LATVIJAS UNIVERSITĀTE
ĶĪMIJAS FAKULTĀTE

LAURA ELĪNA IKKERE

BIOLOĢISKO INDIKATORORGANISMU UN PASĪVO PARAUGU ŅEMŠANAS IEKĀRTU PIELIETOJUMS ĶĪMISKO PIESĀRŅOTĀJU NOTEIKŠANAI AR AUGSTAS IZŠĶIRTSPĒJAS MASSPEKTROMETRIJAS METODĒM

PROMOCIJAS DARBS

Doktora grāda iegūšanai ķīmijas nozarē
Analītiskās ķīmijas apakšnozarē

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Darbs sastāv no kopsavilkuma latviešu un angļu valodās un piecām zinātniskajām publikācijām.
Darba forma: publikāciju kopa ķīmijas nozarē, analītiskās ķīmijas apakšnozarē.

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Ar promocijas darbu un tā kopsavilkumu var iepazīties Latvijas Universitātes Bibliotēkā Rīgā, Raiņa bulv. 19.

LU Ķīmijas zinātņu nozares promocijas padomes

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ANOTĀCIJA

Bioloģisko indikatororganismu un pasīvo paraugu ņemšanas iekārtu pielietojums ķīmisko piesārņotāju noteikšanai ar augstas izšķirtspējas masspektrometrijas metodēm. Ikkere, L. E., zinātniskie vadītāji Dr. Chem., Prof. Bartkevičs, V. un Dr. Chem., Prof. Vīksna, A. Zinātnisko publikāciju kopas kopsavilkums, 54 lappuses, 16 attēli, 2 tabulas, 76 literatūras avoti. Latviešu valodā.

Promocijas darbs ietver pārskatu par dažādu bioloģisko indikatororganismu, tai skaitā sauszemes dzīvnieku, zušu un gliemeņu pielietojumu sauszemes un ūdens ekosistēmu piesārņojuma novērtēšanai. Pētījumā aplūkots plašs piesārņotāju klāsts, tai skaitā noturīgie organiskie piesārņotāji – liesmas slāpētāji un perfluorētie savienojumi, kā arī nesen uzmanību piesaistījuši piesārņotāji – farmaceitiski aktīvie savienojumi. Veikts biotisku un abiotisku paraugu matricu pielietojamības salīdzinājums attiecībā uz ūdens piesārņojuma novērtēšanu. Veikta divu inovatīvu augstas izšķirtspējas masspektrometrijas metožu izstrāde farmaceitiski aktīvu savienojumu noteikšanai. Viena no izstrādātajām metodēm balstīta uz augstefektīvo šķidrumu hromatogrāfiju apvienojumā ar Orbitrap masspektrometrisko detektoru, un tiek pielietota, lai veiktu vienlaicīgu 164 farmaceitisko savienojumu un to metabolītu noteikšanu. Savukārt otrā izstrādātajā metodē pielietots īpaši augstas izšķirtspējas Furjē transformācijas jonu ciklotronu rezonanses masspektrometrs bez hromatogrāfiskas analītu atdalīšanas, tādējādi nodrošinot ātru apstiprinošu analīzes metodi hinolonu grupas antibiotiku noteikšanai. Abas izstrādātās metodes paredzētas turpmākiem piesārņotāju izplatības pētījumiem.

BIOINDIKATORI, AUGSTAS IZŠĶIRTSPĒJAS MASSPEKTROMETRIJA, VIDES PIESĀRŅOJUMS, BROMĒTIE LIESMAS SLĀPĒTĀJI, FARMACEITISKIE SAVIENOJUMI, NESTEROĪDIE PRETIEKAISUMA LĪDZEKĻI

SATURS / CONTENTS

APZĪMĒJUMU SARAKSTS

APPI	atmosfēras spiediena fotojonizācija
BFR	bromētie liesmas slāpētāji
BTBPE	1,2-bis(2,4,6-tribromofenoksi)etāns
DBDPE	dekabromodifeniletāns
dSPE	dispersīvā cietfāzes ekstrakcija
EH-TBB	2-etil-heksiltetrabrombenzoāts
EI	elektrontrieciena jonizācija
FR	liesmas slāpētāji
FT-ICR	Furjē transformācijas jonu ciklotronu rezonanse
GC	gāzu hromatogrāfija
GPC	gēlcaurspiešanas hromatogrāfija
HBB	heksabrombenzols
HBCD	heksabromciklododekāns
HESI	apsildāmas eletroizsmidzināšanas jonizācija
HLB	hidrofīlais-lipofīlais līdzsvars
HPLC	augstefektīvā šķidrumu hromatogrāfija
HRMS	augstas izšķirtspējas masspektrometrija
l.m.	lipīdu masa
LC	šķidrumu hromatogrāfija
m.m.	mitra masa
MRL	maksimāli pieļaujamais līmenis
MS/MS	tandēma masspektometrija
MWCNT	daudzslāņu oglekļa nanocaurulītes
WWTP	notekūdeņu attīrīšanas stacija
POP	noturīgie organiskie piesārņotāji
NSAID	nesteroīdie pretiekaisuma līdzekļi

PBDE	polibromētie difenilēteri
PFC	perfluorētie savienojumi
PFOA	perfluoroktānskābe
PFOS	perfluoroktānsulfonāti
POCIS	pasīvā paraugu ņemšanas iekārta
QqQ	trīskāršā kvadrupola analizators
s.m.	sausa masa
SE	ekstrakcija ar šķīdinātāju
SIM	izvēlētā jona skenēšana
SPE	cietfāzes ekstrakcija
SRM	izvēlētās reakcijas skenēšana
TBBPA	tetrabrombisfenols A
TBP-DBPE	2,4,6-tribromfenil-2,3-dibrompropilēteris
TOF	nolidojuma laika analizators
EQS	vides kvalitātes standarts
Vpp	maksimuma-maksimuma spriegums

IEVADS

Ikdienā gan cilvēka darbības, gan rūpniecisko procesu rezultātā vidē nonāk dažādi bīstami ķīmiskie savienojumi. Noturīgie organiskie piesārņotāji (POP), piemēram, liesmas slāpētāji (FR), ir zināmi gadu desmitiem, taču gadu no gada tiek atklāti arvien jauni piesārņotāji, piemēram, farmaceitiski aktīvie savienojumi. Šīs vielas negatīvi ietekmē ekosistēmu, tostarp dzīvos organismus un cilvēkus. Šo piesārņotāju noteikšana abiotiskos vides paraugos, piemēram, ūdenī, ir sarežģīta zemo koncentrāciju dēļ, līdz ar to ir nepieciešama dažādu indikatoru izmantošana, lai noteiktu ļoti zemu piesārņojuma līmeni. Šobrīd pētnieku vidū ir aktuāli izmantot bioindikatoru pieeju, novērtējot dažādus piemērotus dzīvos organismus [1].

Iepriekšminētajai indikatoru stratēģijai ir augsts potenciāls plaša spektra piesārņotāju analīzei, īpaši kombinācijā ar jutīgām un selektīvām instrumentālām metodēm, piemēram, augstas izšķirtspējas masas spektrometriju (HRMS). Šāda pieeja ļauj ne tikai būtiski pazemināt piesārņojošo vielu noteikšanas robežu, bet arī paver iespējas iegūto datu retrospektīvai analīzei. Turklāt augstās selektivitātes dēļ savienojumus ir iespējams noteikt arī traucējošu matricas komponentu klātbūtnē. Tas savukārt ļauj samazināt kopējo analīzes laiku gan ātrākas parauga sagatavošanas, gan īsākas hromatogrāfijas programmas dēļ, jo ir iespējams identificēt un kvantitatīvi noteikt savienojumus, kas nav pilnībā hromatogrāfiski atdalīti. Tomēr galvenā priekšrocība ir iespēja ievērojami palielināt vienlaikus nosakāmo piesārņotāju klāstu.

Problēmas praktiskā nozīme.

Stokholmas konvencijas jauno POP sarakstā jau ir iekļauti tādi POP kā heksa-, hepta- un dekabromdifenilēteri, kā arī perfluoroktānsulfonāti (PFOS) un perfluoroktānskābe (PFOA). Tas norāda uz to noturību vidē ilgā laika periodā, par to spēju plaši izplatīties apkārtējā vidē dabisku procesu rezultātā, tai skaitā augsnē, ūdenī un, jo īpaši, gaisā. Tāpat šiem savienojumiem ir raksturīga spēja uzkrāties dzīvo organismu, tostarp cilvēku taukaudos, kā rezultātā šie savienojumi augstākos līmeņos ir atrodami pārtikas ķēdes augšgalā esošos organismos. Šiem savienojumiem piemīt augsta toksicitāte gan cilvēkam, gan citiem dzīvajiem organismiem[2].

Vispārējas modernizācijas un labklājības celšanās rezultātā vidē nonāk arvien jaunas piesārņotāju grupas. Vieni no pēdējās desmitgadēs vairāk uzmanību piesaistījušiem piesārņotājiem ir farmaceitiski aktīvi savienojumi. Tiek lēsts, ka cilvēkiem paredzēto medikamentu patēriņš ES ir no 50 līdz 150 g uz vienu cilvēku gadā. Veterinārās zāles tiek lietotas mazākos daudzumos, taču arī tas ir augošs farmaceitisko produktu tirgus segments. Satraucošs ir fakts, ka lielākajā daļā ES dalībvalstu aptuveni 50% neizlietoto cilvēkiem paredzēto zāļu (3 līdz 8% no kopējā pārdotā daudzuma) netiek atbilstoši utilizēti [3]. Līdz ar to dažādu

vides sektoru (ūdens, augsnes un gaisa) piesārņojums ar farmācijas produktiem ir kļuvis par vides problēmu. Ar farmaceitisko piesārņojumu ir saistīti vairāki apdraudējumi un riski, tostarp antibakteriālā rezistence, hormonu līmeņu izmaiņas ūdens organismos un pat populācijas samazināšanās dažu organismu gadījumā [3].

Iepriekš minētie negatīvie efekti uz vidi un organismiem uzsver nepieciešamību veikt šo vielu uzraudzību gan vidē, gan pārtikā. Piesārņotāju noteikšana vidē niecīgos daudzumos sagādā grūtības gan zemā noteikšanas līmeņa, gan piesārņojuma līmeņa svārstību dēļ, kas novērojami dažādos laika periodos. Šīs problēmas iespējams pārvarēt, izmantojot bioindikatoru pieeju. Attiecībā uz pārtikas analīzi nepārtraukti ir nepieciešamas arvien jaunas, ātrākas, uzticamākas metodes ar plašu analītu klāstu, ko iespējams noteikt vienā analītiskajā mērījumā.

Promocijas darba mērķis ir izstrādāt analītiskās metodes un pielietot tās piesārņotāju (dažādu POP un farmaceitiski aktīvo savienojumu) noteikšanai vides un pārtikas matricās, izmantojot HRMS iekārtas un bioloģiskos indikatororganismus, kā arī pasīvās paraugu ņemšanas iekārtas (POCIS). Izstrādātās metodes paredzētas plaša piesārņotāju klāsta vienlaicīgai apstiprinoši kvantitatīvai noteikšanai, lai novērtētu to sastopamību Baltijas reģionā.

Promocijas darba uzdevumi:

i. Novērtēt dažādu sauszemes dzīvnieku pielietojamību kā vides piesārņojuma bioindikatorus attiecībā uz polibromētiem difenilēteriem (PBDE), heksabromciklododekānu (HBCD) un tetrabrombifenolu A (TBBPA);
ii. Pārbaudīt zušu kā indikatororganismu pielietojamību ūdens piesārņojuma novērtēšanai attiecībā uz jaunajiem bromētiem liesmas slāpētājiem (BFR);
iii. Izpētīt gliemeņu kā bioindikatoru pielietojamību PBDE, perfluorēto savienojumu (PFC) un nesteroīdo pretiekaisuma līdzekļu (NSAID) piesārņojuma statusam ūdens ekosistēmā;
iv. Salīdzināt dažādas matricas, tostarp gliemenes, perifitonu, sedimentus un ūdeni kā ūdens piesārņojuma indikatorus;
v. Novērtēt dažādus POCIS sorbentus farmaceitiskā piesārņojuma noteikšanai ūdenī;
vi. Izstrādāt perspektīvas HRMS metodes, izmantojot augstas veiktspējas šķidruma hromatogrāfiju, kas savienota ar Orbitrap HRMS (HPLC-Orbitrap-HRMS) un Furjē transformācijas jonu ciklotronu rezonanses HRMS (FT-ICR-HRMS), lai paplašinātu analītisko kapacitāti turpmākiem padziļinātiem farmaceitiskā piesārņojuma pētījumiem.

Zinātniskā novitāte:

i. Analizējot plaša spektra piesārņotājus (noturīgie organiskie piesārņotāji, farmaceitiskie savienojumi) dažādās matricās, demonstrēta dažādu HRMS analizatoru, tostarp magnētiskā sektora, Orbitrap un FT-ICR, kā arī dažādu jonizācijas metožu, piemēram, elektronu trieciena (EI), apsildāmas elektroizsmidzināšanas (HESI) un atmosfēras spiediena fotojonizācijas (APPI) daudzpusība un pielietojamība.

ii. Veikts plašs Latvijas sauszemes un ūdens biotas piesārņojuma stāvokļa novērtējums, analizējot savvaļas dzīvnieku, zušu, gliemeņu, perifitona, sedimentu un ūdens paraugus.

iii. Izstrādāta efektīva analītiska metode, 164 farmaceitiski aktīvo vielu un to metabolītu vienlaicīgai identificēšanai un kvantificēšanai muskuļu audos.

iv. Izstrādātā ātrā un uzticamā tiešās injekcijas FT-ICR-HRMS metode hinolonu antibiotiku apstiprinošai noteikšanai ir viens no pirmajiem ziņojumiem par šīs progresīvās tehnoloģijas izmantošanu pārtikas nekaitīguma nozarē.

Pētījuma praktiskais pielietojums.

Iegūta plaša informācija par piesārņojuma stāvokli Baltijas reģionā. Optimizētās un izstrādātās analītiskās metodes nodrošina virkni labi raksturotu, praktisku un uzticamu iespēju veikt farmaceitiski aktīvo savienojumu noteikšanu dažādās matricās. Metodes ir viegli pielāgojamas dažādām paraugu matricām, un tās var izmantot šo ķīmisko vielu sastopamības ilgstošai uzraudzībai vai zinātniskiem pētījumiem

Publicētie zinātniskie raksti.

1. **Ikkere, L.E.**; Perkons, I.; Pugajeva, I.; Gruzauskas, R.; Bartkiene, E.; Bartkevics, V. Direct injection Fourier transform ion cyclotron resonance mass spectrometric method for high throughput quantification of quinolones in poultry. *J Pharm Biomed Anal*[1] **2020**, *188*.
2. Pugajeva, I.; **Ikkere, L.E.**; Judjallo, E.; Bartkevics, V. Determination of residues and metabolites of more than 140 pharmacologically active substances in meat by liquid chromatography coupled to high resolution Orbitrap mass spectrometry. *J Pharm Biomed Anal*[1] **2019**, *166*, 252-263.
3. **Ikkere, L.E.**; Perkons, I.; Sire, J.; Pugajeva, I.; Bartkevics, V. Occurrence of polybrominated diphenyl ethers, perfluorinated compounds, and nonsteroidal anti-inflammatory drugs in freshwater mussels from Latvia. *Chemosphere*[2] **2018**, *213*, 507-516.
4. Zacs, D.; **Ikkere, L.E.**; Bartkevics, V. Emerging brominated flame retardants and dechlorane-related compounds in European eels (*Anguilla anguilla*) from Latvian lakes. *Chemosphere*[1] **2018**, *197*, 680-690.
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[1] *Peer reviewed journal, imprint of Elsevier (IF=3.209 (2020)), ISSN: 0731-7085*
[2] *Peer reviewed journal, imprint of Elsevier (IF=7.086 (2020)), ISSN: 0045-6535*
[3] *Peer reviewed journal, imprint of Elsevier (IF=7.963 (2020)), ISSN: 0048-9697*

Zinātnisko konferenču saraksts.

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2. International scientific symposium "Science to strengthen sustainable and safe food systems", Riga, Latvia, 2020. **Ikkere, L.E.**; Perkons, I.; Pugajeva, I.; Gruzauskas, R.; Bartkiene, E.; Bartkevics, V. Direct-injection Fourier-transformation ion cyclotron resonance mass spectrometric method for ultra-fast quantification of quinolones in poultry (poster/in book of abstracts)
3. The 77th International Scientific Conference of the University of Latvia, Riga, Latvia, 2019. **Ikkere, L.E.**; Pugajeva, I.; Judjallo, E.; Bartkevics, V. Determination of residues and metabolites of more than 140 pharmacologically active substances in meat by liquid chromatography coupled to high resolution orbitrap mass spectrometry (oral presentation/ in book of abstracts).
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5. The 76th International Scientific Conference of the University of Latvia, Riga, Latvia, 2018. **Ikkere, L.E.**; Perkons, I.; Bartkevics, V. Persistent organic pollutants in freshwater mussels from the Latvian environment (oral presentation/ in book of abstracts).

1. LITERATŪRAS APSKATS

1.1. Bioloģiskie indikatororganismi

Vides analīzē, īpaši ūdens sistēmās, būtiskus apgrūtinājumus rada vides nepārtrauktā mainība. Izmaiņas var būt straujas un grūti novērtējamas. Fizikālie, ķīmiskie un bakterioloģiskie mērījumi nevar atspoguļot daudzo vides faktoru mijiedarbību un ekosistēmu ilgtermiņa ilgtspējību to momentānās dabas dēļ. Ir pierādīts, ka biomonitorings ir svarīgs papildinājums tradicionālajām monitoringa metodēm.

Biomonitoringu definē kā "sistemātisku dzīvo organismu vai to reakciju izmantošanu, lai noteiktu vides stāvokli vai izmaiņas" [4]. Biomonitoringa veikšanai var izmantot dažādus organismus. Šādus organismus sauc par bioloģiskajiem indikatororganismiem. Bioindikators ir "organisms (vai organisma daļa vai organismu kopiena), kas satur informāciju par vides (vai vides daļas) kvalitāti". Ideālam indikatororganismam jāatbilst šādiem kritērijiem: a) taksonomiskā stabilitāte (viegli atpazīt arī nespeciālistam); b) plaša izplatība; c) zema mobilitāte (lokalizēta informācija); d) labi zināmas ekoloģiskās īpašības; e) pietiekama skaitliskā izplatība; f) piemērotība laboratorijas eksperimentiem; g) augsta jutība pret vides stresa faktoru; h) augsta kvantitatīvās noteikšanas un standartizācijas spēja [5].

Bioloģiskajiem indikatororganismiem ir jāspēj atspoguļot gan dažādu vides apstākļu ilgtermiņa mijiedarbības, gan pēkšņas izmaiņas svarīgos vides rādītājos. Ūdens ekosistēmu biomonitoringam iespējams izmantot dažādas indikatororganismu alternatīvas, tomēr kopumā visplašāk atzītie un biežāk izmantotie organismi ir bentiskie makrobezmugurkaulnieki, perifitons, kā arī dažādu sugu zivis. Šie organismi ir pierādījuši savu efektivitāti, gan izmantojot tos atsevišķi, gan kombinācijās [5].

Perifitons ir autotrofu un heterotrofu mikroorganismu maisījums, kas saistīts organiskā detrīta matricā. Perifitons sedz lielāko daļu iegremdēto substrātu, sākot no smiltīm līdz augiem un beidzot ar akmeņiem [6]. Perifitons ir vērtīgs vides stāvokļa rādītājs strautos un upēs. Perifitonu organismi ir producenti, tāpēc tie nodrošina svarīgu barības ķēdes pamatu ūdens ekosistēmās. Perifitons atspoguļo īstermiņa ietekmi un pēkšņas izmaiņas ekosistēmā, jo tā reprodukcijas ātrums ir augsts un dzīves cikls ir īss. Daudzi autori ir devuši priekšroku perifitonam ūdens sistēmu biomonitoringam [5].

Tādi makrobezmugurkaulnieki, kā gliemeži, tārpi, saldūdens gliemenes, mīdijas un vēži, jau ilgstoši tiek izmantoti biomonitoringa nolūkos daudzās valstīs, lai uzraudzītu ūdens ekosistēmu ekoloģisko stāvokli. Vairākas bentisko makrobezmugurkaulnieku, īpaši gliemeņu, īpašības padara tās par piemērotiem indikatororganismiem ķīmiskā piesārņojuma izplatībai vidē [7]. Pirmkārt, tās ir

mazkustīgi organismi, tādējādi, tās spēj nodrošināt informāciju, kas raksturo konkrēto ievākšanas vietu. Turklāt tās ir izplatītas visā pasaulē gan saldūdens ūdenstilpēs, gan jūrās un okeānos. Otrkārt, īpatņi parasti veido kolonijas seklos ūdeņos, no kurienes ir viegli ievākt paraugus un pieejamais daudzums ir pietiekams analīžu veikšanai. Treškārt, to cietā čaula nodrošina vieglāku pārvadāšanu un saglabāšanu ekotoksiloģiskiem pētījumiem un *in situ* analīzēm. Ceturtkārt, gliemenes barojas, filtrējot apkārt esošo ūdeni lielos apjomos caur skropstiņveida žaunām un mutes lēveriem. Šī īpašība ir īpaši svarīga, jo tādā veidā notiek piesārņojuma akumulēšana organismā, nodrošinot informāciju gan par to koncentrāciju ūdenī, gan biopieejamību. Piektkārt, gliemenes ir ekoloģiski nozīmīgas kā barība vai dzīvesvieta citām sugām. Gliemenes ir primārie patērētāji (barojas no planktona un citiem mikroorganismiem), tādējādi tās nogādā antropogēno piesārņojumu no abiotiskās fāzes uz augstākiem trofiskajiem līmeņiem barības ķēdē [8]. Šo īpašību dēļ gliemenes ir izmantotas kā bioindikatori gandrīz 50 gadus [9]. Gliemenēs analizētie piesārņotāji aptver plašu savienojumu klāstu, sākot no "prioritārajiem piesārņotājiem", piemēram, smagajiem metāliem un halogenētiem ogļūdeņražiem, līdz jaunajiem piesārņotājiem, piemēram, FR, virsmaktīvām vielām, farmaceitiskajiem līdzekļiem un pat narkotiskām vielām [8, 10, 11].

Upju ekosistēmas veselības uzraudzībai jau ilgstoši tikušas izmantotas dažādu sugu zivju kopienas, jo tās ir viegli vizuāli novērtējamas un sniedz vērtīgu informāciju. Zivis ir ūdens barības ķēdes augšgalā, un tās patērē cilvēki, tāpēc tās ir svarīgs komponents piesārņojuma novērtēšanai. Tā kā zivīm ir relatīvi ilgs dzīves cikls, kā arī augsta mobilitāte, tās ir piemēroti indikatororganismi ilgtermiņa piesārņojumam plašākā teritorijā. Zivju kopienas reaģē uz vairākumu vides izmaiņu un traucējumu antropogēnās ietekmes rezultātā, tai skaitā ķīmisko piesārņojumu [5]. Eiropas zutis (*Anguilla anguilla*) ir plēsīga, katadroma zivs, kas ir plaši izplatīta visā Eiropā. Zuši absorbē un koncentrē savā organismā bioakumulatīvos organiskos piesārņotājus, kas nelielā koncentrācijā atrodas to uzturā, ko veido vēžveidīgie, tārpi, gliemeži, kāpuri un mazas zivis. Šo iemeslu dēļ zuši jau sen tiek uzskatīti par piemērotiem bioindikatoriem, kas var norādīt uz piesārņotājiem, kas atrodas vietējos biotopos [12, 13].

Biomonitoringu var veikt ne tikai ūdens ekosistēmās, bet arī sauszemes biotopos [14]. Šim nolūkam ir izmantoti neskaitāmi organismi, tostarp augi, bezmugurkaulnieki, rāpuļi, putni un zīdītāji [15]. Sauszemes zīdītāji ir pazīstami kā labi POP un smago metālu indikatororganismi [16]. Izplatītākie lielie zālēdāji Latvijā ir stirnas (*Capreolus capreolus*) (populācija 2014. gadā ~ 130 000 īpatņu), tām seko staltbrieži (*Cervus elaphus*) un aļņi (*Alces alces*) (populācija 2014. gadā attiecīgi ~ 52 000 un ~ 21 000 īpatņu) [17]. Staltbriežiem un aļņiem ir viens no lielākajiem paredzamajiem dzīves ilgumiem, un tādējādi tie ir visjutīgākie pret POP un smago metālu bioakumulāciju, un tos var uzskatīt par

visjutīgākajiem zālēdāju vides piesārņojuma stāvokļa bioindikatoriem. Staltbriežu un aļņu uzturs galvenokārt sastāv no augiem un pārstāv gaisa/augsnes – augu – zālēdāju sistēmu, savukārt mežacūkas (2014. gadā Latvijā populācija ~ 55 000 īpatņu [17]) ir visēdāji, kas patērē daudzveidīgu pārtiku, tai skaitā augus un kukaiņus, kā arī maitas, zivis un moluskus, kas nodrošina papildu piesārņotāju uzņemšanu.

1.2. Pasīvās paraugu ņemšanas iekārtas

POCIS ir ierīce, kas paredzēta ūdenī šķīstošo organisko ķīmisko vielu paraugu ņemšanai no ūdens vides. POCIS ir integrējošs paraugu ņemšanas līdzeklis, kas nodrošina informāciju par laika svērto vidējo ķīmisko vielu, tostarp piesārņotāju, līmeni. Parasti izvietošanas periodi svārstās no dažām nedēļām līdz vairākiem mēnešiem. Viena no galvenajām POCIS lietošanas priekšrocībām ir tā, ka tai nav mehānisku vai kustīgu detaļu, līdz ar to tam nav nepieciešams enerģijas avots vai uzraudzība lietošanas laikā. Turklāt POCIS integrētie sorbenti adsorbē ķīmiskās vielas no ūdens vidē izšķīdušās fāzes, tādējādi atdarinot iedarbību uz ūdens organismu elpceļiem.

1.1.att. **Četri POCIS uzstādīti uz nerūsējošā tērauda izvietošanas statīva**

POCIS ierīce sastāv no sorbenta, kas atrodas starp divām mikroporainām membrānām (skatīt 1.1.attēlu). Membrānas ļauj ūdenim un izšķīdušajām ķīmiskajām vielām iziet cauri sorbentam, kur ķīmiskās vielas tiek adsorbētas. Lielākas daļiņas, piemēram, nogulsnes un cietās daļiņas, neiekļūst membrānā. Membrāna ir izturīga pret bioloģisko piesārņojumu, kas var ievērojami samazināt adsorbēto ķīmisko vielu daudzumu. POCIS izmantojamo sorbentu iespējams

pielāgot tā, lai tas adsorbētu tikai konkrētas interesējošās ķīmiskās vielas. Savukārt, lai varētu veikt plašāka savienojumu klāsta noteikšanu, vienā izvietošanas tvertnē var izmantot dažāda veida sorbentus [18]. POCIS tehnoloģija ir piemērojama dažādām piesārņotāju klasēm, piemēram, medikamentiem, hormoniem, dažādiem pesticīdiem, kā arī mājsaimniecības un rūpniecības produktiem, tostarp alkilfenoliem, kofeīnam un FR [19].

1.3. Pētījumā iekļautās savienojumu grupas

BFR ietver daudzveidīgu antropogēno ķīmisko vielu grupu, ko izmanto, lai novērstu uzliesmošanu. BFR samazina dažādu produktu, piemēram, tekstilizstrādājumu, plastmasas, būvmateriālu un elektronisko iekārtu, uzliesmojamību. Neskatoties uz nepārprotamajām priekšrocībām, ko sniedz BFR izmantošana, šo ķīmisko vielu visuresošā klātbūtne ir izraisījusi to izplatīšanos vidē ražošanas, lietošanas un iznīcināšanas rezultātā. Savas noturības un lipofilitātes dēļ BFR nonāk sauszemes un ūdens barības ķēdēs [20].

