated biphenyls			2009	Flame retardants, see polybromodiphenylether		
HxCDD	Polychlorinated dibenzo-p-dioxins (PCDD) (Dioxins)	x	x	x	PVC production, industrial bleaching, incineration		
PFOS	Perfluorooctane sulfonic acid and its salts			2009	(Fluoro)Surfactant		
	PAHs						
Benzo(a)anthracene	EU, US EPA				Seven EU PAHs are non-volatile and the most toxic		
Benzo(j)fluoranthene	EU						
Benzo(b)fluoranthene	EU, POPs Protocol indicator, US EPA						
Benzo(k)fluoranthene	EU, POPs Protocol indicator, US EPA						
Benzo(a)pyrene	EU, POPs Protocol indicator, US EPA	x	x				
Dibenzo(a,h)anthracene	EU						
Indeno(1,2,3-cd)pyrene	EU, POPs Protocol indicator, US EPA						
Naphthalene	US EPA						
Acenaphthylene	US EPA						
Acenaphthene	US EPA						
Fluorene	US EPA						
Phenanthrene	US EPA						
Anthracene	US EPA						
Fluoranthene	US EPA						
Pyrene	US EPA						
Chrysene	US EPA						
Dibenzo(a,h,)anthracen	US EPA						
Benzo(g,h,i)perylen	US EPA						

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FOAN L., SIMON V.
Journal of Chromatography A, sous presse, en ligne : http://dx.doi.org/10.1016/j.chroma.2012.07.065.

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