Vieni no svarīgākajiem piesārņotājiem šajā saimē ir PBDE, kuru aplēstā produkcija pasaulē 2000. gadu sākumā sasniedza pat 67 000 tonnas gadā, un tie tika plaši izmantoti vairākas desmitgades [21]. Tomēr, ņemot vērā to, ka šie savienojumi ir POP, un to potenciālai pārnesei lielos attālumos, tika noteikti dažādi ierobežojumi to komerciālajai pieejamībai [22]. Tāpēc, lai apmierinātu tirgus pieprasījumu pēc FR, tika izstrādāti vairāki alternatīvi BFR, starp kuriem visvairāk patērētie bija HBCD un TBBPA. Līdzīgi kā PBDE, arī šiem alternatīvajiem BFR bija POP līdzīgas īpašības, un to izmantošana, kam sekoja utilizācija, izraisīja vides piesārņojumu [20, 23]. Eiropas Komisija klasificējusi HBCD kā bioakumulatīvu un toksisku savienojumu, jo tas ir ļoti noturīgs, slikti šķīst ūdenī un tam piemīt augsta $\log K_{ow}$ vērtība [24], savukārt attiecībā uz TBBPA radītajiem riskiem nav vienprātības. Vecākās atsauces ziņo, ka TBBPA, šķiet, ir salīdzinoši zema toksicitāte salīdzinājumā ar citām BFR grupām [25]. Tomēr jaunākie pētījumi atspoguļo toksikoloģiskās bažas par TBBPA klātbūtni vidē [26], tādējādi piesaistot zinātnisku interesi par šīs ķīmiskās vielas sastopamību vidē. Papildus HBCD un TBBPA ir arī vairākas citas ķīmiskas vielas, kas atzītas par jauniem jeb alternatīvajiem BFR, tostarp 1,2-bis(2,4,6-tribromfenoksi)etāns (BTBPE), heksabrombenzols (HBB) un dekabromdifeniletāns (DBDPE). Informācija par šo jauno BFR izplatību ir skopa, taču, lai īstenotu efektīvas stratēģijas šo savienojumu iespējamās bīstamās ietekmes samazināšanai, nepieciešams veikt datu ievākšanu, tādēļ Eiropas Komisija rekomendējusi veikt šo savienojumu monitoringu [27].

PFC ir liela savienojumu grupa, kas tiek ražoti jau vairāk nekā piecus gadu desmitus. PFC ir pilnībā fluorēti sintētiski savienojumi ar unikālām īpašībām, kas tiek klasificēti kā POP. Oglekļa-fluora saite ir viena no spēcīgākajām saitēm organiskajā ķīmijā. Šī īpašība padara PFC izturīgus pret tipiskiem vides

degradācijas procesiem un tādējādi noturīgus vidē. Visizplatītākās PFC ir perfluorētās karbonskābes un perfluorsulfonskābes, no kurām vispazīstamākās ir PFOA un PFOS. PFOS, tā sāļi un sulfonilfluorīds ir iekļauti Stokholmas konvencijas POP sarakstā. Molekulas fluorogļūdeņraža gals ir hidrofobs, lipofīls un nepolārs, savukārt funkcionālā grupa molekulas otrā galā nodrošina polaritāti. Gan PFOS, gan PFOA tiek klasificēti kā virsmaktīvās vielas, kas krasi samazina virsmas spraigumu. PFC tiek plaši lietoti ražošanā, tostarp tekstilizstrādājumu traipu atgrūdošos līdzekļos, papīra izstrādājumu piedevās un ūdens plēvi veidojošās putās, ko izmanto elektriskās strāvas izraisītu ugunsgrēku dzēšanai [28]. Cilvēkiem novērotā PFC toksiskā iedarbība ietver kancerogenitāti un imūntoksicitāti. Kā liecina jaunākie *in vitro* eksperimenti, PFOA ir toksiskāks nekā PFOS [29].

Farmaceitiskie produkti ir būtiska sastāvdaļa gan cilvēku, gan dzīvnieku medicīnā. Veterinārās zāles tiek izmantotas mūsdienu lopkopībā un pārtikas ražošanā un tiek lietotas dzīvnieku veselības uzturēšanai, infekciju profilaksei un slimību ārstēšanai. Tomēr aizliegto veterināro zāļu nelikumīga vai nepareiza lietošana, piemēram, noteikto periodu neievērošana, kas jāietur pēc zāļu lietošanas, var izraisīt zāļu atlieku klātbūtni dzīvnieku audos un ietekmēt pārtikas nekaitīgumu. Veterināro zāļu atliekas var ietvert pašus pamatsavienojumus, kā arī metabolītus un/vai konjugātus, un tiem var būt tieša toksiska ietekme uz patērētājiem, piemēram, alerģiskas reakcijas paaugstinātas jutības indivīdiem, hormonāla iedarbība, traucējot cilvēka hormonu līdzsvaru vai attīstību, vai antibakteriālās rezistences izplatība nepareizas antibiotiku lietošanas rezultātā [30].

Zāļu plašā patēriņa dēļ farmaceitiski aktīvās vielas ir kļuvušas par vienu no svarīgākajām jauno vides piesārņotāju klasēm. Jaunākie pētījumi ir atklājuši to sastopamību visā pasaulē pētītajos vides paraugos, tostarp dažāda veida ūdens matricās. Plaša zāļu lietošana ir izraisījusi relatīvi nepārtrauktu zāļu un to metabolītu ieplūdi notekūdeņos. Turklāt farmaceitiskie līdzekļi var nonākt ūdens avotos slikti kontrolētu ražošanas iekārtu notekūdeņos, galvenokārt tajās, kas saistītas ar plaši lietotām zālēm. Vairākos pētījumos atklāti farmaceitiskie līdzekļi zemās koncentrācijās dažādos ūdens avotos un dzeramā ūdens paraugos. Varētu domāt, ka farmaceitiskais piesārņojums nerada tiešu kaitējumu cilvēkiem, taču satraucošas norādes ūdens dzīvniekos liecina par preventīvu darbību nepieciešamību [31]. Antibakteriālās rezistences gēnu veicināšana vidē apdraud cilvēku veselību, lietojot dzeramo ūdeni, zivis vai ražu, kas satur aktīvās farmaceitiskās sastāvdaļas, tādējādi apdraudot dzīvību glābjošu antibiotiku pieejamību nākotnē [32].

1.4. Piesārņotāju izplatība apkārtējā vidē

PBDE izplatība dažādos organismos ir plaši pētīta un aprakstīta, tomēr ir pieejami tikai daži ziņojumi par šo BFR sastopamību savvaļas medījamo dzīvnieku vidū. Dažos Skandināvijas pētījumos ziņots, ka kopējais PBDE līmenis aļņu un briežu muskuļu paraugos svārstās no 10 līdz 500 pg g^{-1} (m.m.) Atbilstošajiem aknu paraugiem bija ievērojami augstāks piesārņojuma līmenis, sasniedzot 1700 pg g^{-1} (m.m.). [33-35].

Par HBCD sastopamību zušos ir ziņojuši daudzi autori, savukārt datu par citu jauno BFR izplatību ir maz. Ziņotie HBCD līmeņi zušu audos ir ļoti atšķirīgi – no 0,1 līdz 12 100 ng g^{-1} (l.m.) [36, 37]. Ir ziņots par HBB, 2-etil-heksiltetrabrombenzoāta (EH-TBB) un 2,4,6-tribromfenil-2,3-dibrompropilētera (TBP-DBPE) līmeņiem zušos, tomēr to koncentrācijas bija mazākas par 1 ng g^{-1} (l.m.) [38, 39].

PBDE bieži ir konstatēti gliemenēs. Dažādos avotos ziņotie līmeņi svārstās no 11,3 līdz 12 400 pg g^{-1} (m.m.) ar vislielāko vērtību, kas konstatēta gliemenēs no Nīderlandes [40]. Arī PFOS un PFOA ir konstatēti gliemeņu paraugos. Augstākā vidējā PFOS koncentrācija 72 000 pg g^{-1} (m.m.) tika konstatēta gliemenēs no Portugāles ziemeļu un centrālās daļas sateces baseiniem, kur atrodas papīra, tekstila un ādas rūpnīcas, kas izvada attīrītus notekūdeņus upju sistēmās [10]. Dati par NSAID sastopamību gliemenēs ir maz, jo tie netiek uzskatīti par tradicionāliem piesārņotājiem. Ir ticis ziņots par diklofenaka un ibuprofēna klātbūtni gliemenēs. Ziņotās vērtības bija attiecīgi līdz 16,1 un 93,7 ng g^{-1} (s.m.) [41, 42].

Urbanizācijas, industrializācijas un lauksaimniecības procesu dēļ ūdens ekosistēmas piesārņojums ir kļuvis par nopietnu problēmu. Daži autori ir salīdzinājuši farmaceitiskā piesārņojuma stāvokli ūdenī, izmantojot tādus paraugu veidus kā perifitons, bezmugurkaulnieki un zivis. Ir novērots, ka NSAID mēdz koncentrēties virszemes ūdeņos un ūdens organismos, turpretim antibiotikām ir tendence uzkrāties sedimentos [43, 44]. Ir arī novērots, ka savienojumi ar bāziskām īpašībām (pK_a>7) mēdz saistīties ar suspendētām cietvielām [45]. Tomēr tendences attiecībā uz koncentrāciju ūdenī vai sedimentos ir neskaidras. Piemēram, daži autori ziņojuši par augstāku acetaminofēna koncentrāciju virszemes ūdeņos, salīdzinot ar sedimentiem, citi – tieši pretēji [43, 46]. To saturs sedimentos un perifitona paraugos parasti ir zemāks nekā virszemes ūdens paraugos [44, 47].

1.5. Analīzes metodes

Pētījumam izvēlētajām piesārņotāju grupām ir dažādas ķīmiskās īpašības, tādēļ nepieciešams izmantot dažādas analītiskās pieejas. Mūsdienās svarīgs ķīmiskā piesārņojuma analīzes instruments ir HRMS. Tas ļauj noteikt analizējamās vielas ar precizitāti līdz tuvākajai 0,001 atomu masas vienībai. HRMS pirmo reizi izmantoja Džons Beinons 1950. gados, un tas bija aprīkots ar magnētiskā sektora analizatoru [48]. Kopš tā laika HRMS vairs nav ierobežots tikai ar gāzu hromatogrāfiju (GC), jo ir pieejami tādi instrumenti kā nolidojuma laika analizators (TOF), Orbitrap un FT-ICR. Parasti šie instrumenti mēra precīzu analītu masu bez fragmentēšanas, tomēr tos var kombinēt ar kvadrupolu, tādā gadījumā iespējama arī fragmentācijas spektru iegūšana un tiek panākta vēl lielāka selektivitāte [49]. Šīs metodes galvenā priekšrocība ir tā, ka tā ir ļoti selektīva, jo tā mēra precīzu savienojuma masu, ļaujot atšķirt pat nelielas struktūras izmaiņas. Līdz ar to ir iespējams noteikt arī analītus ar ļoti tuvām *m/z* vērtībām un izvairīties no to savstarpējiem traucējumiem.

Viens no lielākajiem izaicinājumiem BFR analīzē ir paraugu sagatavošana. Lai sasniegtu nepieciešamās noteikšanas robežas zemākas par ppt (pg g^{-1}) augsnē/sedimentos/biotā, parasti ir nepieciešami augsti iekoncentrēšanas faktori. Tas savukārt prasa attīrīšanas procedūras, kas spēj selektīvi atdalīt potenciāli lielus organisko materiālu daudzumus, vienlaikus saglabājot pēc iespējas vairāk vēlamo analītu. Ekstraktu attīrīšanas procedūra parasti sastāv no dažādiem posmiem, piemēram, cietfāzes-šķidruma adsorbcijas hromatogrāfijas kolonnās, izmantojot dažādu sorbentu kombinācijas, piemēram, silīcija dioksīda, Florisil, alumīnija oksīda un dažāda veida aktivētās ogles. Lielas molekulmasas savienojumu (piemēram, lipīdu) atdalīšanai no parauga ekstraktiem bieži izmanto gēlcaurspiešanas hromatogrāfiju (GPC).

EI-HRMS un elektronu satveres zemas izšķirtspējas masas spektrometrija negatīvās jonizācijas režīmā ir visizplatītākās BFR, piemēram, PBDE un HBCD noteikšanas metodes. Polihlorēto bifenilu klātbūtne paraugos var izraisīt selektivitātes problēmas, traucējot PBDE GC-MS analīzi. No šīm problēmām iespējams izvairīties, izmantojot HRMS. Vēl viena problēma BFR analīzē, izmantojot GC, ir termiskā labilitāte, īpaši savienojumiem ar augstu bromēšanas pakāpi (deka-BDE, HBCD un oktabromtrimetilfenillindāns). Šķidruma hromatogrāfijas (LC) izmantošana novērš termiskās labilitātes vai savstarpējās konversijas problēmu analīzes laikā. Daudzos pētījumos ir ziņots par LC-MS balstītu metožu izmantošanu HBCD diastereomēru specifiskā analīzē dažāda veida matricās. Izvēlētais analizators parasti ir Orbitrap-HRMS. Izotopiski marķētie iekšējie standarti ļauj pielietot iekšējo standartizāciju – visuzticamāko pieeju pareizai un precīzai kvantitatīvai noteikšanai [50].

PFOS un PFOA ekstrakciju no bioloģiskajām matricām parasti veic ar ultraskaņas palīdzību vai paātrināto šķīdinātāja ekstrakciju. Izvēlētie šķīdinātāji

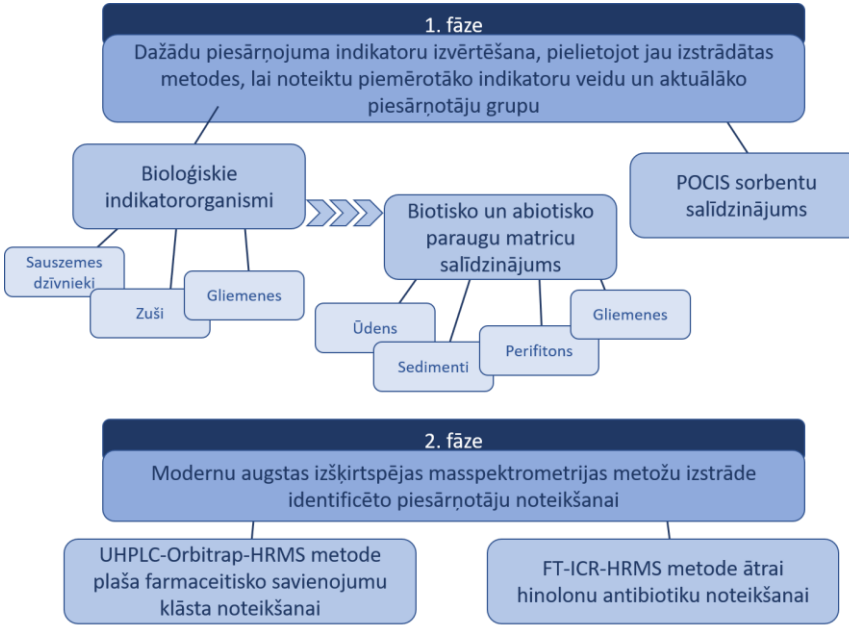
parasti ir metanols, acetonitrils vai metil-*t*-butilēteris. Lai nodrošinātu bāzisku ekstrakcijas vidi, dažkārt izmanto nātrija vai kālija hidroksīdus. Ekstraktu attīrīšanu veic ar dispersīvo aktivēto ogli etiķskābes klātbūtnē vai cietfāzes ekstrakciju (C18 vai *Oasis HLB*). Kvantitatīvo noteikšanu veic, izmantojot šķidruma hromatogrāfijas tandēma masas spektrometriju (LC-MS/MS) [51].

Farmaceitiski aktīvās vielas satur savienojumus ar plašu ķīmisko īpašību spektru, tāpēc arvien pieaug interese par analītiskajām metodēm, kas piemērotas daudzu analītu vienlaicīgai noteikšanai. Piemērotas parauga sagatavošanas procedūras izstrāde ir ievērojams izaicinājums, jo dažādās funkcionālās grupas, analītu amfotērās īpašības, un plašais polaritātes diapazons rada grūtības ekstrakcijas, attīrīšanas un analītiskās atdalīšanas posmos. Tādēļ parauga sagatavošana ir viens no vissvarīgākajiem soļiem. Visbiežāk izmantotās paraugu priekšapstrādes metodes muskuļaudu paraugiem ietver ekstrakciju ar šķīdinātāju (SE) ar vai bez attaukošanas posma, cietfāzes ekstrakciju (SPE), izmantojot SPE kolonnas vai dispersīvo cietfāzes ekstrakciju (dSPE) (QuEChERS pieeja), kam seko tālāka attīrīšana un/vai koncentrēšana [52].

LC-MS nodrošina universālu pieeju, kas piemērojama ļoti plašam farmaceitisko savienojumu klāstam. Visizplatītākā farmaceitiskās analīzes metode ir LC-MS/MS. Tomēr šī brīža tendences analītiskajā ķīmijā ir vērstas uz jaudīgāku HRMS detektoru, piemēram, TOF vai Orbitrap, izmantošanu. Šī tendence ir saistīta ar robustāku, jutīgāku un selektīvāku instrumentu pieejamību. Pēdējos gados Orbitrap sistēma ir guvusi atpazīstamību, jo tai ir labāks dinamiskās koncentrācijas diapazons, lielāka izšķirtspēja, līdz ar to arī labāka masas precizitāte salīdzinājumā ar TOF sistēmu [52].

2. EKSPERIMENTĀLĀ DAĻA

Promocijas darba pētījums tika sadalīts vairākās daļās saskaņā ar 2.1.attēlā attēloto shēmu.



2.1.att. **Darba plānojuma shematisks attēlojums**

2.1. Paraugi

BFR raksturošanai sauszemes ekosistēmā tika izmantoti 24 savvaļas dzīvnieku, tostarp aļņu, staltbriežu un mežacūku audi.

Kopumā 58 dažāda garuma un vecuma zuši, kas pārstāvēja 5 paraugu ņemšanas vietas Latvijā, tika analizēti kā iespējamie ūdens ekosistēmas BFR piesārņojuma rādītāji.

Gliemeņu paraugu partijas, kas pārstāvēja 24 paraugu ņemšanas vietas, tika izmantotas kā indikatororganismi turpmākai ūdens piesārņojuma novērtēšanai, paplašinot darbības jomu, iekļaujot PFC un farmaceitiski aktīvos savienojumus.

Paraugu tipu salīdzināšanas pētījumam tika izmantotas paraugu kopas, kas sastāvēja no gliemenēm, perifitona, sedimentiem un ūdens. Paraugi tika ievākti no trim upēm – Gaujas, Mūsas un Pēterupes. Paraugu ievākšanas vietas Gaujā

tika izvēlētas pēc apdzīvotām vietām – Valmieras, Cēsīm, Siguldas un Ādažiem, kā arī ietekā. Mūsā paraugi tika ņemti Pasvalē (Lietuva) pirms un pēc notekūdeņu attīrīšanas stacijas (WWTP), Latvijas-Lietuvas robežas teritorijā, kā arī no ietekas Lielupē. Pēterupē paraugi tika ievākti no četrām vietām Saulkrastu dzīvojamo ciematu teritorijā, kā arī ietekā.

Sorbentu efektivitātes noteikšanai tika izmantotas POCIS paraugu ņemšanas iekārtas, kas pildītas ar 9 dažādiem sorbentiem. 200 mg sorbenta tika manuāli iepakoti starp divām PES membrānām (0,1 μm, Ø 90 mm). Sorbenti, to ražotāji un īpatnējie virsmas laukumi apkopoti 2.1. tabulā. POCIS iekārtas tika ievietotas zivjaudzētavas "Tome" baseinā, kas darbojas Daugavas ūdens caurteces režīmā, un turētas vienu nedēļu.

2.1. tabula

POCIS izmantoto sorbentu raksturlielumi

Nr.p.k.	Sorbents	Ražotājs	Īpatnējais virsmas laukums, $m^2 g^{-1}$
1	HLB	*AFFINISEP*	190-210
2	Grafēna nanogranulas	*XG Sciences*	750
3	Grafēna nanogranulas	*Alpha Aesar*	500
4	MWCNT	*Chengdu Organic Chemicals Company*	200
5	MWCNTs-COOH	*Chengdu Organic Chemicals Company*	120
6	Oglekļa molekulārais siets	*SUPELCO*	>1000
7	MWCNT-aminopropilimidazols	*Bayer MaterialScience AG*, modificētas ar aminopropilimidazola grupu Materiālzinātnes un lietišķās ķīmijas fakultātes Polimērmateriālu institūtā	120
8	Oglekļa nitrīds	Sintezēts RTU Materiālzinātnes un lietišķās ķīmijas fakultātes Polimērmateriālu institūtā	210
9	Grafēns-oglekļa nitrīds	*XG Sciences* grafēna nanogranulas un sintezētais oglekļa nitrīds (1:1)	-

2.2. Bromēto liesmas slāpētāju analīze

2.2.1. Paraugu sagatavošana

Metode izvēlēto BFR noteikšanai tika pielāgota no iepriekš publicētiem pētījumiem [53-55]. Homogenizētu un liofilizētu vai mitru audu paraugu alikvotām tika pievienots iekšējo standartu šķīdums. Paraugus ekstrahēja ar dihlormetānu/*n*-heksānu (1:1, v/v), izmantojot automātisko Soxtec™ 2055 tauku ekstrakcijas sistēmu vai ultraskaņas veicināto ekstrakciju. Pēc ekstrakcijas šķīdinātājus iztvaicēja līdz sausam atlikumam vieglā slāpekļa plūsmā.

Gravimetriski noteica lipīdu saturu un tauku atlikumu atkārtoti izšķīdināja *n*-heksānā. Ekstrakts tika attīrīts, izmantojot ar skābi modificētu silikagela vai Florisil kolonnas. Pēc šķīdinātāja iztvaicēšanas ekstraktu apstrādāja ar koncentrētu sērskābi. Pirms PBDE GC-HRMS analīzes skābi saturošais apakšējais slānis tika atdalīts un organiskais slānis tika ietvaicēts, pievienojot atgūstamības standarta šķīdumu. Pēc PBDE analīzes šķīdinātājs tika nomainīts pret metanolu, un ekstraktos tika analizēts HBCD, TBBPA un EBFR saturs, izmantojot HPLC-Orbitrap-HRMS instrumentu.

2.2.2. Instrumentālā analīze

PBDE analīze tika veikta, izmantojot GC-HRMS, pielietojot EI jonizāciju pozitīvās jonizācijas režīmā izvēlētā jona skenēšanas (SIM) režīmā. Kvantitatīvā noteikšana tika veikta, izmantojot izotopu atšķaidīšanas metodi, kā iekšējos standartus izmantojot ar $^{13}C_{12}$ iezīmētus analītu surogātus. HBCD, TBBPA un EBFR analīzei tika izmantota HPLC-Orbitrap-HRMS metode. Interesējošie savienojumi tika atdalīti, izmantojot C18 apgrieztās fāzes analītisko kolonnu. HBCD un TBBPA analīzei tika izmantota HESI jonizācija negatīvajā jonizācijā, savukārt jaunie BFR tika noteikti, izmantojot negatīvo APPI režīmu, izmantojot toluolu kā piedevu. Analītu noteikšana tika veikta SIM režīmā, izmantojot divus intensīvākos jonus no attiecīgā molekulāro jonu klastera. Kvantitatīvā noteikšana tika veikta, pamatojoties uz izotopu atšķaidīšanu ar $^{13}C_{12}$-iezīmētiem analītu surogātiem un iekšējo standartizāciju.

2.3. Perfluorēto savienojumu analīze

Parauga sagatavošanas procedūra ietvēra ultraskaņas veicināto ekstrakciju ar metanolu un 0,2 M nātrija hidroksīda šķīdumu ūdenī, kam sekoja analītu koncentrēšana un attīrīšana, izmantojot vājās anjonu apmaiņas SPE kolonnas. Pēc eluāta ietvaicēšanas sausais atlikums tika izšķīdināts metanolā. Instrumentālās analīzes pamatā bija HPLC-Orbitrap-HRMS, izmantojot $HESI^-$ jonizāciju izvēlētās reakcijas skenēšanas (SRM) režīmā.

2.4. Farmaceitiski aktīvo savienojumu analīze

Atkarībā no parauga veida un izvēlētajām analītiem tika izmantotas dažādas analītiskās metodes.

2.4.1. Paraugu sagatavošana

NSAID analīzei 2 g homogenizētu gliemeņu paraugu ekstrahēja ar acetonitrilu un 0,02 M askorbīnskābes ūdens šķīdumu. Analītu koncentrēšana un attīrīšana tika panākta ar Strata C18 SPE kolonnām.

Sorbentus no POCIS ekstrahēja ar metanolu/dihlormetānu (1:1 v/v), izmantojot Soxtec™ automatizēto Soksleta ekstrakciju. Pirms instrumentālās analīzes ekstraktu šķīdinātājs tika aizvietots ar acetonitrila/metanola (9:1, v/v) maisījumu.

24 farmaceitiski aktīvo savienojumu analīzei dažādās matricās, sedimentu un perifitona paraugi tika liofilizēti pirms paraugu sagatavošanas procedūras. Gliemeņu, sedimentu un perifitona paraugi tika ekstrahēti, izmantojot ultraskaņas veicināto ekstrakciju. Ekstrakcija tika veikta divos ciklos pa 15 min, katrā ciklā izmantojot 5 mL 80% metanola šķīdumu ūdenī ar 0,1 % etiķskābi. Abas iegūtās ekstraktu porcijas tika apvienotas un atšķaidītas ar ūdeni. Tika veikta SPE, izmantojot Strata-X kolonnas. Ūdens paraugiem tika veikta tā pati SPE procedūra, pirms analīzes paraugus filtrējot. Visbeidzot, visi ekstrakti pēc ietvaicēšanas tika izšķīdināti ūdens/metanola šķīdumā (80:20, v/v).

Gaļas paraugus ekstrahēja, veicot mehānisku maisīšanu ar 0,1 % skudrskābi acetonitrilā Orbitrap skrīninga metodei un ar tīru acetonitrilu hinolonu analīzei ar FT-ICR-HRMS. Ekstraktu attīrīšana tika panākta, veicot paraugu izsaldēšanu -70°C temperatūrā. Hinolonu analīzei iegūtais ekstrakts tika atšķaidīts ar 0,1% skudrskābi ūdenī. Skrīninga metodei ekstrakti tika iztvaicēti un izšķīdināti 2:1 (v/v) ūdens-acetonitrila šķīdumā, kas satur 5 mM amonija formiāta un 0,01% etiķskābes.

2.4.2. Instrumentālā analīze

NSAID instrumentālo atdalīšanu un analīzi veica ar LC-MS/MS, izmantojot Acquity UPLC sistēmu (Waters, Milford, MA, ASV). Atdalīšana tika veikta uz Phenomenex Luna Omega analītiskās kolonnas (100 × 4,6 mm, 2,6 μm). Mobilās fāzes sastāvēja no 0,01% etiķskābes šķīduma ūdenī (A) un acetonitrila (B), plūsmas ātrums bija 0,6 mL min^{-1} un tika izmantota gradienta programma. Hromatogrāfs bija savienots ar QTrap 5500 (AB Sciex, MA, ASV) masspektrometru, kas aprīkots ar elektroizsmidzināšanas avotu, kas darbojās SRM režīmā negatīvā jonizācijā.

Dažādu farmaceitisko savienojumu un to metabolītu skrīnings tika veikts, izmantojot Dionex UltiMate 3000 HPLC sistēmu (Thermo Fisher Scientific, Sanhosē, CA, ASV) un Phenomenex Luna Omega analītisko kolonnu (100 × 2,1 mm, 1,6 μm). Kustīgā fāze sastāvēja no (A) 0,1% skudrskābes ūdenī, (B) 0,1% skudrskābes acetonitrilā un (C) 0,1% skudrskābes metanolā. Tika izmantota gradienta programma, un plūsmas ātrums bija 0,3 mL min^{-1}. HPLC sistēma bija savienota ar Q-Orbitrap HRMS masas spektrometru (Thermo Fisher Scientific), kas aprīkots ar HESI avotu, kas darbojas pozitīvā un negatīvā jonizācijas režīmā. Pilnas skenēšanas dati gan pozitīvajā, gan negatīvajā jonizācijas režīmā tika iegūti ar masas izšķirtspēju 70 000 FWHM.

24 farmaceitiski aktīvo vielu analīze tika veikta, izmantojot Dionex UltiMate 3000 HPLC sistēmu (Thermo Fisher Scientific, Sanhosē, CA, ASV) un Kinetex C18 analītisko kolonnu (100 × 2,1 mm, 2,6 μm). Kustīgā fāze sastāvēja no (A) 0,01% etiķskābes ūdenī, (B) acetonitrila un (C) metanola. Tika izmantota gradienta programma, un plūsmas ātrums bija 0,3–0,4 mL min^{-1}. HPLC sistēma bija savienota ar Q-Orbitrap HRMS masas spektrometru (Thermo Fisher Scientific), kas aprīkots ar HESI avotu. SRM tika izmantots gan pozitīvajā, gan negatīvajā jonizācijas režīmā.

Hinolonu noteikšana tika veikta ar tiešās injekcijas FT-ICR-HRMS metodi. Pirms analīzes katru dienu tika izmantots nātrija formiāta šķīdums, lai kalibrētu instrumentu, kas aprīkots ar 7,0 T supervadītāja magnētu (Bruker Daltonics, Brēmene, Vācija). Paraugs tika tieši ievadīts ESI avotā ar plūsmas ātrumu 250 μL h^{-1}. Masas spektrometrs tika iestatīts tā, lai tas darbotos masas diapazonā 100–1000 *m/z* pozitīvajā jonizācijas režīmā. Katrs masspektrs tika iegūts, uzkrājot 32 individuālus apakšmērījumus (2 miljoni datu punktu vienā apkašmērījuma datu kopā). Izšķirtspēja $m/\Delta m(50\%)=140\ 000$ un masas precizitāte <3 ppm nodrošināja nekļūdīgu molekulāro formulu piešķiršanu vienvērtīgi lādētiem molekulārajiem joniem. MS/MS eksperimentos tika izvēlēts sadursmes izraisītas disociācijas režīms, kā sadursmes gāze izmantots argons, sadursmes enerģija tika regulēta no 5 eV līdz 25 eV, izolācijas logi iestatīti uz 5 *m/z* un sadursmes RF amplitūda tika iestatīta uz 1500 Vpp.

3. REZULTĀTI UN TO IZVĒRTĒJUMS

3.1. Bioloģisko indikatoru pielietošana piesārņotāju izplatības pētījumos

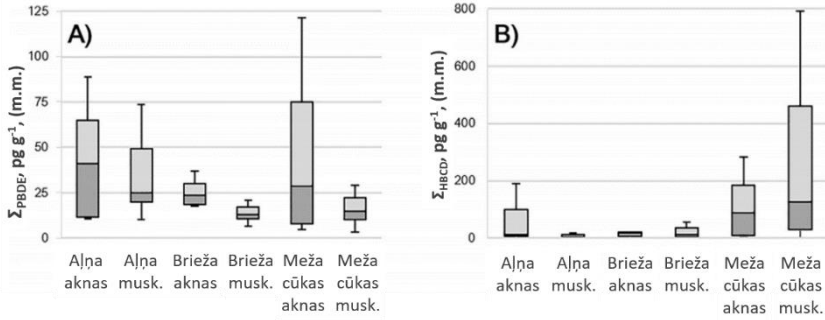
3.1.1. Savvaļas medījumi kā BFR piesārņojuma indikatori[1]

Lai novērtētu savvaļas dzīvnieku kā sauszemes ekosistēmas piesārņojuma indikatoru pielietojamību, tika analizēti astoņu staltbriežu (*Cervus elaphus*), deviņu mežacūku (*Sus scrofa*) un septiņu aļņu (*Alces alces*) muskuļu un aknu audu paraugi, lai noteiktu BFR, tostarp PBDE, HBCD un TBBPA saturu.

Lielākās vidējās PBDE koncentrācijas (46,6 pg g^{-1} (m.m.)) tika novērotas aļņu audos, bet ievērojami zemākas – mežacūku un staltbriežu audos. Iegūtie rezultāti bija zināmā mērā pārsteidzoši, ņemot vērā zālēdāju aļņu un visēdāju mežacūku atšķirīgo uzturu, tādējādi sagaidot augstāku BFR saturu mežacūku audos papildu PBDE ekspozīcijas ceļu dēļ. Tas liecina, ka vecumam ir liela nozīme PBDE akumulācijā, jo aļņu īpatņu vidējais vecums bija 2,7 gadi, bet mežacūku īpatņu vidējais vecums bija 1,4 gadi. Tomēr netika novērota neviena būtiska korelācija starp mūsu pētījumā izmantotā parauga vecumu un BFR saturu (r_s diapazonā no -0,49 līdz 0,64; *p*-vērtības diapazonā no 0,13 līdz 0,84). Meža cūku paraugos bija visaugstākais HBCD līmenis ar vidējo koncentrāciju 264 pg g^{-1} (m.m.) muskuļaudos. Aļņu un staltbriežu paraugos HBCD koncentrācijas bija augstākas aknu audos nekā muskuļaudos, savukārt mežacūkām HBCD koncentrācija bija augstāka muskuļaudos nekā aknās. Neskatoties uz TBBPA plašo pielietojumu Eiropā, kopumā tika novērota zema vidējā koncentrācija no 0,52 līdz 4,54 pg g^{-1} (m.m.). Visticamākais šī novērojuma izskaidrojums varētu būt fakts, ka TBBPA saturošu polimēru ražošanas laikā šis BFR tiek ķīmiski saistīts ar materiālu, samazinot tā izdalīšanās iespējamību vidē. Analizētajos paraugos noteiktie PBDE un HBCD piesārņojuma līmeņi parādīti 3.1. attēlā.

[1]Zacs, D.; Rjabova J.; **Ikkere, L.E.**; Bavrins, K.; Bartkevics, V. Brominated flame retardants and toxic elements in the meat and liver of red deer (*Cervus elaphus*), wild boar (*Sus scrofa*), and moose (*Alces alces*) from Latvian wildlife. *Sci Total Environ* **2018**, *621*, 308-316.

Novērotie piesārņojuma līmeņi ir salīdzināmi vai nedaudz zemāki, nekā ziņots citos pētījumos. Somijas briežu muskuļos novērotā ∑PBDE koncentrācija bija robežās no 10 līdz 180 pg g^{-1} (m.m.), bet Lapzemes reģiona paraugos bija lielāka vērtība – 500 pg g^{-1} (m.m.). Citos veiktajos pētījumos aknu paraugos konstatētie līmeņi ir ļoti atšķirīgi. Briežu aknās Vācijā detektēts saturs 30–120 pg g^{-1} (m.m.), aļņos Norvēģijā – 33–50 pg g^{-1} (m.m), bet ziemeļbriežos Somijā līdz pat 1700 pg g^{-1} (m.m.) [33, 35].



3.1.att. **PBDE (A) un HBCD (B) koncentrācijas analizētajos audos**

Attiecībā uz PBDE pārstāvju profilu, dominējošie bija PBDE tetra- līdz heksabromētie homologi, kas sastādīja no 59 līdz 91% no visiem analizētajiem PBDE. Tas norāda, ka nesen izmantotais "penta-BDE" preparāts ir iespējamais piesārņojuma avots. Neraugoties uz to, ka nesen plaši tika lietoti "okta-BDE" un "deka-BDE" preparāti, kas satur galvenokārt PBDE-209, šis PBDE pārstāvis sastādīja tikai aptuveni 6 % no kopējā daudzuma. Daudz lielāks saturs tika novērots aknu paraugos, sasniedzot vidēji 36% mežacūku aknās. Iegūtie rezultāti atspoguļo atšķirības bioakumulācijas vai biotransformācijas potenciālā PBDE pārstāvjiem ar dažādu bromēšanas pakāpi, kā tas jau ir ticis novērots iepriekšējos pētījumos [33-35].

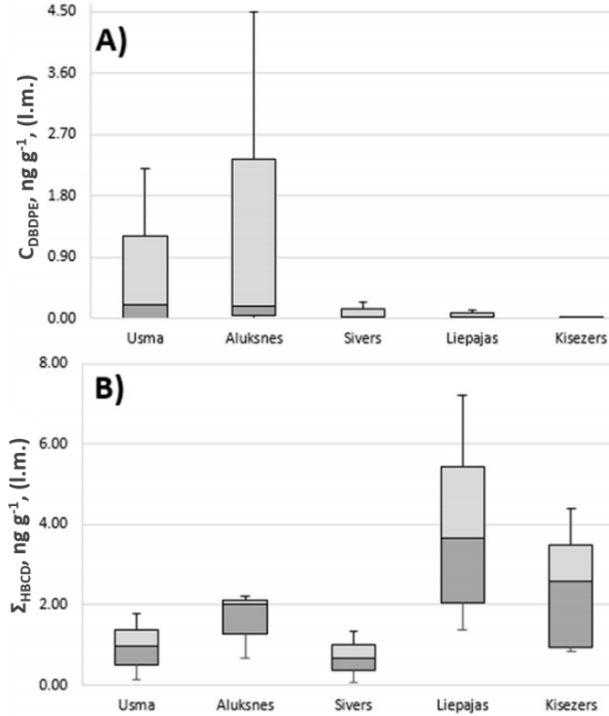
Novērotais HBCD izomēru profils bija līdzīgs ūdens biotā parasti sastopamajam sadalījumam, proti, novērojama izteikta α-HBCD dominance pār β- un γ-HBCD [13], α-HBCD sastādot vidēji 85% no ∑HBCD.

3.1.2. Zuši kā jauno BFR piesārņojuma indikatori[2]

Lai novērtētu zušu kā indikatororganismu pielietojamību ūdens ekosistēmas piesārņojumam, tika noteikts septiņu jauno BFR (tostarp HBCD, DBDPE, TBP-DBPE, HBB, EH-TBB, BTBPE un tetradekabrom-1,4-difenoksibenzola) saturs zušos (*Anguilla anguilla*), kas ievākti no pieciem Latvijas ezeriem. Iegūtie rezultāti apkopoti 3.2. attēlā.

HBCD tika konstatēts visos analizētajos paraugos, apstiprinot HBCD visuresošo izplatību Eiropas ūdens ekosistēmā [56]. α-, β- un γ-HBCD koncentrāciju (∑HBCD) summa bija robežās no 0,05 līdz 6,58 ng g^{-1} (l.m.), ar vidējo vērtību 1,64 ng g^{-1} (l.m.). Noteiktie HBCD līmeņi zušos no citām valstīm bija ievērojami augstāki, augstākā vidējā koncentrācija 4500 ng g^{-1} (l.m.) tika novērota zušos no ļoti piesārņotām teritorijām Beļģijā, kur atrodas vairākas tekstilrūpnīcas [37]. Analizētajos paraugos novērotais HBCD izomēru modelis bija tipisks biotai, tāpat, kā tika novērots jau iepriekšējā pētījumā, ar izteiktu α-HBCD pārsvaru pār β- un γ-HBCD [13]. Galvenie faktori, kas var būt par iemeslu šim novērojumam, ir: 1) β- un γ-HBCD enzimātiska izomerizācija par α-HBCD, kā tas ir ticis novērots zivīm [56]; 2) α-HBCD ir augstāka šķīdība ūdenī (~49 mg L^{-1}) nekā β- un γ-HBCD (~2 mg L^{-1}), un tādējādi tas ir vieglāk pieejams uzņemšanai [57]; 3) *in vitro* eksperimenti ar aknu sub-šūnu frakcijām, kas iegūtas no žurkām un forelēm, parādīja, ka β- un γ-HBCD biotransformācija bija aptuveni trīs reizes ātrāka nekā α-HBCD [58]; un 4) γ-HBCD termiskā un fotolītiskā izomerizācija par α-HBCD [59].

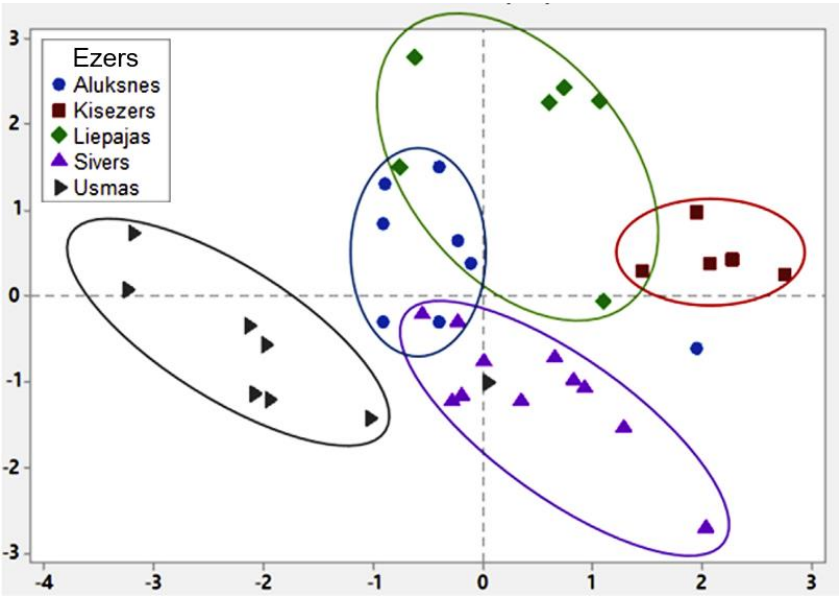
[2]Zacs, D.; **Ikkere, L.E.**; Bartkevics, V. Emerging brominated flame retardants and dechlorane-related compounds in European eels (*Anguilla anguilla*) from Latvian lakes. *Chemosphere* **2018**, *197*, 680-690.



3.2.att. **DPDBE (A) un HBCD (B) koncentrācijas analizētajos zušu paraugos**

DBDPE bija vienīgais BFR, kas, neskaitot HBCD, tika atrasts analizētajos zušu paraugos. 59% paraugu saturēja DBDPE līdz 33 ng g^{-1} (l.m.). Cik zināms, līdz šim nav publicēti citi ziņojumi par DBDPE sastopamību zušos. Saskaņā ar pieejamajām atsaucēm DBDPE netika konstatēts dažādos zivju paraugos no Sentlorensa upes Kanādā un Čīles piekrastes ūdeņos [60, 61]. Savukārt paraugos, kas iegūti no ļoti piesārņotas upes Dienvidķīnā un vairākām upēm Spānijā, tika konstatētas koncentrācijas attiecīgi līdz 230 un 130 ng g^{-1} (l.m.) [62, 63]. DBDPE Eiropā ir uzskaitīts kā maza ražošanas apjoma ķīmiskais savienojums, tomēr Ķīnā tas ir otrais visbiežāk izmantotais BFR [64]. Ņemot vērā novēroto koncentrāciju izteikto neviendabīgumu, galvenie DBDPE piesārņojuma ceļi joprojām nav zināmi. Piesārņojums ar DBDPE var rasties gan no punktveida avotiem, gan no attāliem avotiem, jo BDE-209 gadījumā pētījumos atklāts piesārņojums arī attālos reģionos atmosfēras pārneses rezultātā. Fizikāli ķīmiskās līdzības dēļ tas varētu attiekties arī uz DBDPE [65].

Iegūtie rezultāti atspoguļo, ka Latvijas ezeros ir pieņemams vides stāvoklis attiecībā uz kopējo HBCD saturu, ņemot vērā Direktīvā 2013/39/ES [66] noteiktos EQS. Augstākie HBCD līmeņi tika novēroti zušos no ezeriem, kas atrodas reģionos ar augstāku industrializācijas pakāpi, savukārt galveno komponentu analīzes rezultāti (3.3. att.) liecina, ka HBCD koncentrācija ir atkarīga no konkrētā ezera, atspoguļojot nevienmērīgu jauno BFR piesārņojumu Latvijas ezeros.



3.3.att. **HBCD līmeņu un zušu bioloģisko parametru galveno komponentu analīzes grafiks**

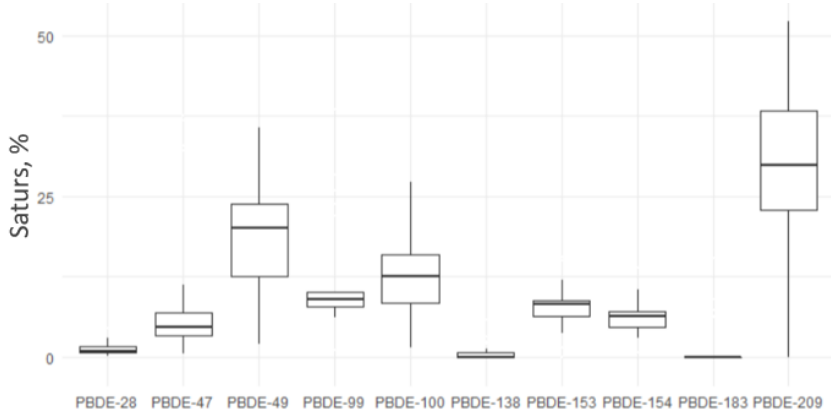
3.1.3. Gliemenes kā BFR, PFC un farmaceitisko savienojumu piesārņojuma indikatori[3]

Tālākais ūdens indikatororganismu novērtējums tika veikts, pētot saldūdens gliemeņu pielietojamību. Papildus jau apspriestajiem BFR saldūdens gliemeņu audos tika noteikta arī cita POP grupa, proti, PFC un jaunie piesārņotāji – farmaceitiski aktīvie savienojumi, konkrēti NSAID.

PBDE tika konstatēti visos analizētajos paraugos, kas vēlreiz apstiprina šo liesmas slāpētāju plašo izplatību vidē. Kopējā ΣPBDE koncentrācija bija robežās no 11,3 līdz 193 pg g^{-1} (m.m.), ar vidējo vērtību 42,1 pg g^{-1} (m.m.). Novērotie līmeņi bija vairāk nekā kārtu zemāki nekā iepriekšējā pētījumā konstatētie līmeņi zušos no Latvijas ezeriem (skatīt 3.1.2. apakšnodaļu). Kā ierosināja Marriusen et al, tas var būt saistīts ar atšķirībām abu organismu lipīdu saturā [35]. Turklāt zuši ir augstākā barības ķēdes posmā, un to kopējais mūžs ir garāks. Salīdzinot novērojumu rezultātus ar citviet gliemenēs konstatētajiem, koncentrācijas Latvijas gliemenēs bija ievērojami zemākas, izņemot gliemenes no Baiyangdian ezera, kas ir līdzīgas šim pētījumam [67]. Tomēr, neskatoties uz to, ka Latvijā konstatētie PBDE līmeņi bija salīdzinoši zemi, 83% paraugu tika pārsniegta EQS vērtība biotā (8,5 pg g^{-1} (m.m.) pārstāvju Nr. 28, 47, 99, 100, 153 un 154 summai), kā noteikts Eiropas Komisijas Direktīvā 2013/39/ES [66].

Attiecībā uz PBDE pārstāvju profilu (skatīt 3.4.attēlā) visaugstākais līmenis tika novērots PBDE-209 gadījumā, kas ir vienīgais dekabromētais BDE. Tas var būt "deka-BDE" preparāta plašās izmantošanas, suspendēto daļiņu radīto piesārņojošo vielu uzkrāšanās un neefektīva attīrīšanas procesa rezultāts. Pārstāvji Nr. 47, 49, 99 un 100, kas pārstāv tetra- un pentabromētu BDE, dominēja lielākajā daļā paraugu. Šis novērojums atbilst citu autoru rezultātiem [68, 69].

[3] **Ikkere, L.E.**; Perkons, I.; Sire, J.; Pugajeva, I.; Bartkevics, V. Occurrence of polybrominated diphenyl ethers, perfluorinated compounds, and nonsteroidal anti-inflammatory drugs in freshwater mussels from Latvia. *Chemosphere* **2018**, *213*, 507-516.



3.4.att. **Gliemenēs konstatēto PBDE pārstāvju sadalījums**

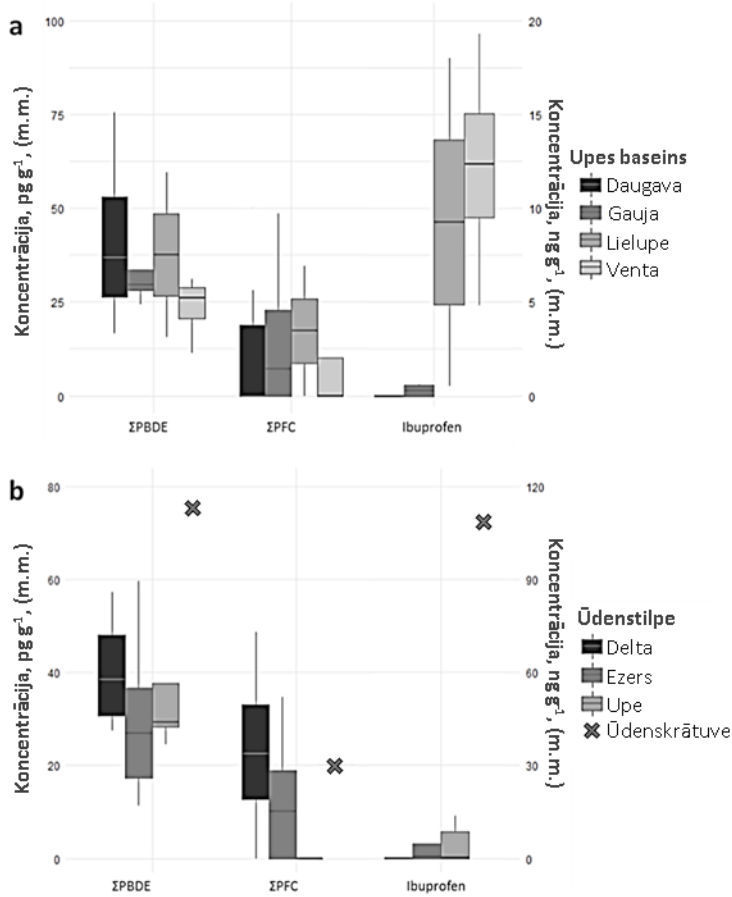
Gliemeņu audos tika noteikti divi savienojumi, kas pārstāv PFC - PFOS un PFOA. PFOS tika konstatēts 3 no 24 (13%) paraugiem ar koncentrāciju robežās no 10 līdz 21 pg g^{-1} (m.m.), savukārt PFOA tika konstatēts biežāk – 10 no 24 (42%) paraugu, diapazonā no 13 līdz 51 pg g^{-1} (m.m.). Novērotās vērtības bija divas līdz trīs kārtas zemākas nekā PFOA un tā atvasinājumu EQS vērtība biotā, kas noteikta Eiropas Komisijas Direktīvā 2013/39/EK [66]. Rezultāti, par kuriem ziņots attiecībā uz gliemenēm no citiem reģioniem, bija ievērojami augstāki – PFOS līmenis Portugāles ziemeļu un centrālās daļas upju sateces baseinos bija līdz 72 000 pg g^{-1} (m.m.) [10].

Gliemenēs tika analizēti arī farmaceitiskie savienojumi – deviņi NSAID, tostarp ibuprofēns, tolfenamīnskābe, meloksikāms, karprofēns, fluniksīns, diklofenaks, fenilbutazons, ketoprofēns un mefenamīnskābe. No deviņiem savienojumiem gliemenēs tika konstatēts tikai ibuprofēns. Ibuprofēns bija 50% paraugu ar koncentrāciju robežās no 0,52 līdz 109 ng g^{-1} (m.m.) vai no 5,1 līdz 1363 ng g^{-1} (s.m.) Ziņojumu par pētījumiem citās valstīs ir maz, ņemot vērā, ka NSAID ir jauni piesārņotāji. Līmenis, kas novērots gliemenēs no Taihu ezera, Ķīnā, ir krietni zemāks, sasniedzot vien 93,7 ng g^{-1} (s.m.) [42].

Augsts ibuprofēna līmenis Latvijas ūdens vidē novērots citos pētījumos [70, 71], kur ibuprofēns konstatēts virszemes ūdeņos un neattīrītos notekūdeņos. Ibuprofēns bija visvairāk patērētais NSAID grupas medikaments Latvijā 2017. gadā [72]. Galvenais ibuprofēna ievadīšanas ceļš virszemes ūdeņos ir notekūdeņu attīrīšanas iekārtu notekūdeņi. Ibuprofēns un tā metabolīti tiek izvadīti kanalizācijas sistēmā gan cilvēka metabolisma rezultātā, gan kopā ar neizlietotajām zālēm, kuras var izmest caur kanalizāciju un tualetēm [73].

Katras piesārņotāju grupas kopējo koncentrāciju diagrammas ir

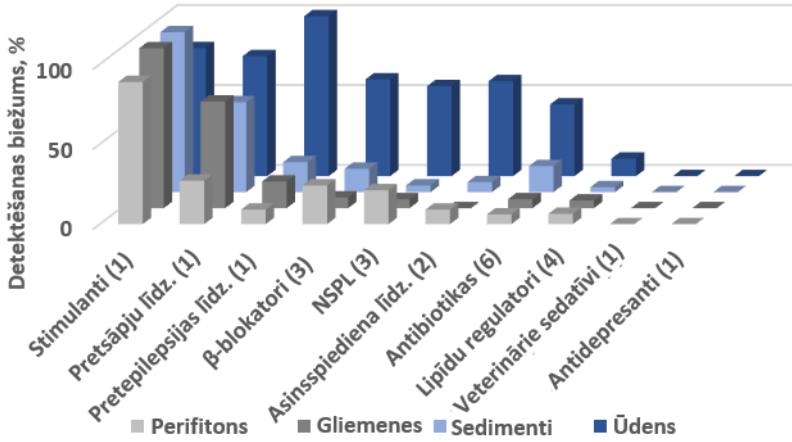
parādītas 3.5. attēlā. Paraugi tika grupēti pēc sateces baseina un ūdenstilpju veidiem – ezers, upe, upes delta vai ūdenskrātuve. PBDE bija biežāk sastopamas Daugavas un Lielupes baseinā. Vislielākā izkliede tika novērota ibuprofēna gadījumā, jo Lielupes un Ventas baseinos tas bija sastopams ievērojami lielākās koncentrācijās nekā Gaujas un Daugavas baseinos. Attiecībā uz piesārņotāju izplatību dažāda veida ūdenstilpēs, paraugā no Rīgas ūdenskrātuves tika konstatēti ievērojami augstāki PBDE un ibuprofēna koncentrāciju līmeņi.



3.5.att. **ΣPBDE, ΣPFC (pg g^{-1} (m.m.)) un ibuprofēna (ng g^{-1} (m.m.)) saturs gliemeņu paraugos dažādos sateces baseinos (a) un ūdenstilpju veidos (b)**

3.1.4. Perifitona, gliemeņu, ūdens un sedimentu salīdzinājums

Lai novērtētu dažādu paraugu tipu piemērotību indikatoru pielietojumam, tika analizēti perifitona, gliemeņu, ūdens un sedimentu paraugi no trim Latvijas upēm. Katra partija, kas sastāvēja no četriem paraugu veidiem, tika ievākta no četrām līdz piecām paraugu ņemšanas vietām katrā upē. Pētījumam tika atlasīti 23 farmaceitiski aktīvi savienojumi. Ķīmisko savienojumu atlase tika veikta, pamatojoties uz dažādu publikāciju informāciju par ūdeņos biežāk sastopamajām zāļu atliekām, un lielākā daļa no tām ir Pasaules Veselības organizācijas sarakstā kā pirmās nepieciešamības zāles. Atlasītie savienojumi pieder dažādām terapeitiskajām grupām, piemēram, NSAID, asins lipīdu līmeni pazeminošiem līdzekļiem, antibiotikām, pretepilepsijas līdzekļiem, β-blokatoriem, antidepresantiem un citiem.



3.6.att. **Analizēto savienojumu detektēšanas biežums dažādos paraugu veidos**

No 23 analizētajiem savienojumiem tikai trīs – lipīdu regulators simvastatīns, veterinārais sedatīvs ksilazīns un antidepresants fluoksetīns netika konstatēti nevienā no paraugiem. Visu analizēto terapeitisko grupu detektēšanas biežums ir parādīts 3.6. attēlā. Visbiežāk konstatētais savienojums bija centrālo nervu sistēmu stimulants kofeīns ar kopējo noteikšanas biežumu 91%. Šis savienojums tika iekļauts pētījumā, jo tas tiek uzskatīts par ķīmisko marķieri sadzīves notekūdeņu radītajam ūdens piesārņojumam. Mūsu iepriekšējā pētījumā kofeīns tika konstatēts notekūdeņos līdz 12 μg L^{-1} [70]. Šajā pētījumā kofeīna koncentrācija virszemes ūdeņos bija zem 50 ng L^{-1}. Augstākais līmenis tika novērots NSAID diklofenakam un pretepilepsijas līdzeklim karbamazepīnam ūdenī – attiecīgi 1138 un 1099 ng L^{-1}. Novērotie

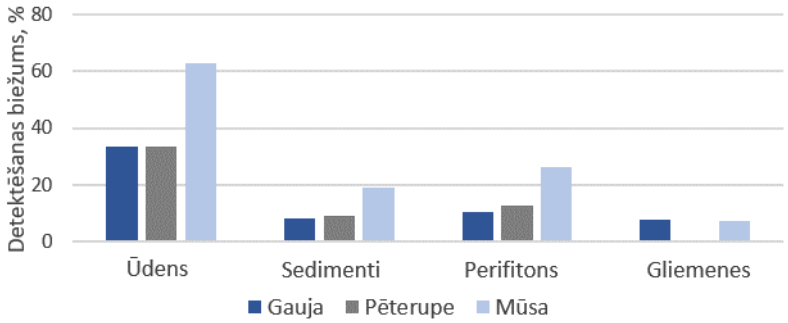
līmeņi ir samērā augsti, piemēram, karbamazepīns līdzīgās koncentrācijās tika konstatēts Vācijas upēs, savukārt citās valstīs līmenis bija ievērojami zemāks [74]. Diklofenaka gadījumā citur ir novērots koncentrācijas līmenis līdz pat 7100 ng L^{-1} [75]. Augstais piesārņojuma līmenis Mūsas upē Lietuvā pie Pasvales, visticamāk, ir saistīts ar tuvumā esošo WWTP. Lai pārbaudītu šo hipotēzi, pirms WWTP ieplūdes tika savākta vēl viena paraugu partija, kas sastāvēja no ūdens un sedimentu paraugiem. Iegūtie rezultāti paraugos pirms un pēc WWTP ieplūdes apkopoti 3.1. tabulā. Lielākajai daļai savienojumu tika novērots ievērojams līmeņu pieaugums, visizteiktāk antibiotikas sulfametoksazola, antihipertensīva metoprolola un NSAID diklofenaka gadījumā – aptuveni 200 reizes. Šis novērojums var liecināt par nepilnīgu WWTP darbību attiecībā uz farmaceitisko savienojumu atdalīšanu. Citi autori ir aprakstījuši, ka tradicionālajās WWTP diklofenaka atdalīšanas efektivitāte ir zema un dažreiz iespējams novērot pat koncentrācijas pieaugumu konjugēto formu hidrolīzes dēļ [75].

3.1. tabula

Farmaceitiski aktīvo savienojumu izplatība Mūsas upē pie Pasvales, Lietuvā

Savienojums	Terapeitiskā grupa	Koncentrācija ūdenī, ng L^{-1}		Koncentrācija sedimentos, ng g^{-1} (s.m.)	
		Pirms NAS	Pēc NAS	Pirms NAS	Pēc NAS
Azitromicīns	Antibiotikas	<1	31	<1	<1
Ciprofloksacīns		<1	100	<1	<1
Eritromicīns		<10	44	<5	<5
Klaritromicīns		1,6	40	<1	<1
Sulfametoksazols		1,0	161	<0,5	<0,5
Trimetoprīms		<1	34	<0,5	<0,5
Losartāns	Antihipertensīvi	0,6	30	<1	<1
Metoprolols		1,4	273	1,8	12
Propranolols		0,1	2,8	<1	<1
Valsartāns		6,0	55	<1	1,3
Atorvastatīns	Lipīdu vielmaiņas regulatori	<1	1,4	<10	<10
Pravastatīns		<1	12	<1	1,5
Diklofenaks	NSAID	6,3	1138	<5	<5
Ibuprofēns		<1	12	<1	<1
Acetaminofēns	Pretsāpju līdzekļi	<1	30	<1	<1
Karbamazepīns	Pretepilepsijas līdzekļi	21	1099	<1	8,2

Trīs analizēto upju salīdzinājums parādīts 3.7. attēlā. Augstākie konstatētie līmeņi, kā arī noteikšanas biežums tika novērots Mūsas upē. Attiecībā uz paraugu ņemšanas vietām tikai Mūsas upē tika novērotas būtiskas atšķirības. Kā minēts iepriekšējā rindkopā, ievērojami augstāki līmeņi tika konstatēti paraugos no Pasvales, Lietuvā. Augsti līmeņi konstatēti arī paraugos no Mūsas grīvas (Lielupes), jo tur raksturīga lielāka antropogēnā slodze. Pārējās divās upēs atšķirības starp paraugu ņemšanas vietām nebija tik izteiktas.

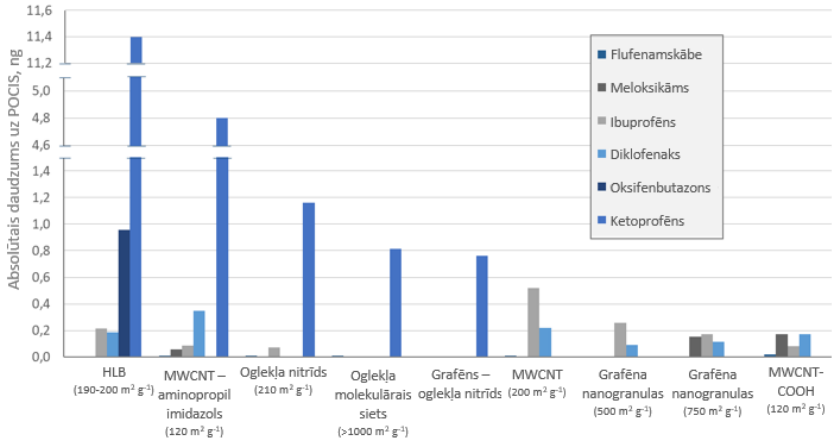


3.7.att. **Savienojumu detektēšanas biežums pētītajās upēs**

Visbeidzot tika salīdzināti četri paraugu veidi. Kā redzams 3.6. un 3.7. attēlā, visaugstākais detektēšanas biežums tika novērots ūdens paraugos. Tas var būt saistīts ar zemāku matricas efektu līmeni nekā citiem paraugu veidiem. Var secināt, ka ūdens ir vispiemērotākā matrica īslaicīgai piesārņojuma novērošanai. Citi paraugu veidi var būt piemērotāki ilgstošai piesārņojuma noteikšanai; tomēr, lai uzlabotu analītisko metodoloģiju pielietojamību, ir nepieciešamas jutīgākas un selektīvākas analītiskās metodes.

3.2. POCIS pielietojums piesārņojuma noteikšanai

Lai novērtētu POCIS pielietojamību, tika pārbaudīti deviņi dažādi sorbenti piecpadsmit NSAID noteikšanai Daugavā. Daudzslāņu oglekļa nanocaurulītes (MWCNT) un TNIM4 bāzes sorbenti iepriekš ir pierādījuši, ka veiksmīgi adsorbē NSAID no virszemes ūdens paraugiem [71]. Šajā pētījumā tika izmantoti gan tradicionāli un bieži lietoti sorbenti, piemēram, hidrofilā-lipofilā līdzsvara sorbents (HLB), gan jauni sorbenti, piemēram, MWCNT un citi. Visi POCIS tika ievietoti zivjaudzētavas "Tome" baseinā, kas darbojas Daugavas ūdens caurplūdes režīmā, uz vienu nedēļu vienā un tajā pašā paraugu ņemšanas vietā, lai nodrošinātu vienādus apstākļus. Rezultāti parādīti 3.8. attēlā. Kā redzams, iegūtie NSAID līmeņi ir ļoti atkarīgi no izmantotā sorbenta.



3.8.att. **Noteiktās nesteroīdo pretiekaisuma līdzekļu koncentrācijas izmantojot dažādus POCIS sorbentus**

Vislabākie rezultāti tika iegūti, izmantojot plaši pielietoto HLB sorbentu. Tika konstatēti pieci no piecpadsmit savienojumiem, tostarp oksifenbutazons, kas netika detektēts neviena cita sorbenta ekstraktos. Turklāt ketoprofēna līmenis bija ievērojami augstāks, nekā tika novērots ar citiem sorbentiem. Tomēr meloksikāms tika detektēts tikai izmantojot grafēna, MWCNT-COOH un MWCNT-aminopropilimidazola sorbentus. Šis novērojums varētu būt saistīts ar to, ka no visiem analītiem meloksikāmam ir viszemākā pK_a vērtība, proti 1,1. Komerciāli pieejamais HLB sorbents ir piemērots plaša klāsta savienojumu sorbcijai, tomēr īpaši skābiem analītiem rekomendē izmantot anjonapmaiņas sorbentus.

Attiecībā uz īpatnējo virsmas laukumu redzams, ka grafēna gadījumā lielāks virsmas laukums nodrošina ketoprofēna sorbciju, kas netika novērota mazāka virsmas laukuma gadījumā. Atšķirīgiem sorbentiem netika novērota īpatnējā virsmas laukuma ietekme uz sorbcijas efektivitāti.

Iegūtie rezultāti norāda, ka, lai sasniegtu labākos rezultātus plašam savienojumu klāstam, optimāli būtu izmantot dažādu sorbentu kombinācijas.

Salīdzinājumā ar iepriekš 1.3.1. apakšnodaļā aprakstītajiem rezultātiem par NSAID izplatību gliemenēs, kur no deviņiem pārstāvjiem tika konstatēts tikai ibuprofēns, eksperimentos ar POCIS izmantošanu konstatēts plašāks savienojumu klāsts – seši pārstāvji. Tas liecina, ka POCIS piemīt augstāka efektivitāte attiecībā uz NSAID piesārņojuma noteikšanu ūdenī, nekā gliemenēm. Šis novērojums varētu būt saistīts notiekošajiem metabolisma procesiem, kas dažādi ietekmē NSAID akumulēšanās spēju gliemeņu

organismā. Tomēr jāņem vērā, ka abas paraugu kopas tika ievāktas atšķirīgās paraugu ņemšanas vietās, tādēļ viennozīmīgus secinājumus izdarīt nav iespējams. Lai veiktu korektu abu indikatoru veidu salīdzinājumu, būtu nepieciešams veikt papildu eksperimentus, ievietojot gliemenes un POCIS vienā paraugu ņemšanas vietā un nodrošinot vienādus apstākļus.

3.3. HRMS metožu izstrāde farmaceitisko vielu noteikšanai

Iepriekš aprakstītie pētījumi ir ziņojuši satraucoši augstu farmaceitisko vielu līmeni ūdens vidē, norādot uz farmaceitisko piesārņojuma pieaugošo aktualitāti. Pētījumos ir demonstrēta arī dažādu organismu muskuļaudu pielietojuma daudzpusība, detektējot dažādu klašu piesārņotājus. Šo iemeslu dēļ turpmākie promocijas darba pētījumi tika veltīti progresīvu HRMS metožu izstrādei plaša spektra farmaceitisko līdzekļu noteikšanai muskuļaudos.

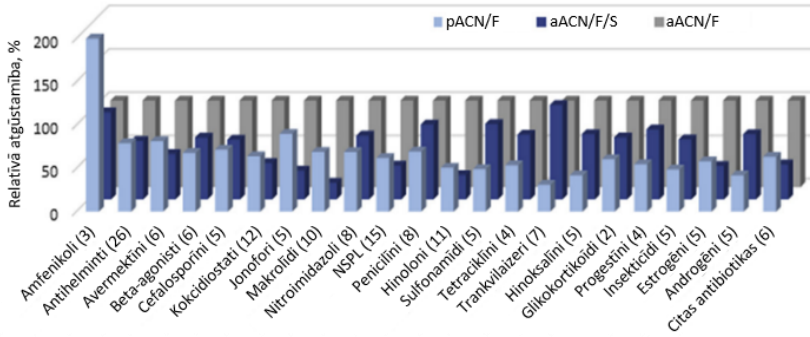
3.3.1. Orbitrap-HRMS metode farmaceitisko savienojumu noteikšanai[4]

Tika izstrādāta HRMS metode 164 farmaceitiski aktīvo savienojumu un to metabolītu skrīningam, izmantojot HPLC-Orbitrap-HRMS. Pētījumā iekļautie savienojumi pieder pie tādām terapeitiskām klasēm kā pretinfekcijas līdzekļi (antibiotikas un ķīmijterapijas līdzekļi), pretiekaisuma un pretparazītu līdzekļi, kortikoīdi un līdzekļi, kas iedarbojas uz nervu un reproduktīvo sistēmu, vielas ar hormonālu un tireostatisku iedarbību un beta agonisti.

Darbā tika salīdzinātas un optimizētas dažādas paraugu sagatavošanas procedūras atlasīto zāļu vielu noteikšanai vistas, cūku un liellopu muskuļaudos. Tika novērtēta šķīdinātāja ekstrakcija ar tīru un paskābinātu acetonitrilu. Pārbaudītas arī dažādas ekstrakta attīrīšanas metodes, tostarp izsaldēšana, izsālīšana, SPE kolonnas un dSPE. Labākie rezultāti tika iegūti ar tīru acetonitrila ekstrakciju, kas apvienota ar izsaldēšanas posmu un SPE, izmantojot fosfolipīdu attīrīšanas kolonnas vai Strata-X kolonnas. dSPE,

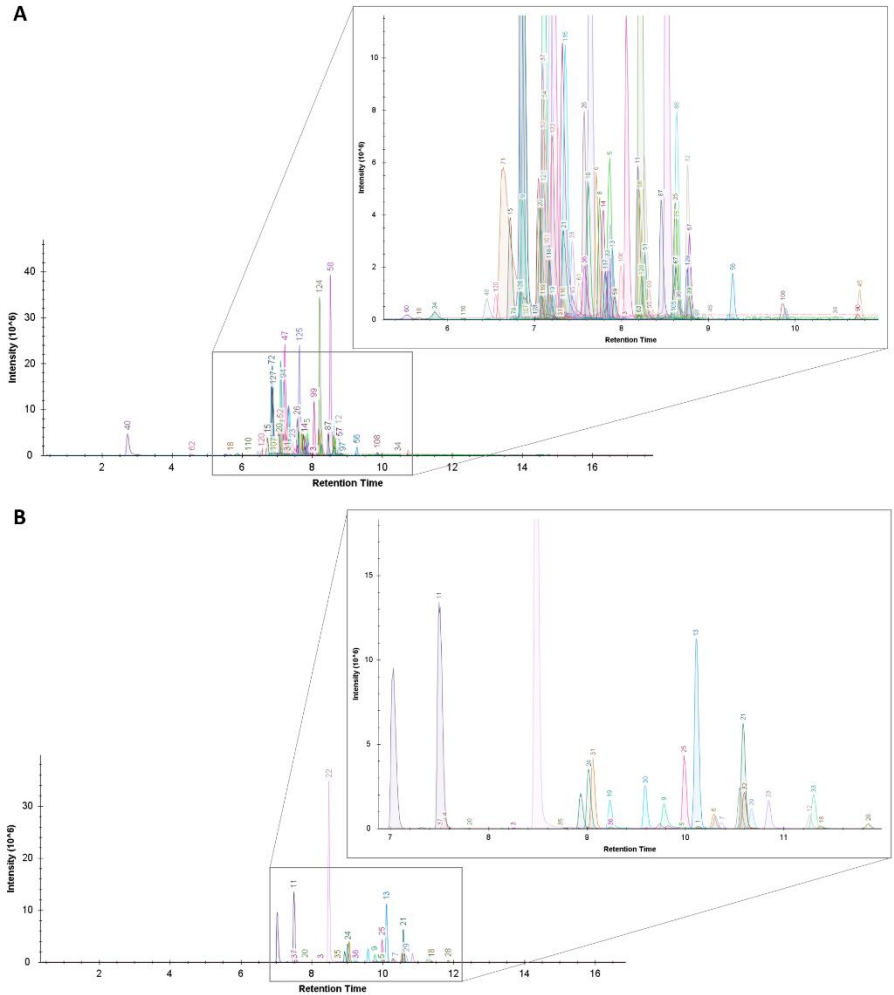
[4]Pugajeva, I.; **Ikkere, L.E.**; Judjallo, E.; Bartkevics, V. Determination of residues and metabolites of more than 140 pharmacologically active substances in meat by liquid chromatography coupled to high resolution Orbitrap mass spectrometry. *J Pharm Biomed Anal* **2019**, *166*, 252-263.

izmantojot primāro un sekundāro amīnu sāļus, nebija piemērota, jo tika zaudēti vairāki analīti. Relatīvo analītu atgūstamību kopsavilkums ir parādīts 3.9. attēlā.



3.9.att. **Relatīvās atgūstamības ar dažādām paraugu sagatavošanas metodēm (iekavās analītu skaits): pACN/F – ekstrakcija ar acetonitrilu un izsaldēšana; aACN/F/S – ekstrakcija ar paskābinātu acetonitrilu, izsālīšana un izsaldēšana; aACN/F – ekstrakcija ar paskābinātu acetonitrilu un izsaldēšana (pieņemts par 100 %)**

Hromatogrāfiskās metodes optimizācijas ietvaros salīdzinātas dažādas kustīgās fāzes un analītiskās kolonnas. Jonu avota parametri, piemēram, temperatūra un slāpekļa gāzes plūsma, tika optimizēti, lai uzlabotu jonizācijas procesa efektivitāti. Izvērtējot dažādas izšķirtspējas vērtības, tika izvēlēta 70 000 FWHM. Visu analītu izvēlēto jonu signālu hromatogrammas paradītas 3.10. attēlā.



3.10.att. **Visu pētījumā iekļauto analītu izvēlēto jonu hromatogrammas pozitīvajā (A) un negatīvajā (B) jonizācijas režīmā**

Optimizētā metode tika validēta, izmantojot tukšos paraugus ar standartpiedevām četros līmeņos (0,5, 1,0, 1,5 un 2 reizes maksimāli pieļaujamā līmenī (MRL)) vai pēc iespējas zemākā koncentrācijā vielām, kurām nav noteikts MRL. Izvērtētie veiktspējas parametri bija selektivitāte, matricas efekts, kvantitatīvas noteikšanas robeža un instrumentālā noteikšanas robeža un

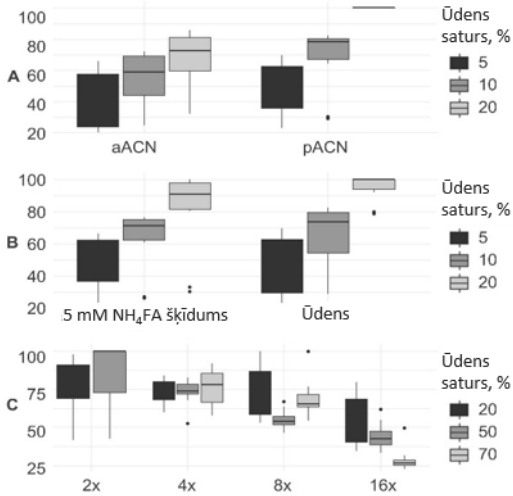
pareizība. Kopā 123 atlasītos savienojumus muskuļaudu paraugos varēja kvantitatīvi noteikt ar pareizību (atgūstamību) no 70 līdz 120% un RSD mazāku par 30%. Visbeidzot, izstrādātā metode tika veiksmīgi pielietota, lai konstatētu un kvantitatīvi noteiktu veterināro zāļu atliekas reālos paraugos, kas atzīti par aizdomīgiem, izmantojot neselektīvo kvalitatīvo testu inhibitoru noteikšanai. Iegūtie rezultāti tika apstiprināti, izmantojot attiecīgas apstiprināšanas metodes. Vairākos paraugos konstatētas koncentrācijas, kas pārsniedz MRL.

3.3.2. FT-ICR-HRMS metode hinolonu noteikšanai[5]

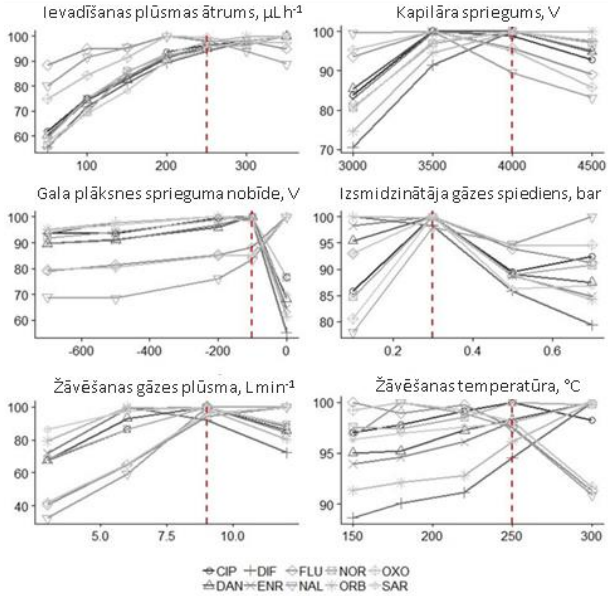
Otra HRMS metode farmaceitiski aktīvo vielu noteikšanai muskuļaudos tika izstrādāta, izmantojot FT-ICR-HRMS. Šim pētījumam kā analīti tika izvēlēti specifiskas antibiotiku grupas savienojumi – hinoloni, jo saistībā ar to izmantošanu ir daudz ziņojumu par augstu detektēšanas biežumu un saistību ar antibakteriālās rezistences attīstību [76]. Šajā pētījumā vienlaicīga desmit hinolonu grupas antibiotiku kvantitatīva apstiprinoša noteikšana tika panākta ar tiešas injekcijas analīzi mazāk nekā stundas laikā.

Paraugu sagatavošanas optimizācija ietvēra tīra un paskābināta acetonitrila ekstrakcijas salīdzinājumu, bufersāļu klātbūtnes ietekmi uz gala šķīdumu un atšķaidīšanu ar ūdeni. Novērtēto parametru salīdzinājums parādīts 3.11.attēlā. Tika optimizēti MS parametri, piemēram, plūsmas ātrums, skenēšanu skaits, kapilāra spriegums, gala plāksnes sprieguma nobīde, izsmidzinātāja spiediens, žāvēšanas gāzes plūsmas ātrums un žāvēšanas temperatūra. MS parametru optimizācijas kopsavilkums ir parādīts 3.12. attēlā.

[5] **Ikkere, L.E.**; Perkons, I.; Pugajeva, I.; Gruzauskas, R.; Bartkiene, E.; Bartkevics, V. Direct injection Fourier transform ion cyclotron resonance mass spectrometric method for high throughput quantification of quinolones in poultry. *J Pharm Biomed Anal*[5] **2020**, *188*.

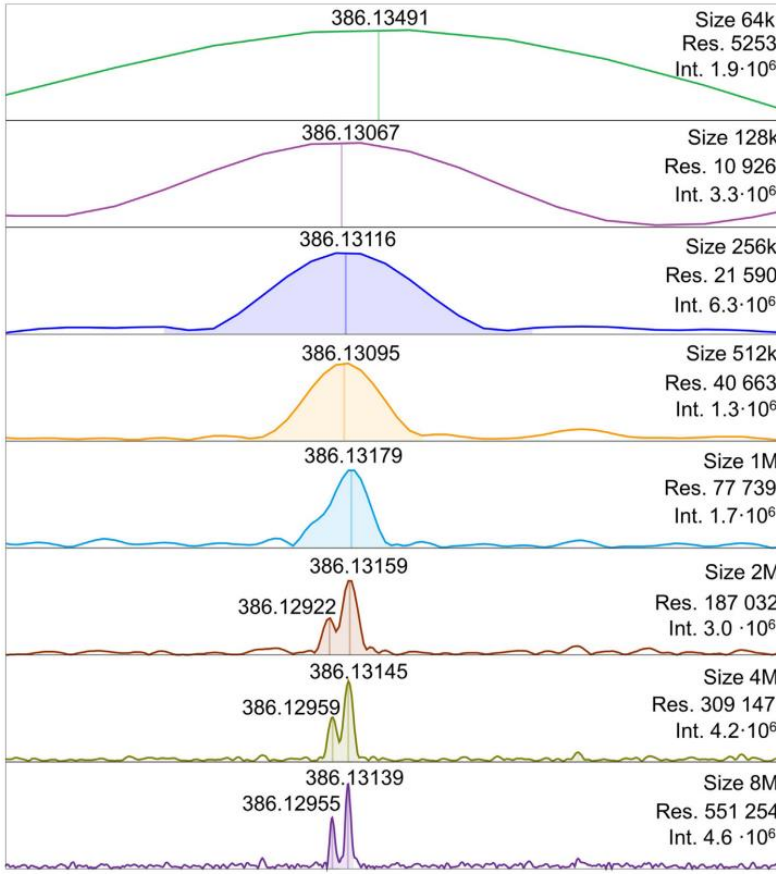


3.11.att. **Relatīvās signālu intensitātes, izmantojot dažādas paraugu sagatavošanas procedūras: A) ekstrakcijas šķīdinātāja un ūdens satura ietekme; B) amonija formiāta un ūdens satura ietekme; C) atšķaidīšanas faktora un ūdens satura ietekme**



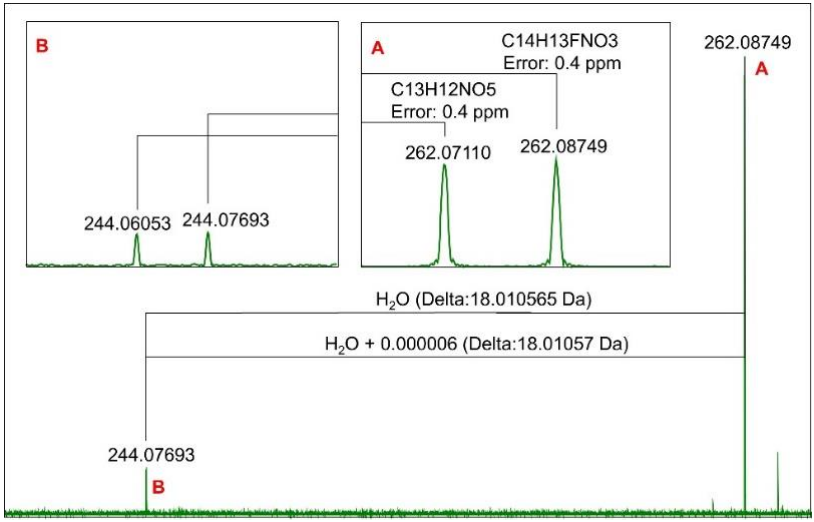
3.12.att. **Optimizēto jonu avota parametru apkopojums**

Īpaši augstās izšķirtspējas priekšrocība tika demonstrēta, atdalot traucējošo signālu no sarafloksacīna signāla (3.13. att.).



3.13.att. **Sarafloksacīna un tam traucējoša matricas signāla masas spektri, izmantojot dažādas izšķirtspējas**

Nepārprotamai hinolonu identificēšanai tika izmantota papildu MS/MS fragmentācijas analīze. Oksolīnskābes un flumekvīna molekulārie un fragmentu jonu attēloti 3.14. attēlā. Neskatoties uz ļoti tuvajām *m/z* vērtībām un hromatogrāfiskās atdalīšanas trūkumu, abi masspektrometriskie signāli ir skaidri atdalīti augstās izšķirtspējas dēļ.



3.14.att. **Oksolīnskābes (*m/z*=262.07110) un flumekvīna (*m/z*=262.08749) masas spektri: A – prekursora joni; B – fragmentu joni**

Izstrādātās metodes pielietojamība tika pārbaudīta, analizējot komerciāli pieejamus vistas gaļas paraugus, kā arī ar hinoloniem apstrādātu vistu paraugus. Neviens no 19 komerciālajiem paraugiem nesaturēja hinolonu antibiotikas. Savukārt apstrādāto vistu paraugi saturēja ciprofloksacīna un enrofloksacīna kopējo līmeni līdz 1064 µg kg^{-1}. Iegūtie rezultāti labi saskanēja ar tiem, kas iegūti ar apstiprinošu HPLC-QqQ-MS/MS metodi.

SECINĀJUMI

1. Pārbaudīti dažādi indikatororganismi un demonstrēta to pielietojamība par piesārņojuma bioindikatoriem:
   a) Aļņu, staltbriežu un mežacūku audu analīzē konstatēts, ka aļņu audos ir visaugstākie PBDE līmeņi, savukārt mežacūku – HBCD. Aknu audos detektētas augstākas koncentrācijas, nekā muskuļaudos. PBDE izomēru sastāvs liecina par "penta-BDE" kā iespējamo piesārņojuma avotu.
   b) Zušu audos no analizētajiem alternatīvajiem liesmas slāpētājiem konstatēti HBCD izomēri un DBDPE. HBCD piesārņojums zušos nepārsniedza vides kvalitātes standartu, kas norādīts direktīvā 2013/39/EU. Salīdzinot ar citviet ziņotiem piesārņojuma līmeņiem ūdens organismos, Latvijā konstatētais piesārņojuma līmenis ir zems.
   c) Visos gliemeņu paraugos tika konstatēta PBDE klātbūtne, taču piesārņojuma līmenis bija zemāks par citviet detektēto. Neskatoties uz to, 83 % paraugu tika pārsniegts direktīvā 2013/39/EU noteiktais vides kvalitātes standarta slieksnis. Perfluorēto savienojumu līmenis bija relatīvi zems. Konstatētais ibuprofēna piesārņojums gliemenēs bija būtiski augstāks, nekā ziņots citviet.
2. Salīdzinot gliemenes, perifitonu, sedimentus un ūdens paraugus kā ūdens piesārņojuma indikatorus, tika konstatēta 86 % analizēto farmaceitisko savienojumu klātbūtne. Augstākā koncentrācija detektēta ūdenī nesteroīdā pretiekaisuma līdzekļa diklofenaka un pretepilepsijas līdzekļa karbamazepīna gadījumā – ap 1 μg L^{-1}. Par piemērotāko paraugu matricas veidu īslaicīga ūdens piesārņojuma noteikšanai tika konstatēts ūdens.
3. Vislabākos rezultātus POCIS izmantošanā nesteroīdo pretiekaisuma līdzekļu noteikšanai ūdenī uzrādīja HLB sorbents. Tika detektēti pieci no piecpadsmit savienojumiem, tai skaitā oksifenbutazons, kas netika detektēts ar citiem sorbentiem. Tomēr meloksikāms tika detektēts tikai ar grafēna, MWCNT-COOH un MWCNT-aminopropilimidazola sorbentiem. Tas liecina, ka optimālai POCIS izmantošanai piesārņojuma novērtēšanai nepieciešams izmantot dažādu sorbentu kombinācijas.
4. Izstrādātas divas HRMS metodes farmaceitiski aktīvu savienojumu noteikšanai:
   a) Izstrādāta un validēta HPLC-Orbitrap-HRMS metode vienlaicīgai 164 farmaceitisko savienojumu identificēšani un kvantificēšanai muskuļaudos. Optimizētā plašam analītu klāstam piemērotā paraugu

sagatavošanas metode sniedz iespēju iegūt informāciju par plaša spektra farmaceitisko savienojumu klātbūtni paraugos.

b) Izstrādātā tiešās injekcijas FT-ICR-HRMS metode nodrošina hinolonu antibiotiku apstiprinošu noteikšanu muskuļaudos īsā laikā, kas ir viens no galvenajiem antibakteriālās rezistences cēloņiem.

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Es vēlos izteikt vislielāko atzinību manam zinātniskajam vadītājam profesoram Dr. chem. Vadimam Bartkevičam par viņa sniegtajiem vērtīgajiem un konstruktīvajiem padomiem šī darba plānošanas un izstrādes gaitā, kā arī viņa veltīto dārgo laiku. Esmu īpaši pateicīga par mana otrā zinātniskā vadītāja profesora Dr. chem. Artura Vīksnas sniegtajiem noderīgajiem padomiem promocijas darba izstrādes beigu posmā.

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UNIVERSITY OF LATVIA
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APPLICATION OF BIOLOGICAL INDICATOR ORGANISMS AND POLAR ORGANIC CHEMICAL INTEGRATIVE SAMPLERS FOR THE DETERMINATION OF CHEMICAL CONTAMINANTS USING HIGH-RESOLUTION MASS SPECTROMETRY TECHNIQUES

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ABSTRACT

Application of biological indicator organisms and polar organic chemical integrative samplers for the determination of chemical contaminants using high-resolution mass spectrometry techniques. Ikkere, L. E., supervisors Dr. Chem., Prof. Bartkevičs, V. and Dr. Chem., Prof. Vīksna, A. Summary of the collection of scientific articles in analytical chemistry, 53 pages, 16 figures, 2 tables, 76 literature references. In English.

The thesis presents an overview on the application of various biological indicator organisms, including terrestrial animals, eels and mussels for the assessment of both terrestrial and aquatic contamination. A wide range of contaminants has been investigated, including persistent organic pollutants, such as flame retardants and perfluorinated compounds, as well as contaminants of emerging concern – pharmaceutically active substances. A comparison of the applicability of biotic and abiotic sample matrixes regarding aquatic ecosystem contamination has been carried out. Nine types of sorbents have been investigated regarding the determination of non-steroidal anti-inflammatory drugs in water by utilising polar organic chemical integrative sampler approach. Finally, two advanced mass spectrometric methodologies have been developed for the analysis of pharmaceuticals. The method based on high performance liquid chromatography coupled to Orbitrap mass spectrometry allows simultaneous determination of 164 pharmaceutically active substances and their metabolites, whereas direct injection Fourier transformation ion cyclotron resonance method is characterised by rapid detection and confirmation of quinolone class antibiotics. Both methodologies ensure the further extension of environmental contamination research.

BIOINDICATORS, HIGH-RESOLUTION MASS SPECTROMETRY, ENVIRONMENTAL CONTAMINATION, BROMINATED FLAME RETARDANTS, PHARMACEUTICAL RESIDUES, NONSTEROIDAL ANTI-INFLAMMATORY DRUGS

ABBREVIATIONS

APPI	atmospheric pressure photoionization
BFR	brominated flame retardant
BTBPE	1,2-bis(2,4,6-tribromophenoxy)ethane
DBDPE	decabromodiphenyl ethane
dSPE	dispersive solid phase extraction
d.w.	dry weight
EH-TBB	2-ethyl-hexyl tetrabromobenzoate
EI	electron impact
EQS	environmental quality standard
FR	flame retardant
FT-ICR	Fourier transformation ion cyclotron resonance
GC	gas chromatography
GPC	gel permeation chromatography
HBB	hexabromobenzene
HBCD	hexabromocyclododecane
HESI	heated electrospray ionisation
HLB	hydrophilic-lipophilic balance
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
LC	liquid chromatography
l.w.	lipid weight
MRL	maximum residue level
MS/MS	tandem mass spectrometry
MWCNT	multi-walled carbon nanotubes
NSAID	nonsteroidal anti-inflammatory drug
PBDE	polybrominated diphenyl ethers
PFC	perfluorinated compound

PFOA	perfluorooctanoic acid
PFOS	perfluorooctane sulfonic acid
POCIS	polar organic chemical integrative sampler
POP	persistent organic pollutant
QqQ	triple quadrupole mass analyser
SE	solvent extraction
SIM	selected ion monitoring
SPE	solid phase extraction
SRM	selected reaction monitoring
TBBPA	tetrabromobisphenol A
TBP-DBPE	2,4,6-tribromophenyl 2,3-dibromopropyl ether
TOF	time of flight
Vpp	Peak-to-peak voltage
w.w.	wet weight
WWTP	wastewater treatment plant

INTRODUCTION

Various hazardous chemical compounds are released into the environment daily as a result of both human activities and industrial processes. Persistent organic pollutants (POPs), such as flame retardants (FRs), have been known for decades, but year after year, new emerging pollutants, such as pharmaceutically active compounds, are being discovered. These substances adversely affect the ecosystem, including living organisms and humans. The detection of these contaminants in abiotic environmental samples, such as water, is difficult due to the low concentrations, consequently various indicators are needed to detect very low levels of contamination. At present, it is important for the scientific community to use the bioindicator approach when assessing suitable living organisms [1].

The above-mentioned indicator strategy has a high potential for the analysis of a wide range of contaminants, especially in combination with sensitive and selective instrumental methods such as high-resolution mass spectrometry (HRMS). This approach not only allows to significantly lower the limit of detection for pollutants, but also opens up opportunities for retrospective analysis of the data obtained. In addition, due to the high selectivity, it is also possible to detect compounds in the presence of interfering matrix components. This, in turn, allows reduction of the total analysis time due to both faster sample preparation and shorter chromatographic program, as it is possible to identify and quantify compounds that are not completely chromatographically separated. However, the main advantage is the possibility to significantly increase the range of simultaneously detectable pollutants.

The practical relevance of the problem.

POPs such as hexa-, hepta- and deca-bromodiphenyl ethers, as well as perfluorooctane sulfonic acid (PFOS) and perfluoroctanoic acid (PFOA) are already listed under the Stockholm Convention as new POPs. This indicates their persistence in the environment for exceptionally long periods of time, availability to become widely distributed throughout the environment as a result of natural processes involving soil, water and, most notably, air, accumulation in the fatty tissue of living organisms including humans, being found at higher levels in the food chain, and being toxic to both humans and wildlife [2].

However, due to modernization and increase in welfare new emerging pollutants are becoming a source of concern. Consumption of human medicines has been estimated between 50 to 150 g per person per year in the EU. Veterinary drugs are used in smaller quantities, nevertheless it is a growing segment of the pharmaceutical products market. In the majority of EU Member States, about 50% of unused human medicinal products (3 to 8% of the total amounts sold) are not collected [3]. Consequently, the pollution of the various compartments of the environment (water, soil and air) with pharmaceutical residues has become an

environmental concern. Several hazards and risks have been associated with pharmaceutical pollution, including antimicrobial resistance, altered hormone levels in aquatic organisms, and even decline of a population [3].

The aforementioned negative effects highlight the necessity for monitoring of these substances both in the environment and food. The environmental analysis of trace contaminants presents difficulties due to low levels and fluctuations over short periods of time. These problems could be overcome by employing the bioindicator approach. Regarding food analysis, continuous efforts are necessary in order to develop faster, more reliable methods with a wide range of analytes included in a single run.

The aim of the work is related to the development and application of mass spectrometric methods for the determination of pollutants (various POPs and pharmaceuticals) in environmental and food matrices using HRMS equipment and biological indicator-organisms, as well as polar organic chemical integrative samplers (POCIS). The developed methods are to be able to simultaneously confirm and quantify a wide range of contaminants. The elaborated methods for the determination of contaminants are foreseen for the validation and application in order to assess their occurrence in the Baltic region.

The approach used to achieve the aim of the work:

i. Assess the applicability of several terrestrial animals as bioindicators of environmental contamination regarding polybrominated diphenyl ethers (PBDEs), hexabromocyclododecane (HBCD) and tetrabromobisphenol A (TBBPA);
ii. Examine the applicability of eels as indicator-organisms for estimation of aquatic contamination regarding emerging brominated flame retardants (BFRs);
iii. Investigate the applicability of mussels as bioindicators for the contamination status of PBDEs, perfluorinated compounds (PFCs) and nonsteroidal anti-inflammatory drugs (NSAIDs) in the aquatic ecosystem;
iv. Compare various matrices, including mussels, periphyton, sediments, and water as aquatic contamination indicators;
v. Evaluate different POCIS sorbents for pharmaceutical contamination determination;
vi. Develop advanced HRMS methodologies employing high performance liquid chromatography coupled to Orbitrap HRMS (HPLC-Orbitrap-HRMS) and Fourier transformation ion cyclotron resonance HRMS (FT-ICR-HRMS) in order to extend the analytical scope for further in-depth studies of pharmaceutical contamination.

Scientific novelty.

i. The versatility and applicability of different HRMS analysers, including magnetic sector, Orbitrap-HRMS, and FT-ICR-MS, as well as various ionization techniques, such as electron impact (EI), heated electrospray (HESI) and atmospheric pressure photoionization (APPI) was demonstrated by analysing a wide range of both POPs and emerging contaminants in various matrices;
ii. New information was gathered about the contamination status of Latvian terrestrial and aquatic biota was carried out by analysing wild game, eels, mussels, periphyton, sediments, water and commercially available meat samples;
iii. An effective analytical method for simultaneous identification, screening and quantification of 164 residues and metabolites of pharmacologically active substances in muscle tissue was developed;
iv. The developed fast and reliable quantitative confirmatory direct injection FT-ICR-HRMS method is one of the first reports of employing this advanced technology in the food safety sector.

Practical application of the work.

Widespread knowledge of contamination status in the Baltic region has been obtained. The optimised and developed analytical methods provided a range of well characterised, practical and reliable options for performing measurement of pharmaceuticals in various matrices. The methods are highly adaptable and could be applied for extended monitoring of the occurrence or scientific studies on these chemicals.

Scientific publications.

1. **Ikkere, L.E.**; Perkons, I.; Pugajeva, I.; Gruzauskas, R.; Bartkiene, E.; Bartkevics, V. Direct injection Fourier transform ion cyclotron resonance mass spectrometric method for high throughput quantification of quinolones in poultry. *J Pharm Biomed Anal*[1] **2020**, *188*.
2. Pugajeva, I.; **Ikkere, L.E.**; Judjallo, E.; Bartkevics, V. Determination of residues and metabolites of more than 140 pharmacologically active substances in meat by liquid chromatography coupled to high resolution Orbitrap mass spectrometry. *J Pharm Biomed Anal*[1] **2019**, *166*, 252-263.
3. **Ikkere, L.E.**; Perkons, I.; Sire, J.; Pugajeva, I.; Bartkevics, V. Occurrence of polybrominated diphenyl ethers, perfluorinated compounds, and nonsteroidal anti-inflammatory drugs in freshwater mussels from Latvia. *Chemosphere*[2] **2018**, *213*, 507-516.
4. Zacs, D.; **Ikkere, L.E.**; Bartkevics, V. Emerging brominated flame retardants and dechlorane-related compounds in European eels (*Anguilla anguilla*) from Latvian lakes. *Chemosphere*[1] **2018**, *197*, 680-690.
5. Zacs, D.; Rjabova J.; **Ikkere, L.E.**; Bavrins, K.; Bartkevics, V. Brominated flame retardants and toxic elements in the meat and liver of red deer (*Cervus elaphus*), wild boar (*Sus scrofa*), and moose (*Alces alces*) from Latvian wildlife. *Sci Total Environ*[3] **2018**, *621*, 308-316.

[1] *Peer reviewed journal, imprint of Elsevier (IF=3.209 (2020)), ISSN: 0731-7085*
[2] *Peer reviewed journal, imprint of Elsevier (IF=7.086 (2020)), ISSN: 0045-6535*
[3] *Peer reviewed journal, imprint of Elsevier (IF=7.963 (2020)), ISSN: 0048-9697*

List of conferences.

1. The 78th International Scientific Conference of the University of Latvia, Riga, Latvia, 2020. **Ikkere, L.E.**; Perkons, I.; Pugajeva, I.; Gruzauskas, R.; Bartkiene, E.; Bartkevics, V. Direct-injection Fourier-transformation ion cyclotron resonance mass spectrometric method for ultra-fast quantification of quinolones in poultry (in book of abstracts).
2. International scientific symposium "Science to strengthen sustainable and safe food systems", Riga, Latvia, 2020. **Ikkere, L.E.**; Perkons, I.; Pugajeva, I.; Gruzauskas, R.; Bartkiene, E.; Bartkevics, V. Direct-injection Fourier-transformation ion cyclotron resonance mass spectrometric method for ultra-fast quantification of quinolones in poultry (poster/in book of abstracts)
3. The 77th International Scientific Conference of the University of Latvia, Riga, Latvia, 2019. **Ikkere, L.E.**; Pugajeva, I.; Judjallo, E.; Bartkevics, V. Determination of residues and metabolites of more than 140 pharmacologically active substances in meat by liquid chromatography coupled to high resolution orbitrap mass spectrometry (oral presentation/ in book of abstracts).
4. IV Pasaules latviešu zinātnieku kongress, Rīga, Latvija, 2018. **Ikkere L.E.**, Pērkons I., Bartkevičs, V. Bioindikatoru izmantošana Latvijas apkārtējās vides ķīmiskā piesārņojuma novērtēšanai. (poster/in book of abstracts)
5. The 76th International Scientific Conference of the University of Latvia, Riga, Latvia, 2018. **Ikkere, L.E.**; Perkons, I.; Bartkevics, V. Persistent organic pollutants in freshwater mussels from the Latvian environment (oral presentation/ in book of abstracts).

1. LITERATURE REVIEW

1.1. Biological indicator organisms

In environmental analysis, especially aquatic systems, one of the main difficulties is that changes in hydrology are rapid and difficult to estimate. Long-term sustainability of river ecosystems is of instantaneous nature. Physical, chemical and bacteriological measurements cannot reflect the integration of numerous environmental factors and long-term sustainability of ecosystems for their instantaneous nature. Biomonitoring has been proven to be necessary supplementary to traditional monitoring techniques.

Biomonitoring is defined as "the systematic use of living organisms or their responses to determine the condition or changes of the environment" [4]. Various organisms can be used in order to carry out biomonitoring. Such organisms are called biological indicator organisms. A bioindicator is "an organism (or part of an organism or a community of organisms) that contains information on the quality of the environment (or a part of the environment)". An "ideal" indicator should have the characteristics as follows: (a) taxonomic soundness (easy to be recognized by non-specialist); (b) wide distribution; (c) low mobility (local indication); (d) well-known ecological characteristics; (e) numerical abundance; (f) suitability for laboratory experiments; (g) high sensitivity to the environmental stressor; (h) high ability for quantification and standardisation [5].

Bioindicator organisms need to indicate both the long-term interaction of several environmental conditions and sudden changes of the important factors. There are several alternatives for indicators of biomonitoring in the aquatic environment, however benthic macroinvertebrates, periphyton and various fish species are the most frequently utilised. These organisms have shown efficacy when used separately and combined [5].

Periphyton is a mixture of autotrophic and heterotrophic microorganisms embedded in a matrix of organic detritus. Periphyton covers most submerged substrates, ranging from sand to plants to rocks [6]. Periphyton is a valuable indicator of environmental status in streams and rivers. Periphyton organisms are primary producers, hence they provide the important foundation of food webs in water ecosystems. Periphyton reflects short-term impacts and sudden changes in the ecosystem, since the reproduction rates are rapid and life cycles are short. Periphyton has been preferred for aquatic system biomonitoring purposes by many authors [5].

Numerous countries have a long history of using macroinvertebrates, such as snails, worms, freshwater clams, mussels, and crayfish to monitor the ecological status of water ecosystems. Several characteristics of benthic macroinvertebrates, especially mussels make them suitable as an indicator organism for the occurrence of chemical contamination in the environment [7].

They are sessile, thus providing location-specific information. Furthermore, mussels are filter-feeders that mainly consume phytoplankton by pumping and filtering large volumes of water. This water filtration behaviour also enables them to effectively accumulate chemical pollutants from water, thereby providing an integrative measure of the concentration and bioavailability of water pollutants. As lower members of the aquatic food chain, they transfer anthropogenic pollutants from the abiotic phase and the primary production level to the higher trophic levels in the food chain, such as mussel-eating invertebrates, birds, and mammals [8]. Due to these qualities, bivalve organisms have been used as bioindicators for nearly 50 years [9]. The range of contaminants analysed in mussels ranges from "priority pollutants" like heavy metals and halogenated hydrocarbons to chemicals of emerging concern like FRs, surfactants, pharmaceuticals, and even drugs of abuse [8, 10, 11].

Fish communities have been applied to monitor river ecosystem health for a long time as highly visible and valuable components of the water ecosystems. Fish are the top of the aquatic food web and are consumed by humans, making them important for assessing contamination. They can be good indicators of long-term (several years) effects and broad habitat conditions due to their relatively long life cycle and mobility. Fish communities respond significantly and predictably to almost all kinds of anthropogenic disturbances, including chemical pollution [5]. The European eel (*Anguilla anguilla*) is a carnivorous, catadromous fish, which is widely distributed throughout Europe. Eel absorbs and concentrates the bioaccumulative organic pollutants that are present in low concentrations in its diet consisting of crustaceans, worms, snails, larvae, and small fish. For these reasons, eels have long been considered as bioindicator species that can point to the contaminants present in local habitats [12, 13].

Biomonitoring can be carried out not only in aquatic ecosystems, but also in terrestrial habitats [14]. For this purpose, a myriad of organisms has been used, including plants, invertebrates, reptiles, birds and mammals [15]. Terrestrial mammals are known as good indicator species for POPs and heavy metals [16]. Red deer (*Cervus elaphus*) and moose (*Alces alces*), being the two most dominant species (population of ~52,000 and ~21,000 animals in 2014, respectively) after roe deer (*Capreolus capreolus*, population of ~130,000 in 2014), are the largest herbivores among Latvian wildlife [17]. Red deer and moose have the longest life expectancy and thus are the most susceptible to bioaccumulation of POPs and heavy metals, and can be considered to be the most sensitive herbivore bioindicators of environmental contamination status. While the diet of red deer and moose consists generally of plants and represents the air/soil – plant – herbivore system, wild boar (population of ~ 55,000 in Latvia in 2014 [17]) is known to be an omnivore consuming a wide variety of plants and insects, as well as carrion, fish, and molluscs that provide an additional uptake of contaminants.

1.2. Polar organic chemical integrative samplers

POCIS is a device for sampling water-soluble organic chemicals from aqueous environments. The POCIS is an integrative sampler that provides time-weighted average levels of chemicals, including contaminants. Usually, deployment periods are ranging from few weeks to several months. One of the main advantages of the use of the POCIS, is that it has no mechanical or moving parts, consequently it requires no power nor supervision during use. Furthermore, the sorbents integrated in the POCIS adsorb chemicals from the dissolved phase of the aquatic environment, mimicking the respiratory exposure of aquatic organisms.

Fig.1.1. **Four POCIS mounted on a stainless-steel deployment canister**

The POCIS device consists of a sorbent contained between two microporous membranes (see Fig.1.1). The membranes allow water and dissolved chemicals to pass through the sorbent where the chemicals are adsorbed. Larger particles such as sediment and particulate matter are excluded. The membrane is resistant to biofouling that can significantly reduce the amount of chemicals adsorbed. The type of sorbent utilized can be changed to specifically target certain chemicals of interest. Different kinds of sorbent can be used on a single deployment canister in order to sample a wider range of chemicals [18]. POCIS technology is applicable to various classes of contaminants such as pharmaceuticals, hormones, various pesticides and household and industrial products, including alkyl phenols, caffeine and FRs [19].

1.3. Compound classes included in the scope of the study

BFRs comprise a diverse group of anthropogenic chemicals that are used to prevent fire incidents. BFRs lower the flammability of a wide array of products, such as textiles, plastics, building materials, and electronic equipment. Despite the clear benefits provided by the use of BFRs, the ubiquitous presence of these chemicals has resulted in their diffusion into the environment during manufacturing, use, and disposal. Due to their persistency and lipophilicity, BFRs enter the terrestrial and aquatic food chains [20].

Some of the most important contaminants in this family are PBDEs with the estimated worldwide production up to 67,000 tons per year in the early 2000s, and extensive use over several decades [21]. However, considering that these compounds are POPs and taking into account their potential for long-range transport, certain restrictions were imposed on their commercial availability [22]. Therefore, in order to meet the market demand for FRs, several alternative BFRs were developed, among which the most consumed were HBCD and TBBPA. Similar to PBDEs, these alternative BFRs also showed POP-like properties and their use followed by disposal led to environmental contamination [20, 23]. While HBCD was classified by the European Commission as a bioaccumulative and toxic compound because of its high persistence, low water solubility, and high $\log K_{ow}$ value [24], there is no consensus on the risks due to TBBPA. Older references report that TBBPA seems to have a comparatively low toxicity compared to the other BFR groups [25]. Nevertheless, the most recent studies reflect toxicological concerns regarding the environmental presence of TBBPA [26], therefore attracting scientific interest to the occurrence of this chemical in the environment. Besides HBCD and TBBPA there are a number of other chemicals recognised as emerging BFRs, including 1,2-bis(2,4,6-tribromophenoxy) ethane (BTBPE), hexabromobenzene (HBB) and decabromodiphenyl ethane (DBDPE). Although very scarce data is available about the occurrence of the majority of these compounds, and in order to implement effective strategies for the minimisation of possible hazardous effects of these compounds, such information was requested by the relevant authorities [27].

PFCs are a large group of compounds that are being produced for over five decades. PFCs are fully fluorinated synthetic compounds with unique properties, that are classified as POPs. Carbon-fluorine bond is one of the strongest bonds in organic chemistry. This property makes PFCs resistant to typical environmental degradation processes and hence persistent in the environment. The most common PFCs are the perfluorinated carboxylic acids and perfluorosulfonic acids of which PFOA and PFOS are the most well-known. PFOS, its salts and sulfonyl fluoride are on the Stockholm Convention on Persistent Organic Pollutants list. The fluorocarbon end of the molecule is hydrophobic, lipophilic and non-polar, whereas functional group on the other end of the molecule ensures polarity. Both PFOS and PFOA are classified as

surfactants that drastically reduce surface tension. PFCs are used in numerous applications, including stain repellents for textiles, additives to paper products, and aqueous film forming foams used to fight electrical fires [28]. The toxic effects of PFCs observed in humans include carcinogenicity and immunotoxicity. As shown in recent in vitro experiments, PFOA is more toxic than PFOS [29].

Pharmaceuticals are essential in both human and animal medicine. Veterinary drugs are used in modern animal husbandry and food production and are applied to maintain animal health, prevent infection, and treat diseases. However, illegal use of prohibited veterinary drugs or improper use, such as ignoring the required withdrawal periods may result in the presence of drug residues in animal tissues and affect food safety. Veterinary drug residues may include the parent compounds themselves, as well as metabolites and/or conjugates, and may have direct toxic effects on consumers, such as allergic reactions in hypersensitive individuals, hormonal effects by interfering with the balance of human hormones, or the development of antibiotic-resistant bacteria as a result of misusing antibiotics [30].

Due to the widespread use of drugs, pharmaceutically active substances have become one of the most important emerging classes of environmental pollutants. Recent studies have discovered their occurrence in environmental samples investigated worldwide, including different types of aqueous matrices. The ubiquitous use of pharmaceuticals has resulted in a relatively continuous discharge of the pharmaceuticals and their metabolites into wastewater. In addition, pharmaceuticals may be released into water sources in the effluents from poorly controlled manufacturing or production facilities, primarily those associated with generic medicines. A number of studies found trace concentrations of pharmaceuticals in wastewater, various water sources and drinking water samples. Pharmaceutical pollution does not appear to be harming humans directly, but disturbing indications from aquatic life suggest the necessity for preventive actions [31]. The fostering of antimicrobial resistance genes in the environment endangers human health through consumption of drinking water, fish or crops containing active pharmaceutical ingredients, by jeopardizing the availability of life-saving antibiotics in the future [32].

1.4. Occurrence in the environment

The occurrence of PBDEs in various organisms is widely reported. However, only a handful of reports on the occurrence of BFRs in wild game are available. Few Scandinavian studies reported total levels of PBDEs in moose and deer muscle samples ranging from 10 to 500 pg g^{-1} (w.w.). Corresponding liver samples had significantly higher contamination levels reaching 1700 pg g^{-1} (w.w.) [33-35].

The occurrence of HBCD in eels has been reported by numerous authors, whereas data about other emerging BFRS is scarce. Levels of HBCD in eel tissue varied greatly from 0.1 up to 12 100 ng g^{-1} (l.w.) [36, 37]. Levels of HBB, 2-

ethyl-hexyl tetrabromobenzoate (EH-TBB) and 2,4,6-tribromophenyl 2,3-dibromopropyl ether (TBP-DBPE) have been reported in eels, however the concentrations were below 1 ng g^{-1} (l.w.) [38, 39].

PBDEs have been frequently detected in mussels. The levels varied from 11.3 to 12 400 pg g^{-1} (w.w.) with the highest value detected in mussels from the Netherlands [40]. PFOS and PFOA have been detected in mussel samples as well. The highest average concentration of PFOS of 72 000 pg g^{-1} (w.w.) was detected in mussels from north-central Portuguese estuaries, where paper, textile, and leather factories discharge their effluents [10]. Data on NSAID occurrence in mussels is scarce, since they are not considered as traditional contaminants. Presence of diclofenac and ibuprofen was reported in mussels. Reported values were ranging up to 16.11 and 93.7 ng g^{-1} (d.w.) respectively [41, 42].

Aquatic system contamination has become a serious problem due to urbanisation, industrialisation, and agricultural activities. Few authors have compared pharmaceutical contamination status in water and such sample types as periphyton, invertebrates and fish. It has been observed that NSAIDs tend to concentrate in surface water and aquatic organisms, whereas antibiotics are prone to accumulate in sediments [43, 44]. It has also been observed that compounds with basic characteristics (pK_a>7) tend to bind to suspended solids [45]. However, the trends regarding concentration in water or sediments are ambiguous. For example, some authors reported higher acetaminophen concentrations in surface water compared to sediments, others just the opposite [43, 46]. Concentrations in sediments and periphyton samples are generally lower compared to those in surface water samples [44, 47].

1.5. Analytical approaches

Contaminant groups selected for the study have diverse chemical properties hence various analytical approaches have to be applied. An important tool in modern analysis is HRMS. HRMS allows detection of analytes to the nearest 0.001 atomic mass units. The HRMS was first applied by John Beynon in the 1950s and it was equipped with magnetic sector analyser [48]. Since then, HRMS is no longer limited to gas chromatography (GC), because such instruments as time-of-flight (TOF), Orbitrap and FT-ICR are available. Generally, these instruments measure the exact mass of analytes without fragmentation, however, they can be combined with a quadrupole in which case fragmentation is also possible and even higher selectivity is achieved [49]. The major advantage of this method is that it is very selective since it measures the exact mass of a compound allowing even minor changes in structure to be distinguished. Consequently, analytes of very close *m/z* values can be determined and interferences are avoided.

One of the greatest challenges in the analysis of BFRs is sample preparation. In order to achieve the necessary detection limits of sub-ppt (picogram per gram) for soils/sediments/biota high concentration factors are

typically needed. This in turn requires the clean-up procedures that are capable of removing potentially large quantities of organic material selectively, while retaining as much of the desired analytes as possible. The clean-up procedure of extracts usually consists of various steps such as solid-liquid adsorption chromatography in open columns utilising the combination of different sorbents, such as silica, Florisil, alumina, and various types of activated carbon. Gel permeation chromatography (GPC) is also frequently used for the removal of the bulk of high molecular weight compounds (e.g., lipids) from the sample extracts.

For BFRs such as PBDEs or HBCD, the most common detection techniques are EI-HRMS and electron capture negative ionization low resolution mass spectrometry. The presence of polychlorinated biphenyls in the samples could cause selectivity problems by interfering with the GC-MS analysis of PBDEs. These problems could be avoided by the use of HRMS. Another issue in the analysis of BFRs using GC is thermal lability, especially for highly brominated compounds (deca-BDE, HBCD and octabromotrimethylphenyllindane). The application of liquid chromatography (LC) eliminates the problem of thermal lability or interconversion during the analysis. Numerous studies have reported the utilisation of LC-MS based methods in diastereomer-specific analysis of HBCDs in various types of matrices. The analyser of choice generally is Orbitrap-HRMS. Isotopically labelled standards allow applying internal standardisation – the most powerful approach for a reliable and accurate quantification [50].

Extraction of PFOS and PFOA from biological matrixes is usually performed by ultrasound assisted extraction or accelerated solvent extraction. Solvents of choice are methanol, acetonitrile or methyl-*t*-butyl ether. Sodium or potassium hydroxides are sometimes used to ensure basic extraction conditions. Clean-up of extracts is performed by dispersed active carbon in presence of acetic acid or solid phase extraction (C18 or *Oasis HLB*). Quantitative determination is achieved by means of liquid chromatography tandem mass spectrometry (LC-MS/MS) [51].

Pharmaceutically active substances comprise compounds of a wide range of chemical properties, therefore analytical methods for multi-analyte determination are of great interest. Development of the suitable sample preparation procedure is a considerable challenge since the different functional groups, often encountered amphoteric properties of analytes, and the wide range of polarity pose difficulties for extraction, clean-up, and analytical separation. Therefore, sample preparation is one of the most critical steps. The most frequently used sample pre-treatment methods for meat samples include solvent extraction (SE) with or without defatting step, solid phase extraction (SPE), and dispersive solid phase extraction (dSPE) method (QuEChERS approach) for further purification and/or concentration [52].

LC-MS provides a universal approach applicable to the broadest range of pharmaceutical compounds. The most common technique for pharmaceutical

analysis has been LC-MS/MS. The current trend is focused towards the use of powerful HRMS detectors like TOF and Orbitrap. This development is due to the availability of more rugged, sensitive, and selective instrumentation. In the last years, the Orbitrap system has become more recognized because of its better dynamic concentration range, higher resolving power, consequently better mass accuracy compared to the TOF system [52].

2. EXPERIMENTAL PART

The research of the thesis was divided in several parts, according to the scheme depicted in Fig.2.1.

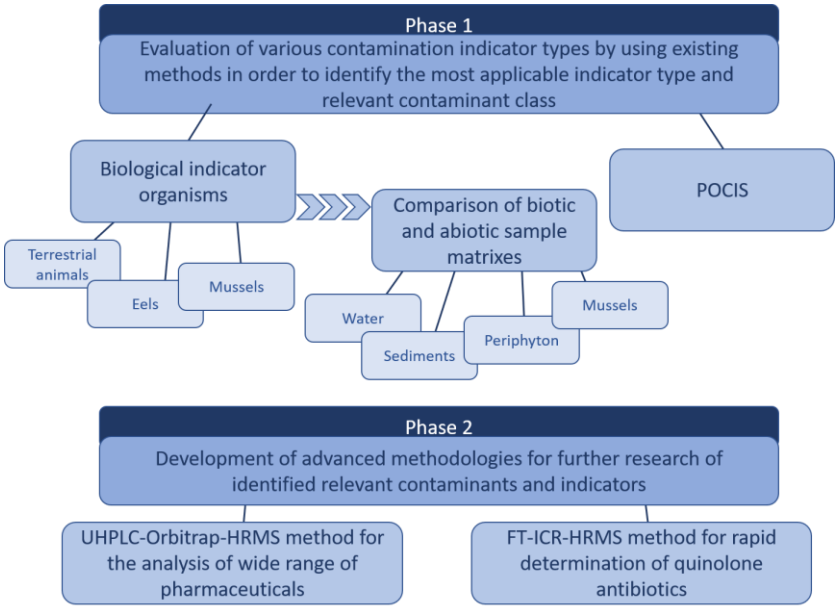

Fig.2.1. **Schematic representation of the experiment design of the thesis**

2.1. Samples

Tissue of 24 wild animals including moose, red deer, and wild boar was used for characterisation of BFRs in terrestrial ecosystems.

A total of 58 eels of various lengths and ages representing 5 sampling locations in Latvia were analysed as emerging BFR contamination indicators of the aquatic ecosystem.

Batches of mussel specimens representing 24 sampling sites were used as indicator-organisms for further evaluation of aquatic contamination, extending the scope to include PFCs and pharmaceuticals.

Sample sets consisting of mussels, periphyton, sediments and water from 15 sampling sites were used for the sample type comparison study. Sample sets were collected from three rivers – Gauja, Mūsa and Pēterupe. Samples from Gauja River were taken at sites located after four cities – Valmiera, Cēsis, Sigulda, and Ādaži, as well as at the estuary. Sampling sites in Mūsa River were near Pasvale (Lithuania) before and after wastewater treatment plant (WWTP),

on the Latvia-Lithuania border, and at the river mouth (Lielupe). Samples from Pēterupe River were collected at four sampling sites in the populated territory of Saulkrasti, as well as at the estuary.

For investigation of sorbent efficiency, POCIS samplers packed with 9 different sorbents were used. 200 mg of the sorbent was manually packed between two PES membranes (0.1 μm, Ø 90 mm). Sorbents used in the study as well as their manufacturers and specific surface areas are summarised in Table 2.1. The samplers were placed in a tank at the "Tome" fishery, that was operated in flow-through mode (water from Daugava River) and held for one week.

Table 2.1.

Sorbents used in POCIS samplers

No.	Sorbent	Manufacturer	Specific surface area, $m^2 g^{-1}$
1	HLB	*AFFINISEP*	190-210
2	Graphene nanoplatelets	*XG Sciences*	750
3	Graphene nanoplatelets	*Alpha Aesar*	500
4	MWCNT	*Chengdu Organic Chemicals Company*	200
5	MWCNTs-COOH	*Chengdu Organic Chemicals Company*	120
6	Carbosieve®	*SUPELCO*	>1000
7	MWCNT-amino propyl imidazole	*Bayer MaterialScience AG*, modified with amino propyl imidazole functional group at RTU Faculty of Materials Science and Applied Chemistry Institute of Polymer Materials	120
8	Carbon nitride	Synthesised at RTU Faculty of Materials Science and Applied Chemistry Institute of Polymer Materials	210
9	Graphene-carbon nitride	*XG Sciences* graphene nanoplatelets and synthesised carbon nitride (1:1)	-

2.2. Analysis of brominated flame retardants

2.2.1. Sample preparation

The method for the determination of selected BFRs was adopted from previously published studies [53-55]. Briefly, aliquots of homogenised and freeze dried or wet tissue samples were spiked with an internal standard solution. The samples were extracted with dichloromethane/*n*-hexane (1:1, v/v) using automatic Soxtec™ 2055 Fat Extraction System or ultrasound assisted extraction. The solvents were evaporated to dryness under a gentle stream of nitrogen, the lipid content was determined gravimetrically and the fatty residue was redissolved in *n*-hexane. The extract was purified using acid-modified silica gel or Florisil columns. After solvent evaporation the extract was treated with concentrated sulfuric acid. The acidic bottom layer was discarded and the organic layer was evaporated with the addition of recovery standard solution prior to the

GC-HRMS analysis of PBDEs. After the analysis of PBDEs, the solvent was exchanged to methanol and the extracts were analysed for the content of HBCDs, TBBPA, and EBFRs using an HPLC-Orbitrap-HRMS instrument.

2.2.2. Instrumental analysis

The analysis of PBDEs was performed using GC-HRMS by applying EI ionization in positive ion mode and selective ion monitoring (SIM) detection mode. Quantification was carried out using isotope dilution method by applying $^{13}C_{12}$-labeled surrogates as internal standards. For the analysis of HBCDs, TBBPA, and EBFRs an HPLC-Orbitrap-HRMS method was used. The compounds of interest were separated using a C_{18} reversed-phase analytical column. For analysis of HBCDs and TBBPA HESI in negative ion mode was used, whereas emerging BFRs were determined using negative APPI mode by applying toluene as dopant. The detection of analytes was performed in targeted SIM mode using the two most abundant ions of the respective molecular ion clusters. The quantification was carried out based on isotope dilution with $^{13}C_{12}$-labeled surrogates and internal standardisation.

2.3. Analysis of perfluorinated compounds

The sample preparation procedure included ultrasound assisted extraction with methanol and 0.2 M sodium hydroxide water solution, followed by concentration and purification of the analytes using solid phase extraction on a weak anion exchange cartridge. After evaporation of the eluate, the dry residue was reconstituted in methanol. Instrumental analysis was based on HPLC-Orbitrap-HRMS using $HESI^-$ operated in selected reaction monitoring (SRM) mode.

2.4. Analysis of pharmaceutically active substances

Various analytical methods were used depending on sample type and analytes of choice.

2.4.1. Sample preparation

For the analysis of NSAIDs, 2 g of homogenised bivalve samples were extracted with acetonitrile and aqueous 0.02 M ascorbic acid solution. Concentration and purification of the analytes was achieved by solid phase extraction with Strata C18 cartridges. Sorbents from POCIS were extracted with methanol/dichloromethane (1:1 v/v) using automated Soxtec™ extraction. Extracts were reconstituted in acetonitrile/methanol (9:1, v/v) prior instrumental analysis.

For the analysis of 24 pharmaceutically active compounds in various matrices, sediments and periphyton samples were lyophilised prior sample

preparation procedure. Mussel, sediment and periphyton samples were extracted using ultrasound assisted extraction. Extraction was performed in two cycles, 15 min each, using 5 mL of 80 % methanol in water with 0.1 % acetic acid. Obtained extract portions were combined and diluted with water. SPE using *Strata-X* columns were performed. Water samples were filtered and the same SPE procedure was performed. Finally, all extracts were reconstituted in water/methanol solution (80:20, v/v).

Meat samples were extracted by mechanical shaking with 0.1 % formic acid in acetonitrile for the Orbitrap screening method and with pure acetonitrile for the analysis of quinolones. Clean-up of extracts was achieved by freezing out samples at -70ºC. For analysis of quinolones obtained extract was diluted with 0.1 % formic acid in water. For the screening method extracts were evaporated and reconstituted in 2:1 (v/v) water–acetonitrile solution containing 5 mM of ammonium formate and 0.01% of acetic acid.

2.4.2. Instrumental analysis

Instrumental separation and analysis of NSAIDs was performed by LC-MS/MS using Acquity UPLC system (Waters, Milford, MA, USA). Separation was performed on a Phenomenex Luna Omega analytical column (100 × 4.6 mm, 2.6 μm). Mobile phase consisted of 0.01 % acetic acid solution in water (A) and acetonitrile (B) that was delivered at 0.6 mL min^{-1} using a gradient program. Chromatograph was coupled to a QTrap 5500 (AB Sciex, MA, USA) mass spectrometer equipped with an electrospray source operated in SRM mode with negative ionization.

Screening of various pharmaceutical residues and metabolites was performed using a Dionex UltiMate 3000 HPLC system (Thermo Fisher Scientific, San Jose, CA, USA) on a Phenomenex Luna Omega analytical column (100 × 2.1 mm, 1.6 m). The mobile phase consisted of (A) 0.1% formic acid in water, (B) 0.1%formic acid in acetonitrile, and (C) 0.1% formic acid in methanol. The gradient program was used and flow rate was 0.3 mL min^{-1}. The HPLC system was coupled to a Q-Orbitrap HRMS mass spectrometer (Thermo Fisher Scientific) equipped with a heated electrospray ionisation probe operating in the positive and negative ionisation modes. Full scan data both in the positive and negative ionisation modes were acquired at a mass resolving power of 70,000 FWHM.

Analysis of 24 pharmaceutically active substances was performed using a Dionex UltiMate 3000 HPLC system (Thermo Fisher Scientific, San Jose, CA, USA) on a Kinetex C18 analytical column (100 × 2.1 mm, 2.6 m). The mobile phase consisted of (A) 0.01% acetic acid in water, (B) acetonitrile, and (C) methanol. The gradient program was used and flow rate was 0.3 – 0.4 mL min^{-1}. The HPLC system was coupled to a Q-Orbitrap HRMS mass spectrometer (Thermo Fisher Scientific) equipped with a heated electrospray ionisation probe. SRM both in the positive and negative ionisation modes was used.

Determination of quinolones was performed by direct injection FT-ICR-HRMS. Prior to the first analysis, a sodium formate solution was used to calibrate the FT-ICR-HRMS instrument equipped with a 7.0 T superconducting magnet (Bruker Daltonics, Bremen, Germany). The sample was directly introduced at a flow rate of 250 μL h^{-1} into the ESI source. The mass spectrometer was set to operate over the mass range of *m/z* 100–1000 in the positive ion mode. Each spectrum was acquired by accumulating 32 scans of time-domain transient signals in 2 mega-point time-domain data sets. The resolving power $m/\Delta m(50\%)$=140,000 and mass accuracy of <3 ppm provided for unambiguous molecular formula assignments of singly charged molecular ions. In the MS/MS experiments, collision-induced dissociation mode was selected, argon was used as the collision gas, the collision energy was adjustable from 5 eV to 25 eV, the isolation windows were set at *m/z* 5 and the collision RF amplitude was set at 1500 Vpp.

3. RESULTS AND DISCUSSION

3.1. Application of biological indicators for the occurrence studies

3.1.1. Wild game as an indicator of BFR contamination[1]

In order to evaluate the applicability of wild animals as indicators for terrestrial ecosystem contamination, muscle and liver tissues of eight red deer (*Cervus elaphus*), nine wild boar (*Sus scrofa*), and seven moose (*Alces alces*) specimens were analysed for the content of BFRs including PBDEs, HBCD, and TBBPA.

The highest mean concentrations of PBDEs (46.6 pg g^{-1} (w.w.)) were observed in the tissues of moose, while significantly lower values were observed in wild boar and red deer tissues. The obtained results were somewhat surprising, taking into account the different diets of herbivore moose and omnivore wild boar, for which higher BFR content was expected due to additional PBDE exposure pathways. This would suggest that age plays important role in the accumulation of PBDEs, since the average age of moose specimens was 2.7 years, whereas the wild boar specimens had the average age of 1.4 years. However, no significant correlations were observed between the age of any of the specimens used in our study and the content of BFRs (r_s ranging from -0.49 to 0.64; *p*-values ranging from 0.13 to 0.84). The wild boar samples contained the highest levels of HBCD, with the mean concentration equal to 264 pg g^{-1} (w.w.) in muscle tissues. A prevalence of HBCD concentrations in liver tissue exceeding the levels found in musculature was observed for moose and red deer, while in wild boar the HBCD concentrations were higher in muscle than in liver. Generally low mean concentrations of TBBPA from 0.52 to 4.54 pg g^{-1} (w.w.) were observed despite its wide application in Europe. The most probable explanation for this fact could be based on the fact that during the manufacture of TBBPA-containing polymers this BFR is chemically bonded to the material, reducing the potential for its release into the environment. Contamination levels of PBDEs and HBCDs determined in analysed samples are shown in Fig.3.1.

[1]Zacs, D.; Rjabova J.; **Ikkere, L.E.**; Bavrins, K.; Bartkevics, V. Brominated flame retardants and toxic elements in the meat and liver of red deer (*Cervus elaphus*), wild boar (*Sus scrofa*), and moose (*Alces alces*) from Latvian wildlife. *Sci Total Environ* **2018**, *621*, 308-316.

Observed contamination levels are comparable or somewhat lower than reported in other studies. Concentrations of ∑PBDE observed in muscle of Finnish deer ranged from 10 to 180 pg g^{-1} (w.w.) with higher values of 500 pg g^{-1} (w.w.) in specimens of Lapland region. Levels in liver samples varied greatly, German deer containing 30 – 120 pg g^{-1} (w.w.), Norwegian moose 33 – 50 pg g^{-1} (w.w.) and Finnish reindeer reaching up to 1700 pg g^{-1} (w.w.) [33-35].

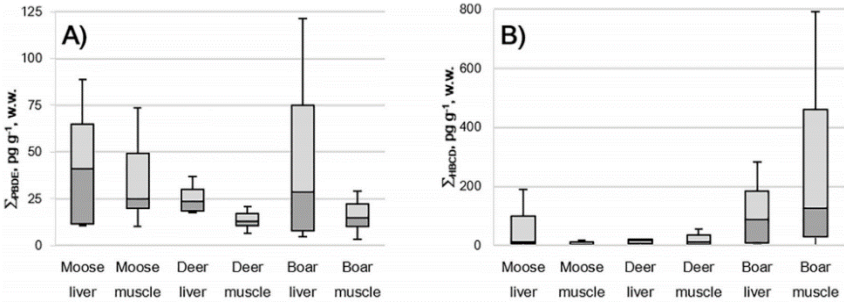

Fig. 3.1. **Box plots of the concentrations of PBDEs (A) and HBCDs (B) in the liver and muscle tissues of the analysed species**

Regarding the congener profile of the PBDEs, the tetra- through hexabrominated homologs of PBDEs were the predominant congeners contributing from 59 to 91% of the total load of the selected PBDEs. This indicates that the recently used "penta-BDE" formulation is a probable source of contamination. Despite the recent widespread application of "octa-BDE" and "deca-BDE" formulations containing mainly PBDE-209, the contribution of this congener to the sum of PBDEs in muscle samples accounted for only about 6%, while a much higher contribution was observed in liver samples, reaching 36% on average for wild boar. Obtained results reflect the differences in bioaccumulation or biotransformation potential of congeners having the different extent of bromination, as already observed in previous studies [33-35].

The observed diastereomer profile of HBCD was similar to the pattern typically found in aquatic biota, including a pronounced domination of α-HBCDs over β- and γ-HBCDs [13], with α-HBCDs contributing 85% of the ∑HBCD on average.

3.1.2. Eels as indicators of emerging BFR contamination[2]

In order to evaluate the applicability of eels as indicator-organisms for aquatic ecosystem contamination, the content of seven emerging BFRs (including HBCD, DBDPE, TBP-DBPE, HBB, EH-TBB, BTBPE and tetradecabromo-1,4-diphenoxybenzene) was evaluated in eels *(Anguilla anguilla)* sampled from five Latvian lakes. The summary of obtained results is shown in Fig.3.2.

HBCD was found in all of the analysed samples, confirming the ubiquitous distribution of HBCD in European aquatic environments [56]. The sum of α-, β-, and γ-HBCD concentrations (∑HBCD) ranged from 0.05 to 6.58 ng g^{-1} (l.w.), with the average value of 1.64 ng g^{-1} (l.w.). The determined levels of HBCD in European eels from other countries were significantly higher, with the highest average concentrations of 4500 ng g^{-1} (l.w.) observed in eels from highly polluted areas in Belgium where a number of textile factories are located [37]. The pattern of HBCD diastereomers observed in the analysed samples was typical for biota already observed in the previous study, with strongly pronounced predominance of α-HBCDs over β- and γ-HBCD [13]. The main factors that may be responsible for this observation are: 1) enzymatic isomerization of β- and γ-HBCD to α-HBCD, as previously observed in fish [56]; 2) α-HBCD has a higher water solubility (~49 mg L^{-1}) than β- and γ-HBCD (~2 mg L^{-1}), and thus is more readily available for uptake [57]; 3) *in vitro* experiments with hepatic sub-cellular fractions obtained from rat and trout showed that the biotransformation of β- and γ-HBCD was approximately three times faster than that of α-HBCD [58]; and 4) thermal isomerisation of γ-HBCD incorporated in materials containing flame retardants to α-HBCD during the disposal of electronic waste, as well as the photolytic conversion of γ- to α-HBCD [59].

[2]Zacs, D.; **Ikkere, L.E.**; Bartkevics, V. Emerging brominated flame retardants and dechlorane-related compounds in European eels (*Anguilla anguilla*) from Latvian lakes. *Chemosphere* **2018**, *197*, 680-690.

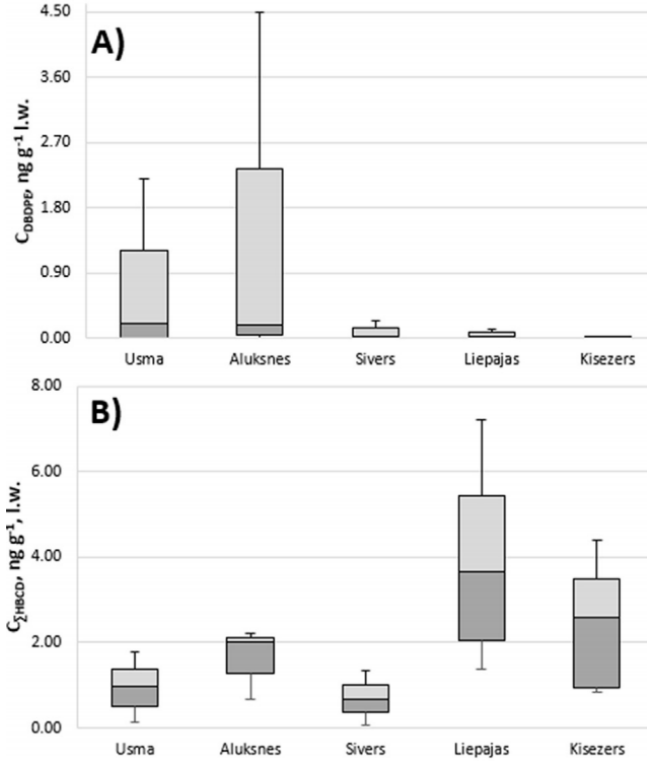

Fig.3.2. **Box plots of the concentrations of DPDBE (A) and ∑HBCD (B)**

DBDPE was the only other BFR found in analysed eel samples. 59% of the samples contained DBDPE in levels up to 33 ng g^{-1} (l.w.). To our knowledge, there are no reports on the occurrence of DBDPE in eels until now. According to the available references, DBDPE was not detected in various fish samples from St. Lawrence River, Canada and coastal waters of Chile [60, 61], while the levels reported for samples originating from a highly polluted river in South China and various rivers in Spain showed concentrations up to 230 and 130 ng g^{-1} (l.w.), respectively [62, 63]. DBDPE is listed as low volume chemical in Europe, however, it is the second most used BFR in China [64]. Taking into account the high heterogeneity of the observed concentrations, the main contamination pathways of DBDPE are still unknown. Contamination with DBDPE may originate both from point sources and long-range transport, since studies of BDE-209 have revealed that long-range transport in the air leads to contamination of distant regions. Due to their physicochemical similarity, this could be true for DBDPE as well [65].

The obtained results reflect the acceptable environmental status of Latvian lakes with regard to the total content of HBCD (∑HBCD), considering the EQS stated in the Directive 2013/39/EU [66]. The highest HBCD levels were observed in eels from lakes corresponding to the industrialisation of these areas, while the results of principal component analysis (Fig. 3.3.) showed that the concentration of HBCD depended on the particular sampling lake, reflecting non-uniform contamination of the Latvian environment with this emerging BFR.

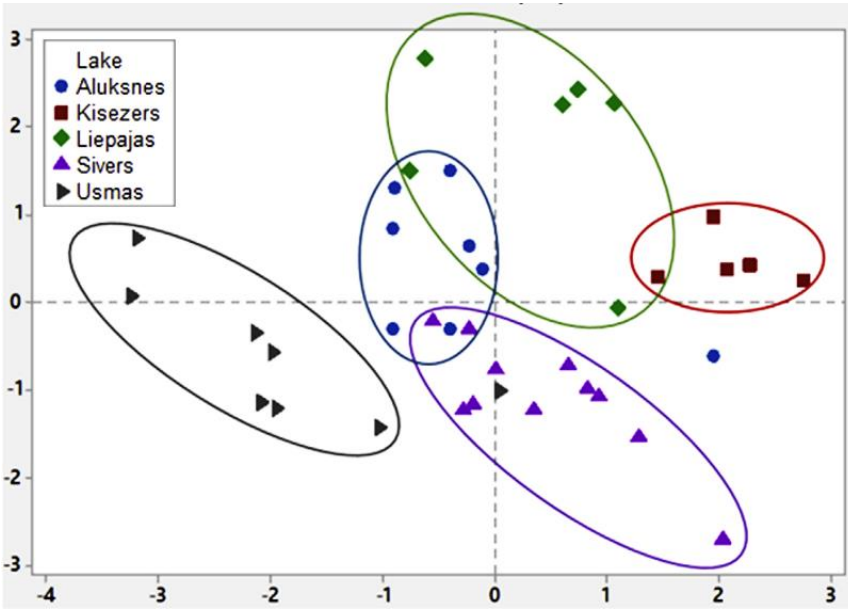

Fig.3.3. **Score plot of principal component analysis of HBCD concentrations and biological parameters**

3.1.3. Mussels as indicators of BFR, PFC and pharmaceutical contamination[3]

Further evaluation of aquatic indicator-organisms was carried out by investigating applicability of freshwater mussels. In addition to already discussed BFRs, another POP group, namely PFCs and emerging pollutants - NSAIDs were determined in the tissue of freshwater mussels.

PBDEs were found in all of the analysed samples, once more confirming the wide distribution of these flame retardants in the environment. The total concentrations of ΣPBDE ranged from 11.3 to 193 pg g^{-1} (w.w.), with the average value of 42.1 pg g^{-1} (w.w.). The observed levels were more than a magnitude lower than determined in eels from Latvian lakes in our previous study (see chapter 3.1.2). As proposed by Marriusen et al, this may be due to differences in lipid content of both organisms [35]. Furthermore, eels are at a higher trophic level and their overall lifespan is longer. When comparing observe results with those detected in mussels elsewhere, our concentrations were significantly lower, with exception of mussels from Baiyangdian Lake, China being similar to our study [67]. However, despite the relatively low levels detected in Latvia, the concentration of PBDEs in 83% of the samples exceeded the EQS value in biota (8.5 pg g^{-1} (w.w.) for the sum of congeners 28, 47, 99, 100, 153, 154), as stated in the European Commission Directive 2013/39/EU [66].

Regarding the congener profile of PBDE (shown in Fig.3.4) the highest levels were observed in the case of PBDE-209, which is the only deca-brominated BDE. This may be the result of widespread usage of deca-BDE formulation, accumulation of pollutants originating from suspended particulate matter and ineffective depuration process. Congeners No. 47, 49, 99, and 100 which represents tetra- and penta-brominated BDE were dominant in most of the samples. This observation corresponds to other authors' results [68, 69].

[3] **Ikkere, L.E.**; Perkons, I.; Sire, J.; Pugajeva, I.; Bartkevics, V. Occurrence of polybrominated diphenyl ethers, perfluorinated compounds, and nonsteroidal anti-inflammatory drugs in freshwater mussels from Latvia. *Chemosphere* **2018**, *213*, 507-516.

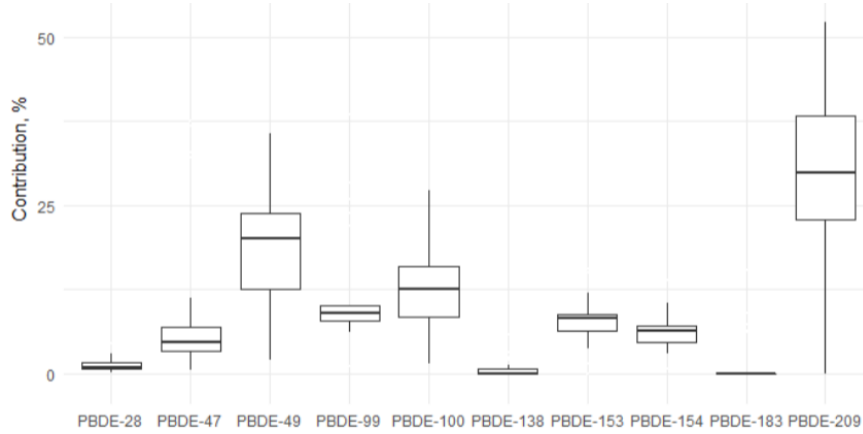

Fig. 3.4. **Box plot of the contributions (%) of selected PBDEs in the analysed mussel samples.**

Two compounds representing the PFCs were determined in the mussel tissue – PFOS and PFOA. PFOS was detected in 3 out of 24 (13%) samples, with concentrations ranging from 10 to 21 pg g^{-1} (w.w.) PFOA, however, was detected more frequently, as 10 out of 24 (42%) samples were above the limit of quantification, ranging from 13 to 51 pg g^{-1} (w.w.) The observed values were two or three magnitudes lower than the EQS value for PFOA and its derivatives in biota stated in the European Commission Directive 2013/39/EC [66]. Results reported in mussels from other regions were considerably higher – levels of PFOS were up to 72 000 pg g^{-1} (w.w.) in north-central Portuguese estuaries [10].

Nine NSAIDs were analysed in mussels, including ibuprofen, tolfenamic acid, meloxicam, carprofen, flunixin, diclofenac, phenylbutazone, ketoprofen, and mefenamic acid. Out of the nine compounds, only ibuprofen was found in mussels. Ibuprofen was present in 50% of the samples, with concentrations ranging from 0.52 to 109 ng g^{-1} (w.w.) or from 5.1 to 1363 ng g^{-1} (d.w.) Reports from elsewhere are scarce, taking into account that NSAIDs are emerging contaminants. Levels observed in mussels from Taihu Lake, China were reaching 93.7 ng g^{-1} (d.w.) [42].

The high levels of ibuprofen in the aquatic environment of Latvia are observed in other studies [70, 71], where ibuprofen was detected in surface waters and untreated wastewater. Ibuprofen was the most consumed medication of the NSAID group in Latvia in 2017 [72]. The primary route for the discharge of ibuprofen into surface waters is through the effluent of wastewater treatment plants. Ibuprofen and its metabolites are excreted into the sewage system along with unused drugs that may be disposed of via drains and toilets [73].

The box plots for the total concentrations of each contaminant group are provided in Fig. 3.5. The samples were grouped by the drainage basin and types

of the water bodies – lake, river, river delta or water reservoir. PBDEs were more common in the Daugava and Lielupe basins. The highest variations were observed in the case of ibuprofen, as it was present in the Lielupe and Venta basins at significantly higher concentrations than in the Gauja and Daugava basins. As for the different bodies of water, the only sample from the Riga water reservoir contained remarkably high concentrations of PBDEs and ibuprofen.

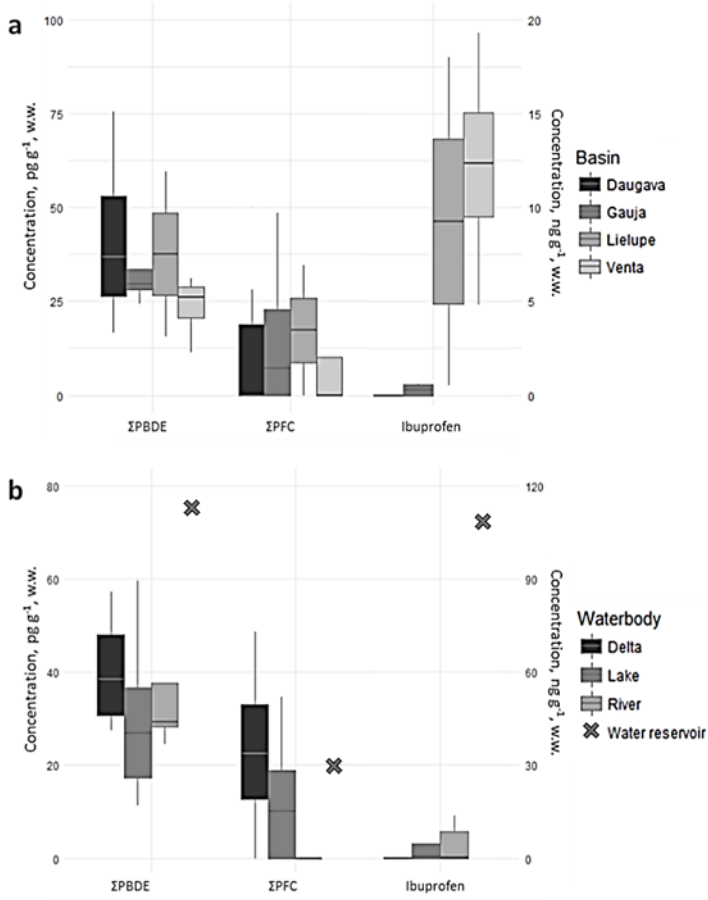

Fig.3.5. **Box plots of the concentrations of selected contaminants (pg g^{-1} (w.w.) (ΣPBDE and ΣPFC) or ng g^{-1} (w.w.) (ibuprofen)), grouped by the drainage basins (a) and types of water bodies (b)**

3.1.4. Comparison of periphyton, mussels, water and sediments

In order to evaluate the suitability of various sample types as indicators, periphyton, mussel tissue, water and sediment samples from three rivers in Latvia were analysed. Each batch consisting of four sample types was collected from four or five sampling sites from each river. 23 pharmaceutically active compounds were selected for the study. The selection of chemical compounds was based on the information from various publications about the most frequently found pharmaceutical residues in wastewater and the majority of them are listed as essential medicines by the World Health Organization. The selected compounds represent different therapeutic groups such as NSAIDs, blood lipid lowering agents, antibiotics, antiepileptic drugs, β-blockers, antidepressants, and others.

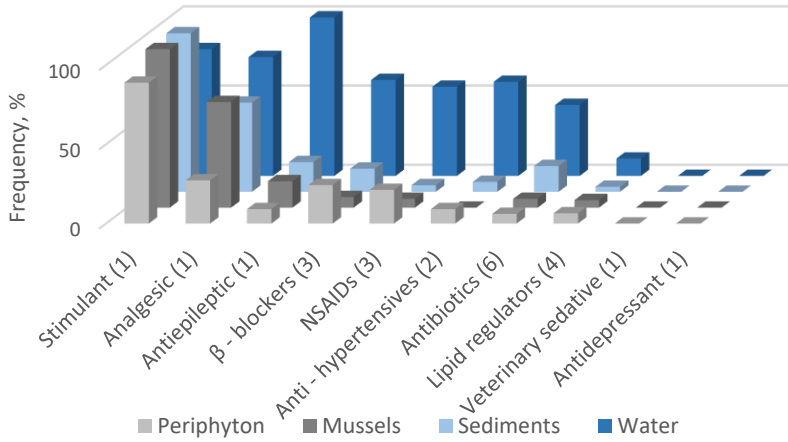

Fig.3.6. **Detection frequencies of compounds from various therapeutic groups**

Out of 23 analysed compounds only three – lipid regulator simvastatin, veterinary sedative xylazine and antidepressant fluoxetine were not detected in any of the samples. The detection frequencies of all analysed therapeutic classes are shown in Fig.3.6. The most frequently detected compound was central nervous system stimulant caffeine with an overall detection frequency of 91%. This compound was included in the study since it is considered a chemical marker for water pollution by domestic wastewaters. In our previous study caffeine was found in wastewaters in levels up to 12 μg L^{-1} [70]. In present research caffeine concentrations in surface waters were below 50 ng L^{-1}. The highest levels were observed for NSAID diclofenac and antiepileptic agent carbamazepine in water – 1138 and 1099 ng L^{-1} respectively. The observed levels are considerably high, for example carbamazepine was found in similar concentrations in rivers from Germany, whereas in other countries levels were considerably lower [74].

In the case of diclofenac, levels up to 7100 ng L^{-1} have been observed elsewhere [75]. The high contamination levels in the Mūsa River in Lithuania near Pasvale is most likely due to nearby WWTP. In order to examine this hypothesis, another batch consisting of water and sediment samples was collected before WWTP. The obtained results are summarised in Table 3.1. For the most of the compounds significant increase in levels was observed. The most pronounced increase was detected in the cases of antibiotic sulfamethoxazole, antihypertensive metoprolol and NSAID diclofenac – approximately 200-fold. This observation might indicate insufficient removal efficiency of described pharmaceuticals from wastewater. Other authors have described that diclofenac is poorly removed in traditional WWTP and sometimes even an increase in concentrations may be observed due to hydrolysis of conjugated forms [75].

Table 3.1.

Occurrence of pharmaceuticals in Mūsa River near Pasvale, Lithuania

Compound	Therapeutic group	Concentration in water, ng L^{-1}		Concentration in sediments, ng g^{-1} (d.w.)	
		Before WWTP	After WWTP	Before WWTP	After WWTP
Azithromycin	Antibiotics	<1	31	<1	<1
Ciprofloxacin		<1	100	<1	<1
Clarithromycin		1.6	40	<1	<1
Erythromycin		<10	44	<5	<5
Sulfamethoxazole		1.0	161	<0.5	<0.5
Trimethoprim		<1	34	<0.5	<0.5
Losartan	Anti-hypertensives	0.6	30	<1	<1
Metoprolol		1.4	273	1.8	12
Propranolol		0.1	2.8	<1	<1
Valsartan		6.0	55	<1	1.3
Atorvastatin	Lipid regulators	<1	1.4	<10	<10
Pravastatin		<1	12	<1	1,5
Diclofenac	NSAID	6.3	1138	<5	<5
Ibuprofen		<1	12	<1	<1
Acetaminophen	Anti-analgesics	<1	30	<1	<1
Carbamazepine	Anti-epileptics	21	1099	<1	8.2

Comparison between the three rivers is shown in Fig.3.7. The highest detection frequencies and detected levels were observed in Mūsa River. Regarding sample sites, only in Mūsa River significant differences were observed. As discussed in the previous paragraph, considerably higher levels were detected in samples from Pasvale, Lietuva. High levels were detected in

samples from Mūsa estuary (Lielupe) as well due to the higher anthropogenic load. In the other two rivers the differences were not that pronounced.

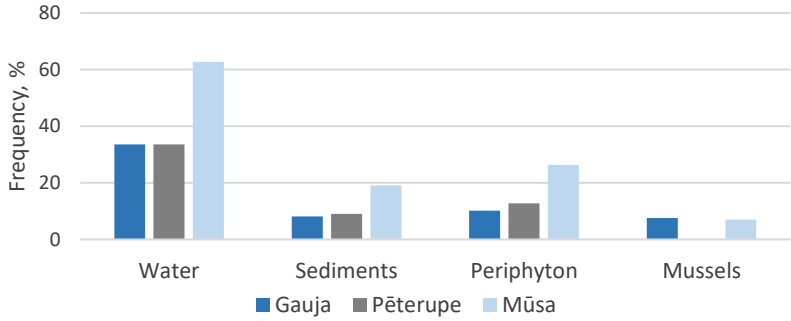

Fig.3.7. **Compound detection frequencies in the three rivers included in the study**

Finally, the four sample types were compared. As can be seen from Fig.3.6 and Fig.3.7, the highest detection frequencies were observed for water samples. This may be due to the lower level of matrix effects than for other sample types. It can be concluded that water is the most suitable matrix for temporal contamination observation. Other sample types may be more suitable for long-term contamination monitoring; however, more sensitive and selective analytical methods are necessary in order to improve the applicability of analytical methodologies.

3.2. Application of POCIS for contamination assessment

In order to evaluate the applicability of POCIS, nine different sorbents were tested for the determination of fifteen NSAIDs in the Daugava River. Multi-walled carbon nanotubes (MWCNTs) and $TNIM_4$ based sorbents have previously proven to successfully adsorb NSAIDs from surface water samples [71]. In the present study we used both commonly used sorbents such as hydrophilic-lipophilic balance (HLB) and novel sorbents such as MWCNTs and others. All of the samplers were placed in the same tank at the “Tome” fishery, that was operated in flow-through mode (water from Daugava River) and held for one week in order to ensure uniform conditions. The results are shown in Fig.3.8. As it can be seen, the levels of the NSAIDs obtained are highly dependent upon the sorbent used.

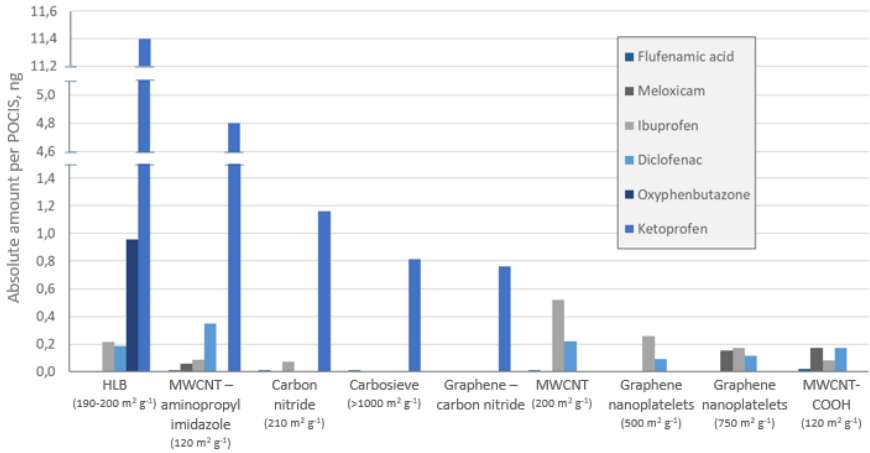

Fig.3.8. **Levels of detected NSAIDs using different sorbents**

The best overall results were obtained when the most common sorbent HLB was used. Five out of fifteen compounds were detected, including oxyphenbutazone which was not present in the extracts of any other sorbent. Moreover, the level of ketoprofen was significantly higher than observed with other sorbents. Nevertheless, meloxicam was only detected when graphene, MWCNT-COOH and MWCNT-aminopropyl imidazole sorbents were used. This observation might be due to the fact that meloxicam has the lowest pK_a value of all the selected analytes, i.e. 1.1. Commercially available HLB sorbent is suitable for sorption of wide range of compounds, however anion exchange sorbents are recommended for sorption of strong acids in order to achieve the best results.

Regarding the specific surface area, it can be noted that graphene nanoplatelets with bigger surface area ensured sorption of ketoprofen, that was not observed with smaller surface area. No effect of surface area was observed between different types of sorbents.

The obtained results suggest, that in order to achieve the best results for a wider scope of compounds, combinations of different sorbents must be used.

POCIS approach has demonstrated higher detection rates of NSAIDs, compared to previously described (section 1.3.1.) obtained NSAID results in mussels, where only one out of nine compounds were detected. This might indicate, that POCIS approach is more efficient regarding NSAID contamination estimation in water ecosystems. This observation may be related to the ongoing metabolic processes that have a different effect on the accumulation of NSAIDs in bivalve molluscs. However, it should be noted that the two sets of samples were collected at different sampling sites, so it is not possible to draw unambiguous conclusions. In order to make an accurate comparison between the

two types of indicators, additional experiments of placing both sample sets in the same location would be necessary.

3.3. Development of HRMS methods for the determination of pharmaceuticals

Aforementioned studies have demonstrated concerning levels of pharmaceuticals in the aquatic environment, indicating the emerging concern regarding pharmaceutical contamination. The studies have also shown the versatility of muscle tissue of various organisms as indicators. Therefore, further efforts were made in order to develop advanced HRMS methods for the determination of a wide range of pharmaceuticals in muscle tissue.

3.3.1. Orbitrap-HRMS multi-residue method[4]

HRMS method for the screening of residues and metabolites of 164 pharmaceutically active substances using HPLC-Orbitrap-HRMS was developed. The compounds included in the study belong to such therapeutic classes as anti-infectious (antibiotics and chemotheurapeutics), anti-inflammatory and antiparasitic agents, corticoids and agents acting on the nervous and reproductive systems, substances with hormonal and thyreostatic action, and beta agonists.

Different sample preparation procedures were compared and optimised for the detection of selected veterinary drugs in chicken, porcine and bovine meat. Solvent extraction with pure and acidified acetonitrile was evaluated. Various extract purification techniques including freezing out, salting-out, solid phase extraction columns and dispersive solid phase extraction were tested. The best results were obtained with pure acetonitrile extraction combined with a freezing out step and SPE using Phospholipid removal column or a Strata-X column. dSPE using primary and secondary amine salts was not suitable since several analytes were lost. Summary of relative analyte recoveries is given in Fig.3.9.

[4]Pugajeva, I.; **Ikkere, L.E.**; Judjallo, E.; Bartkevics, V. Determination of residues and metabolites of more than 140 pharmacologically active substances in meat by liquid chromatography coupled to high resolution Orbitrap mass spectrometry. *J Pharm Biomed Anal* **2019**, *166*, 252-263.

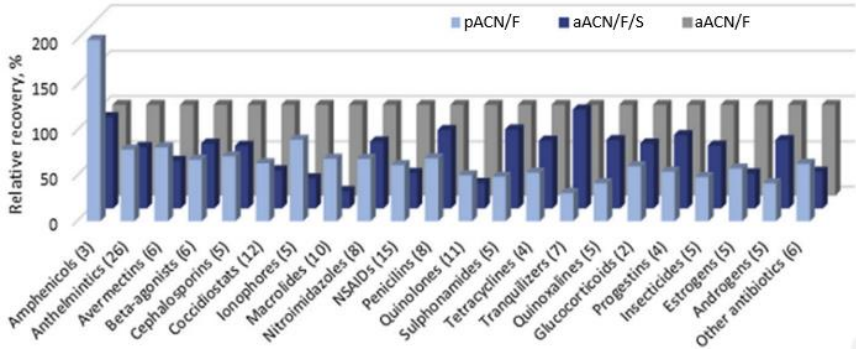

Fig.3.9. **Summary of relative recoveries per therapeutic group (number of analytes in brackets): pACN/F – SE with pure acetonitrile and freezing out; aACN/F/S – SE with acidic acetonitrile, salting out and freezing out; aACN/F – SE with acidic acetonitrile and freezing out (assumed as 100% of recovery)**

Chromatographic method optimisation included evaluation of mobile phases and columns. Ion source parameters like temperature and nitrogen gas flow were optimised for improved efficiency of the ionisation process. Resolution of 70 000 FWHM was proven to be sufficient. Chromatograms containing selected ion peaks are shown in Fig.3.10.

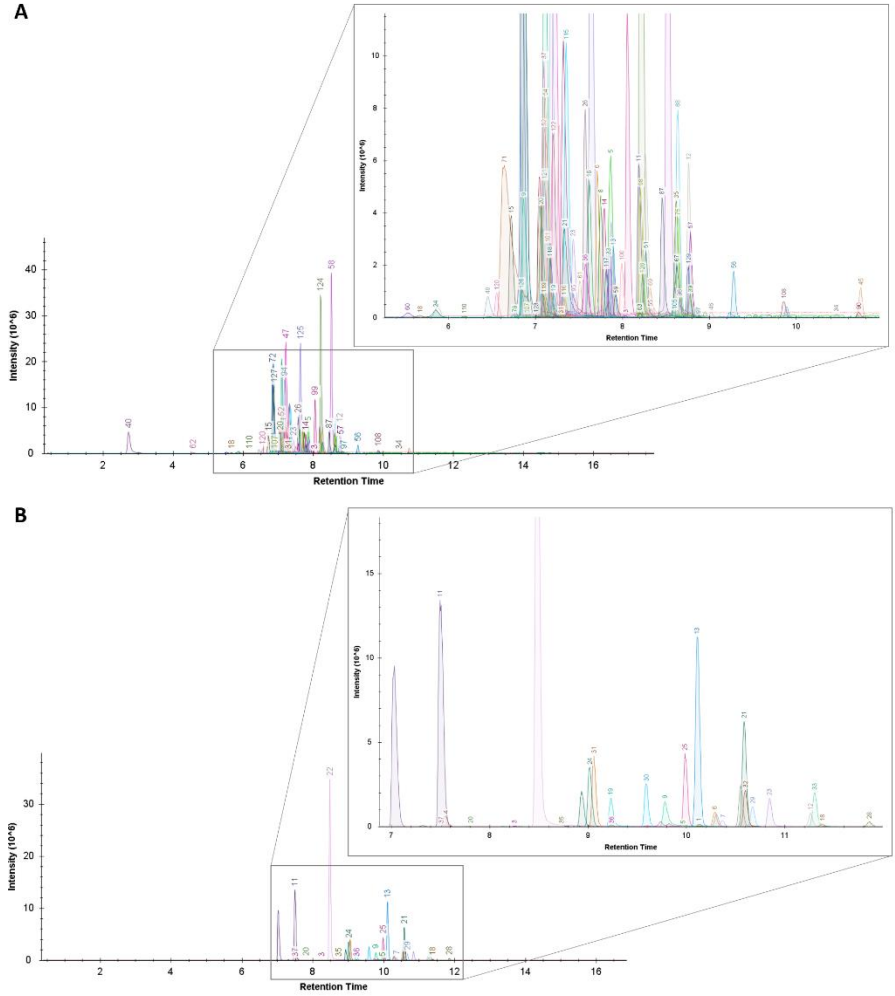

Fig.3.10. **Selected ion chromatograms of all analytes included in the study in positive (A) and negative ionization (B)**

The optimised method was validated by fortifying the blank matrix at four levels (0.5, 1.0, 1.5, and 2 times the maximum residue limit (MRL)), or at concentrations as low as possible for substances without an MRL. The evaluated performance parameters were selectivity, matrix effect, method and instrument limits of quantification, and accuracy. A total of 123 selected compounds in muscle tissue samples could be quantified with accuracy (recovery) ranging from

70 to 120% and RSDs less than 30%. Finally, the method was successfully used to detect and quantify veterinary drug residues in real samples found to be suspect using the non-selective qualitative test for the detection of inhibitor substances. The results were confirmed using the relevant one-residue confirmatory methods revealing concentrations of residues higher than MRLs established for several samples.

3.3.2. FT-ICR-HRMS method for high throughput analysis of quinolones[5]

Another HRMS method for the determination of pharmaceutically active substances in muscle tissue was developed utilising FT-ICR-HRMS. For this study quinolones – a group of antibiotics – was selected as target analytes due to numerous reports of high detection frequencies and link to the development of antimicrobial resistance [76]. In our study simultaneous detection, quantification and confirmation of ten quinolone group antibiotics was achieved by direct injection analysis in less than an hour.

Sample preparation optimisation included evaluation of pure and acidified acetonitrile extraction, addition of buffer salts to final extract and dilution with water. The comparison of evaluated parameters is shown in Fig.3.11. MS parameters such as flow rate, scan count, capillary voltage, end-plate offset, nebuliser pressure, dry gas flow rate, and drying temperature were optimised. The summary of MS parameter optimisation is shown in Fig. 3.12.

[5]**Ikkere, L.E.**; Perkons, I.; Pugajeva, I.; Gruzauskas, R.; Bartkiene, E.; Bartkevics, V. Direct injection Fourier transform ion cyclotron resonance mass spectrometric method for high throughput quantification of quinolones in poultry. *J Pharm Biomed Anal*[5] **2020**, *188*.

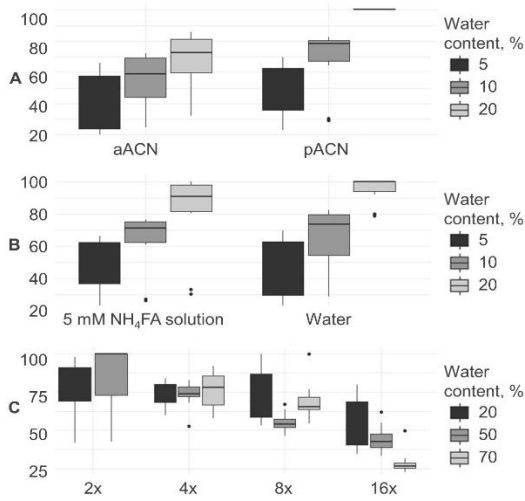

Fig.3.11. **The relative signal heights of ten quinolones during sample preparation optimisation: A) the effect of extraction solvent and water content; B) the effect of ammonium formate and water; C) the effect of dilution and water content**

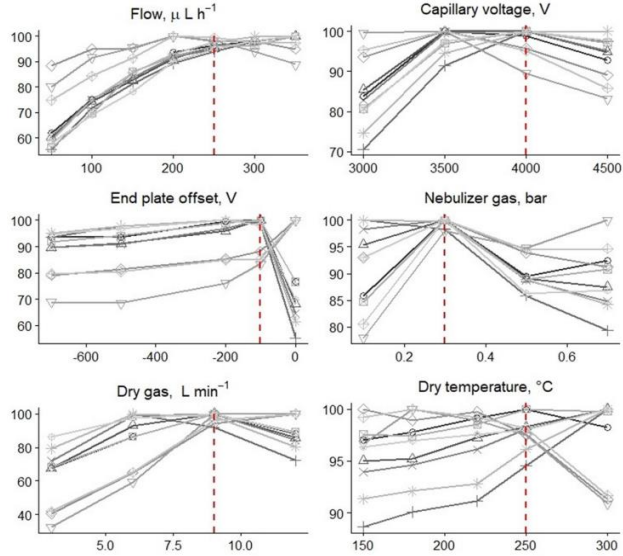

Fig.3.12. **The relative signal heights of ten quinolones during optimization of MS parameters**

The effect of resolution was demonstrated by separating interfering signal from sarafloxacin signal (Fig.3.13.).

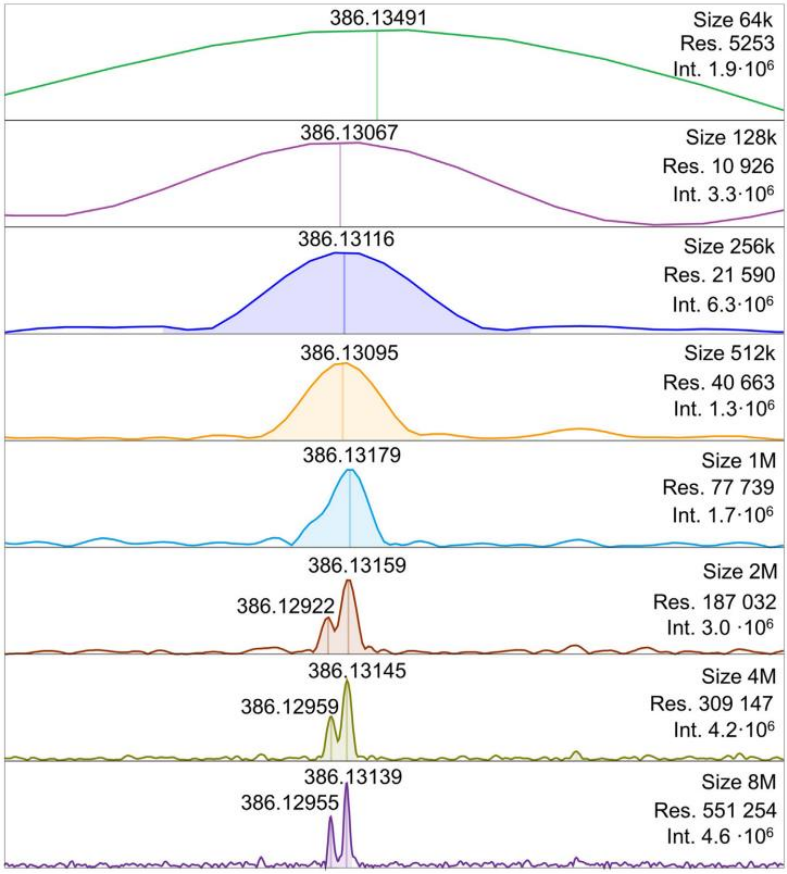

Fig.3.13. **Mass spectra of sarafloxacin and the interfering signal obtained using different resolutions**

Unambiguous identification of quinolones was achieved by additional MS/MS analysis. Distinction between oxolinic acid and flumequine is shown in Fig.3.14. Despite very close *m/z* values and the lack of chromatographic separation, the two peaks are clearly separated due to the high resolution.

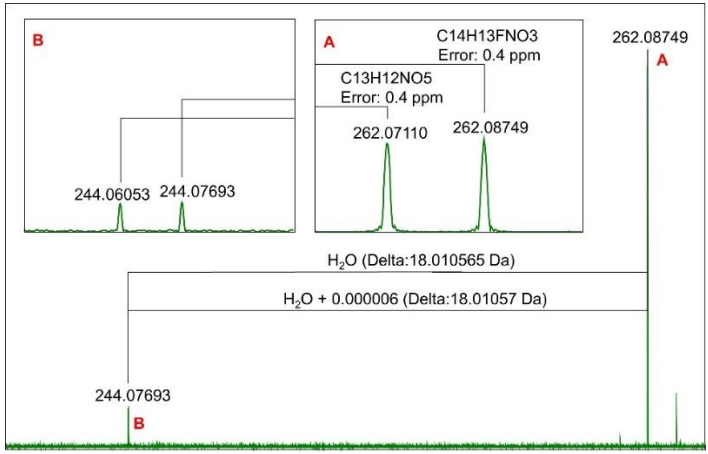

Fig.3.14. **MS/MS spectra of oxolinic acid (*m/z*=262.07110) and flumequine (*m/z*=262.08749): a) parent ions; b) fragment ions ($[M-H_2O+H]^+$)**

The applicability of the developed method was tested by analysing commercially available chicken meat and treated chicken samples. None of the 19 commercial samples contained residues of quinolones. The results were confirmed by a confirmatory LC-MS/MS method. Treated chicken samples contained levels of ciprofloxacin and enrofloxacin up to 1064 $\mu g\ kg^{-1}$. Results were in a good agreement with those obtained by HPLC-QqQ-MS/MS method.

CONCLUSIONS

1. Various organisms have been successfully tested and demonstrated as suitable contamination bioindicators:
   a. Analysis of moose, red deer, and wild boar tissues revealed the highest mean concentrations of PBDEs in the tissues of moose, while wild boar contained the highest levels of HBCDs. For all of the analysed species, liver was found to contain higher concentrations of contaminants compared to the muscle tissue. The congener profile of PBDEs in the tissues of animals indicates probable contamination from the recently used "penta-BDE" formulations.
   b. Among the selected emerging BFRs in European eels, HBCD and DBDPE were found. The environmental status of Latvian lakes with regards to the contamination with HBCD is acceptable according to the EQS criteria stated in the Directive 2013/39/EU. Considering the data available in reports from other regions, it can be concluded that the aquatic biota in Latvia reflects a lower degree of environmental contamination.
   c. PBDEs were detected in all of the mussel samples, but the concentrations were generally lower than those observed in mussels from other regions. Regardless, 83% of the samples exceeded the EQS threshold for the sum of six PBDEs stated in the Directive 2013/39/EU. The levels of PFCs in mussels were low throughout the territory of Latvia. The levels observed for ibuprofen were significantly higher than those detected by other authors.
2. Comparison of mussels, periphyton, sediments and water indicated the presence of numerous pharmaceuticals, revealing detection of 86% of all analytes. The highest levels were observed for NSAID diclofenac and antiepileptic agent carbamazepine at approximately 1 $\mu g\ L^{-1}$. Water was found to be the most suitable matrix for temporal contamination observation.
3. For the POCIS the best overall results were obtained when the most common sorbent HLB was used. Five out of fifteen compounds were detected, including oxyphenbutazone which was not present in the extracts of any other sorbent. Moreover, the level of ketoprofen was significantly higher than observed with other sorbents. Nevertheless, meloxicam was only detected when graphene, MWCNT-COOH and MWCNT-aminopropyl imidazole sorbents were used. This suggests, that

in order to achieve the best results for a wider scope of compounds, combinations of different sorbents must be used.

4. Two advanced HRMS methods have been developed for determination of pharmaceutically active substances:
    a. The developed method for the simultaneous identification and quantification of 164 residues and metabolites of pharmacologically active substances was evaluated and validated by HPLC-Orbitrap-HRMS. The main advantage of the proposed method is the relatively quick and generic sample preparation procedure with a wide scope of analytes detectable in a single analytical run.
    b. The analytical method for the simultaneous detection, quantification and confirmation of ten quinolones in muscle tissue by direct injection FT-ICR-HRMS was evaluated and validated. The elaborated analytical method provides a high throughput determination of quinolones, which is a major source of concern regarding the development of antibiotic resistance.

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Brominated flame retardants and toxic elements in the meat and liver of red deer (Cervus elaphus), wild boar (Sus scrofa), and moose (Alces alces) from Latvian wildlife

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Brominated flame retardants and toxic elements in the meat and liver of red deer (*Cervus elaphus*), wild boar (*Sus scrofa*), and moose (*Alces alces*) from Latvian wildlife

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HIGHLIGHTS

- Occurrence of BFRs and heavy metals in wild game samples from Latvia was evaluated.
- Liver showed higher levels of contaminants in comparison with the musculature.
- Levels of selected BFRs in Latvian terrestrial biota are lower than in other regions.

GRAPHICAL ABSTRACT

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ABSTRACT

In order to evaluate the contamination status of terrestrial biota in Latvia, muscle and liver tissues of red deer (*Cervus elaphus*), wild boar (*Sus scrofa*), and moose (*Alces alces*) were analyzed for the content of polybrominated diphenyl ethers (PBDE), hexabromocyclododecane (HBCD), tetrabromobisphenol A (TBBPA), as well as cadmium and lead. The highest mean concentrations of PBDEs (46.6 pg g^{-1} wet weight (w.w.)), cadmium (0.95 mg kg^{-1} w.w.), and lead (0.22 mg kg^{-1} w.w.) were observed in the tissues of moose, while the wild boar samples contained the highest levels of HBCD, with the mean concentration equal to 264 pg g^{-1} w.w. in muscle tissues. Generally low mean concentrations of TBBPA from 0.52 to 4.54 pg g^{-1} w.w. were observed. The liver tissue of all analyzed specimens was found to contain higher concentrations of contaminants, compared to muscle tissue. The congener profile of PBDEs in the analyzed tissues indicated that the recently used "penta-BDE" formulation was a probable source, while components of HBCD, "octa-BDE", and "deca-BDE" technical mixtures are likely to undergo congener-specific or diastereomer-specific bioaccumulation or metabolic degradation. Considering the reports from other regions, it can be concluded that the terrestrial biota in Latvia is less affected by the studied contaminants.

1. Introduction

Brominated flame retardants (BFRs) comprise a diverse group of anthropogenic chemicals that are used to prevent fire incidents. BFRs lower the flammability of a wide array of products, such as textiles,

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plastics, building materials, and electronic equipment (D'Silva et al., 2004). Despite the clear benefits provided by the use of BFRs, the ubiquitous presence of these chemicals has resulted in their diffusion into the environment during the manufacturing, use, and disposal. Due to their persistency and lipophilicity, BFRs enter the terrestrial and aquatic food chains (D'Silva et al., 2004). Some of the most important contaminants in this family are polybrominated diphenyl ethers (PBDEs) with the estimated worldwide production up to 67,000 tons per year in the early 2000s, and extensive use over several decades (U.S. EPA, 2010). However, considering that these compounds are persistent organic pollutants (POPs) and taking into account their potential for long-range transport (Hites, 2004), certain restrictions were imposed on their commercial availability (European Union, 2003). Therefore, in order to meet the market demand for FRs, several alternative BFRs were developed, among which the most consumed were hexabromocyclododecane (HBCD) and tetrabromobisphenol A (TBBPA). Similarly to PBDEs, these alternative BFRs also showed POP-like properties and their use followed by disposal led to environmental contamination (D'Silva et al., 2004; Abdallah, 2016). While HBCD was classified by the European Commission (EC) as a bioaccumulative and toxic compound because of its high persistence, low water solubility, and high log K_{ow} value (European Chemicals Agency, 2008), there is no consensus on the risks due to TBBPA. Older references report that TBBPA seems to have a comparatively low toxicity compared to the other BFR groups (Darnerud, 2003). Nevertheless, the most recent studies reflect toxicological concerns regarding the environmental presence of TBBPA (Lai et al., 2015), therefore attracting scientific interest to the occurrence of this chemical in the environment.

Atmospheric transport delivers significant quantities of BFRs from contaminated areas into other aquatic and terrestrial environments where these POPs are readily bioaccumulated through food chains (Law et al., 2014). Considering the fact that terrestrial animals are generally known to be less susceptible to the bioaccumulation of POPs compared to aquatic animals, because aquatic ecosystems are affected by POPs through additional pathways (e.g., via TOC-rich sediments and particulate matter suspended in water and by further bioaccumulation and biomagnification of POPs) (Law et al., 2003; Law et al., 2006), there has been less scientific interest towards the investigation of BFRs in terrestrial wildlife and only a handful of reports are available on this topic, with a focus on such carnivore species as fox and bobcat (Corsolini et al., 2000; Boyles et al., 2017; Fuglei et al., 2007). Less information is available on the occurrence of BFRs in herbivores and omnivores (Suutari et al., 2009; Mariussen et al., 2008; Christensen et al., 2005), even though such data would provide a better understanding of environmental transport, deposition patterns, and the ultimate fate of BFRs.

Another important group of toxicants is heavy metals, among which cadmium and lead are of major concern (Flora and Agrawal, 2017). Urbanization, industrialization, intensive agriculture and aquaculture can affect the environment with heavy metal emissions. Such factors as metal smelting and reclamation, fossil fuel combustion, overuse of mineral fertilizers or improper waste disposal are considered to be the most important sources of undesirable metallic elements in the global environment (Falandysz et al., 2005). Despite the substantial efforts to minimize anthropogenic environmental pollutants since the 1970s, the levels of heavy metal contamination in wildlife still may be significant (Cooper et al., 2017).

Red deer (*Cervus elaphus*) and moose (*Alces alces*), being the two most dominant species (population of ~52,000 and ~21,000 animals in 2014, respectively) after roe deer (*Capreolus capreolus*, population of ~130,000 in year 2014), are the largest herbivores among Latvian wildlife (NeoGeolv, 2014). Red deer and moose have the longest life expectancy and thus are the most susceptible to bioaccumulation of BFRs and heavy metals, and can be considered to be the most sensitive herbivore bioindicators of environmental contamination status. While the diet of red deer and moose consists generally of plants and represents the air/soil – plant – herbivore system, wild boar (population of ~ 55,000 in Latvia in 2014 (NeoGeolv, 2014)) is known to be an omnivore consuming a wide variety of plants and insects, as well as carrion, fish, and mollusks that provide an additional uptake of contaminants. It has been shown that the accumulation of heavy metals in plants and soil may increase the risk of transfer to herbivorous wild mammals, including game animals (Falandysz et al., 2005; Bilandzic et al., 2012). In contrast to pure herbivores, wild boars have a highly adaptable diet and are exposed to contaminants via the multiple pathways. Therefore, the differences in nutritional habits of herbivores (red deer and moose) and an omnivore (wild boar) could provide an insight into the exposure routes and bioaccumulation of BFRs and heavy metals.

In this study, we carried out a broad analysis of the most commonly used chemicals from the BFR family, namely, PBDEs, HBCDs, and TBPPA, as well as two heavy metals – lead and cadmium. Considering the bioaccumulation of the selected contaminants, as well as previous recommendations for field studies (Lazarus et al., 2014; Neila et al., 2017; Van den Brink et al., 2016), meat and liver were selected for the analysis within the framework of this study. In order to assess the geographical differences in the distribution of contaminants, the specimens were sourced from different districts of Latvia.

2. Materials and methods

2.1. Samples

A total of twenty-four wild animals were analyzed in the current study, including seven specimens of moose, eight specimens of red deer, and nine wild boars. The individual specimens were killed by local hunters in different districts of Latvia (Fig. 1), according to the relevant Latvian legislation for game hunting during the period from September to December 2016. The animals were shot by hunting rifle and their age and sex were determined. The dissection of the animal carcasses and sampling of the musculature and liver was carried out in the field. Animal tissue samples were packed in polyethylene bags with ice, uniquely coded and delivered to the laboratory within 24 h. In order to minimize the influence of possible contamination of the samples during transportation, approximately one centimeter thick layer was removed from the surfaces of samples after receiving at the laboratory, the samples were homogenized and stored at −18 °C until analysis. The appropriate sample amounts (5–20 g) were analyzed for the content of twenty-four PBDE congeners, three HBCD diastereomers, TBBPA, lead, and cadmium. Detailed information about the analyzed specimens is presented in Table 1.

2.2. Chemicals and materials

All of the solvents used were at least of pesticide purity grade. Silica gel for column chromatography, nitric acid, hydrogen peroxide, and sulfuric acid were purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland) or from Acros Organics (Morris Plains, NJ, USA). The native and isotopically labeled standards for the analyzed BFRs were purchased either from Cambridge Isotope Laboratories (Tewksbury, MA, USA) or from AccuStandard (New Haven, CT, USA). Standard solutions for lead and cadmium were obtained from Merck (Darmstadt, Germany).

Along with the analysis of lead and cadmium, the following groups of brominated flame retardants were determined (for compounds given in bold, $^{13}C_{12}$-labeled surrogates were available and were used as internal or recovery standards):

1) Di- through decabrominated diphenyl ethers (PBDEs): IUPAC numbers 7, 15, 17, **28**, **47**, 49, 66, 71, 77, 85, **99**, **100**, 119, 126, 138, **139**, **153**, **154**, 155, 166, 181, **183**, 190, **209**.
2) Tetrabromobisphenol A and hexabromocyclododecane diastereomers: **TBBPA**, **α- HBCD**, **β- HBCD**, and **γ-HBCD**

Fig. 1. The distribution of sampling locations throughout Latvia.

2.3. Chemical analysis

2.3.1. Sample preparation for the analysis of selected BFRs

The method for the determination of selected BFRs was adopted from previously published studies (Zacs and Bartkevics, 2015; Zacs et al., 2014a; Zacs et al., 2015). Briefly, aliquots of homogenized and freeze dried tissue samples were spiked with an internal standard solution containing $^{13}C_{12}$-labeled α-, β-, and γ-HBCD diastereomers, $^{13}C_{12}$-labeled TBBPA, and $^{13}C_{12}$-labeled PBDE. After equilibration for at least 1 h, the samples were packed into extraction thimbles for the automatic Soxtec™ 2055 Fat Extraction System (Hillerød, Denmark) and extracted with dichloromethane/*n*-hexane (1:1, v/v) solvent system in a pre-weighed glass vessel. The solvents were evaporated to dryness under a gentle stream of nitrogen, the lipid content was determined gravimetrically and the fatty residue was redissolved in ~2 mL of *n*-hexane. The extract was quantitatively transferred to the top of a column packed with acid-modified silica gel (containing 44% of sulfuric acid) and the analytes were eluted with 1:1 *n*-hexane–dichloromethane mixture. The collected eluate was carefully evaporated on a rotary evaporator to about 0.2 mL, the extract was transferred to a 2 mL chromatographic vial, treated with 37 N sulfuric acid (30 μL), and vigorously stirred on a vortex stirrer. The mixture was allowed to stand for 20 min and centrifuged at 3000 rpm to separate the acid and organic layers. The acidic bottom layer was discarded and the organic layer was evaporated with the addition of recovery standard solution in toluene ($^{13}C_{12}$-PBDE-139 (100 pg μL^{-1}) prior to the GC-HRMS analysis of PBDEs. After the analysis of PBDEs, the solvent was exchanged to methanol and the extracts were analyzed for the content of HBCDs and TBBPA using an HPLC-Orbitrap-MS instrument.

Sample preparation for the analysis of heavy metals was performed using microwave–assisted digestion method as described elsewhere (Rudovica and Bartkevics, 2015). In brief, homogenized muscle tissues were placed in a Teflon digestion vessel and treated with concentrated nitric acid and hydrogen peroxide. The digestion procedure was performed by using microwave oven according to the following program: the solutions were heated for 40 min at 150 °C and then for 40 min at 180 °C. After cooling, the solutions were diluted to 50 mL with deionized water and subjected to the instrumental analysis.

2.4. Instrumental analysis

The analysis of PBDEs was performed using gas chromatography coupled to high resolution mass spectrometry (GC-HRMS) by applying electron impact ionization in positive ion mode (EI+) and selective ion monitoring (SIM) detection mode. Quantification was carried out using isotope dilution method by applying $^{13}C_{12}$-labeled surrogates as internal standards. Detailed description of the instrumental analysis of PBDEs can be found elsewhere (Zacs et al., 2015).

Elemental analysis of heavy metals was carried out by using inductively coupled plasma mass spectrometry (ICP-MS) under conditions described elsewhere (Rudovica and Bartkevics, 2015).

For the analysis of HBCDs and TBBPA, an HPLC-Orbitrap-MS method was used. Detailed description of this method is available in the Supporting Information file. In brief, the compounds of interest were separated using a C_{18} reversed-phase analytical column and heated electrospray ionization in negative ion mode ($HESI^-$) was used. The detection of analytes was performed in targeted selected-ion monitoring (t-SIM) mode using the two most abundant ions of the respective molecular ion clusters. The quantification was carried out based on isotope dilution with $^{13}C_{12}$-labeled surrogates and internal standardization.

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

In the analysis of BFRs, all stages of analytical procedure, including the sample extraction, purification, and handling of the final extracts were performed under conditions protected from ultraviolet (UV) radiation (i. e., using amber colored glassware or wrapping the glassware with aluminum foil). The quality control criteria for positive identification of analytes of interest included the retention time of the native compounds within a window of +3 to 0 s compared to the corresponding $^{13}C_{12}$-labeled surrogates for PBDEs, and +5 to 0 s for HBCDs and TBBPA, respectively. The acceptable deviation of the isotopic ratio for the two monitored ions (target/confirmation) was set as ±15% of the theoretical value for PBDEs and ±25% of the value obtained for the calibration mid-point for HBCDs and TBBPA. Five-point calibration curves were used for the quantification of analyte concentrations in each sample run. Procedural blanks and quality control samples were included in the routine quality control (QC) protocol and were analyzed in each sample sequence. The concentrations of contaminants determined in

Table 1
Sampling data for wild animals investigated in the present study.

Sample*	Sex	Age, years	Sampling site	Hunting date	Tissue	Lipid content in tissue,%
Wild boar samples						
WB1	M	1.5	Ērgļi district	Sept 1, 2016.	Liver	10.2
					Muscle	2.2
WB2	M	1.5	Madona district	Sept 2, 2016.	**	**
					Muscle	2.4
WB3	F	1	Kocēni district	Oct 2, 2016.	**	**
					Muscle	1.5
WB4	M	1	Raiskums parish	Oct 10, 2016.	Liver	0.9
					Muscle	10.3
WB5	F	2	Talsi district	Oct 15, 2016.	Liver	2.0
					Muscle	24.4
WB6	F	1	Kocēni district	Oct 10, 2016.	Liver	1.9
					Muscle	19.5
WB7	F	1	Dundaga district	Oct 8, 2016.	Liver	2.0
					Muscle	14.6
WB8	M	2	Burtnieki district	Oct 9, 2016.	**	**
					Muscle	1.3
WB9	F	2	Roja district	Nov 21, 2016.	Liver	7.2
					muscle	18.0
Moose samples						
M1	F	1	Jeri parish	Oct 2, 2016.	Liver	3.0
					Muscle	10.1
M2	M	1	Vaidava parish	Sept 16, 2016.	Liver	2.1
					Muscle	2.0
M3	M	2	Talsi district	Oct 8, 2016.	Liver	1.9
					Muscle	2.8
M4	F	4	Daugavpils district	Oct 17, 2016.	Liver	4.9
					Muscle	1.2
M5	F	5	Gulbene district	Dec 3, 2016.	Liver	2.9
					Muscle	2.3
M6	M	5	Roja district	Nov 5, 2016.	Liver	1.8
					Muscle	0.7
M7	M	1	Daugavpils district	Nov 8, 2016.	Liver	3.6
					Muscle	5.3
Red deer samples						
D1	F	2	Valmiera district	Sept 20, 2016.	Liver	1.4
					Muscle	2.0
D2	F	3	Valmiera district	Sept 20, 2016.	Liver	6.6
					Muscle	2.7
D3	M	3	Asare parish	Sept 26, 2016.	Liver	18.9
					Muscle	1.6
D4	M	5	Blome parish	Sept 10, 2016.	Liver	11.5
					Muscle	16.4
D5	M	2	Īle parish	Nov 28, 2016.	**	**
					Muscle	1.7
D6	M	3	Valdemārpils rural territory	Nov 28, 2016.	**	**
					Muscle	0.8
D7	F	3	Ugāle parish	Nov 28, 2016.	**	**
					Muscle	1.1
D8	F	5	Ventspils district	Oct 12, 2016.	Liver	5.2
					Muscle	3.7

* - Abbreviations: WB - wild boar; M - moose; D – red deer;
** - The tissue was not available.

real samples were corrected by taking into account the analyte concentrations found in procedural blanks. A list of average blanks is presented in the Supporting information (Table S1). The QC samples were in-house reference materials (fortified meat and liver homogenates), and the results obtained for QC samples were in good agreement with the fortification levels (the recovery ranged from 75 to 117%; relative standard deviation (RSD) ranged from 7 to 22%). For elemental detection, daily analyses of blank and QC materials (Multielement standard solution 5 for ICP (Sigma-Aldrich, St. Louis, MO, USA) were performed to monitor the efficiency and accuracy of ICP-MS analysis.

2.6. Statistics

The regression analyses, correlation analyses, and principal component analyses (PCA) were performed by using the Minitab 18 statistical software. Microsoft Excel 2013 was used to construct boxplots for data sets. The horizontal line within the box represents the distribution median. The ends of the box represent the 25th and 75th percentiles, while the interquartile range (IQR) is the difference between the 25th and 75th percentiles. The lines that extend from each end are whiskers, from the ends of the box to the outermost data point that falls within the distances computed as follows: 1st quartile $-1.5 \times$ (IQR) 3rd quartile $+1.5 \times$ (IQR).

3. Results and discussion

3.1. BFRs in the tissues of selected species

The overview of wet weight-based concentrations of selected BFRs in the tissues of moose, red deer, and wild boar is shown in Table S4,

while more detailed results for each individual sample are presented in Tables S5–S10. In the case of estimating the total concentrations of selected PBDEs ($\sum_{PBDE}$) and HBCDs ($\sum_{HBCD}$), the lower bound (LB) concentrations were used as a more pragmatic indicator for the content of BFRs. Data evaluation on wet weight basis was chosen as the more appropriate approach due to the large differences in the obtained percentages of lipids (0.27–24.4%) in the analyzed samples and the fact that the lipid content in wild animals varies seasonally, while the excretion of POPs is much less pronounced (Hakk et al., 2001). Therefore, wet weight-normalized concentrations could provide a more objective contamination status of tissues, since such results are less affected by the concentration or dilution of contaminants in the lipid fraction, compared to the results derived from lipid-normalized concentrations. An additional reason to discuss the wet weight-normalized contaminant concentrations was the inclusion of data on heavy metals, for which comparative assessments typically involve wet weight-based concentrations. Therefore, in order to facilitate the interpretation of results, wet weight-normalized concentrations of selected contaminants were discussed. A wide range of PBDE concentrations were observed in the analyzed samples, although none of the samples had exceptionally high PBDE content. There were no significant correlations between the lipid content and BFR concentrations in the analyzed samples. The $\sum_{PBDE}$ concentrations in the analyzed muscle tissue samples varied between 3.63 and 77.5 pg g^{-1} (w.w.). The muscle samples of moose showed the highest mean PBDE content of 36.0 pg g^{-1} (w.w.) among the tested species, while red deer and wild boar musculature showed significantly lower mean $\sum_{PBDE}$ concentrations of 16.7 and 18.0 pg g^{-1} (w.w.), respectively. The obtained results were somewhat surprising, taking into account the different diets of herbivore moose and omnivore wild boar, for which higher BFR content was expected due to additional PBDE exposure pathways. Some studies report that, besides the feeding ecology, also the age and sex could play an important role in the accumulation of contaminants in mammals (Kim et al., 2013). However, there was no proof that the higher concentrations of PBDEs in moose samples were due the fact that the average age of moose specimens was 2.7 years (ranging from 1 to 5 years), while the wild boar specimens had the average age of 1.4 years (ranging from 1 to 2 years), as no significant correlations were observed between the age of any of the specimens used in our study and the content of BFRs (r_s ranging from −0.494 to 0.636; p-values ranging from 0.125 to 0.842). It was found that the liver of the investigated specimens contained significantly higher mean concentrations of PBDEs, in line with the previous studies about the distribution of POP-like substances in different tissues of pigs and lamb (Fernandes et al., 2010; Shen et al., 2012). Similarly to muscle tissue samples, moose liver samples showed the highest mean $\sum_{PBDE}$ concentration of 46.6 pg g^{-1} (w.w.), but wild boar liver and red deer liver samples had the mean $\sum_{PBDE}$ concentrations of 40.3 and 29.8 pg g^{-1} (w.w.), respectively. Among the selected species, the highest HBCD levels were observed in the tissues of wild boar, with the mean $\sum_{HBCD}$ concentrations of 274 and 125 pg g^{-1} (w.w.) in muscle and liver, respectively. A prevalence of HBCD concentrations in liver tissue exceeding the levels found in musculature was observed for moose and red deer, while in wild boar the HBCD concentrations (w.w.) were higher in muscle than in liver (Table S4). Despite the wide application of TBBPA in Europe, this compound was found in the tissues at low levels, with the mean concentrations ranging from 0.52 to 4.54 pg g^{-1} (w.w.), in accordance with other studies on the occurrence of this BFR in environmental objects (Abdallah, 2016). The highest detection frequency of 71% and maximum concentration of 14.6 pg g^{-1} (w.w.) were observed in moose liver samples. The most probable explanation for the low concentrations of TBBPA in terrestrial animals could be based on the fact that during the manufacture of TBBPA-containing polymers this BFR is chemically bonded to the material (used as a reactive BFR), reducing the potential for its release into the environment, as well as the susceptibility of this compound to metabolic transformation. Similarly to PBDEs, there were no significant correlations between the biological parameters of the analyzed specimens and the concentrations of HBCD and TBBPA.

In order to assess the effects of geographical distribution on BFR levels, PCA was applied for the treatment of the raw data. However, no data clustering was observed on the geostatic map, reflecting the random distribution of BFR concentrations in samples (data not shown). At the same time, the significant differences in the observed contaminant levels could reflect the heterogeneity of the pollution in the habitats of wild animals and probable presence of areas with elevated background contamination, despite the relatively small sampling territory covered (~70,000 km^2), which is comparable in size to the distances covered by individual animals. There are no electronic waste processing facilities and no major waste incinerators in Latvia that have been shown to be significant emitters of BFRs into the environment elsewhere (Liagkouridis et al., 2014), thus the main sources of pollution could be uncontrolled disposal of BFR-containing waste and atmospheric emissions of these compounds through small-scale incineration. Nevertheless, further research can be recommended in order to identify highly contaminated localities.

There are only a handful of reports on the occurrence of BFRs in wild game from other regions, and while there have been no data published on the occurrence of BFRs in the tissues of wild boar, some indicative comparisons can be provided with reindeer and moose. The $\sum_{PBDE}$ concentrations for muscle of red deer from 6.62 to 44.6 pg g^{-1} (w.w.) observed in this study were somewhat lower compared to those obtained in the study on Finnish reindeer, in which the total concentrations of selected PBDEs in the muscle samples of adult animals were in the range from ~10 to 180 pg g^{-1} (w.w.) (Suutari et al., 2011a). The results of the same study for deer liver samples showed significantly higher levels of PBDEs, up to ~1700 pg g^{-1} (w.w.) compared to the current study where $\sum_{PBDE}$ in red deer liver ranged from 17.7 to 59.2 pg g^{-1} (w.w.). In a study from Germany (Päpke et al., 2011), deer liver contained selected PBDEs (excluding PBDE-209) with the total concentration ranging from 30 to 120 pg g^{-1} (w.w.), which also was higher than in our study. Regarding the PBDE content in moose musculature, another research group (Suutari et al., 2011b) reported concentrations that were similar to this study, with $\sum_{PBDE}$ of 20 pg g^{-1} (w.w.) in adult moose specimens sampled in the middle region of Finland, while significantly higher mean $\sum_{PBDE}$ concentrations of 500 pg g^{-1} (w.w.) were observed for the musculature of moose specimens from the Lapland region of Finland. The liver of moose specimens analyzed in the current study contained similar $\sum_{PBDE}$ concentrations to those observed in the study by Mariussen et al. (Mariussen et al., 2008), in which the mean $\sum_{PBDE}$ concentrations were in the range from 33 to 50 pg g^{-1} (w.w.). It can be concluded from the aforementioned studies that herbivores in the wilderness of Latvia are generally less exposed to PBDEs, compared to those in Finland and Norway, which can be attributed to the higher degree of industrialization and economic activity in the Scandinavian countries. The lower susceptibility of terrestrial animals to the accumulation of BFRs in comparison with aquatic biota was confirmed by the fact that the mean concentration of HBCDs found in the musculature of selected wild animals (90.9 pg g^{-1} w.w.) was significantly lower in comparison to the mean concentrations of HBCDs previously found in eel samples (312 pg g^{-1} w.w.) collected from Latvian lakes and in Baltic wild salmon (1590 pg g^{-1} w.w.) (Zacs et al., 2014a; Zacs et al., 2014b).

3.2. The contamination pattern with PBDE congeners/HBCD diastereomers

Despite the different patterns of PBDE bioaccumulation and biotransformation in various species, the congener profiles could substantially reflect the probable sources of PBDE contamination. The tetra- through hexabrominated homologs of PBDEs were the predominant congeners in liver and muscle samples of the studied animals, contributing from 59 to 91% of the total load of the selected PBDEs (Fig. 3). Among the individual congeners, PBDE-47 and PBDE-99 were

predominant in musculature, most likely reflecting that the contamination source was the widely used and recently banned commercial "penta-BDE" formulation, in which the latter congeners are among the most predominant (D'Silva et al., 2004). Despite the widespread application of "octa-BDE" and "deca-BDE" formulations containing mainly PBDE-209, the contribution of this congener to the sum of PBDEs in muscle samples accounted for only about 6%, while a much higher contribution of PBDE-209 to the $\sum_{PBDE}$ was observed in liver samples, reaching 36% on average for wild boar. The observed PBDE distribution between the tissues of selected animals probably reflected the differences in bioaccumulation or biotransformation potential of congeners having different extent of bromination, as already observed in previous studies for non-aquatic organisms (Huwe and Smith, 2007; Kierkegaard et al., 2007; Thuresson et al., 2006). Fig. 4A illustrates significant correlations ($r^2 = 0.98$) between the mean concentrations of PBDEs in the musculature and liver of the selected species, if only tetra- through hexa-BDE congeners are taken into account, while much weaker correlation was observed when considering the hepta- and deca-BDEs ($r^2 = 0.29$ in the case of wild boar if highly brominated congeners are included in the evaluation (data not shown)). The observed data could reflect species-selective bioaccumulation and biotransformation of PBDEs in terrestrial biota and support the earlier reports about species-selective bioaccumulation and biotransformation, probably via debromination of different PBDE congeners (Fernandes et al., 2009; Zhou et al., 2002). A notable observation was also made with regard to the concentrations of individual tetra-brominated, penta-brominated, and hexa-brominated PBDE congeners with high detection rates (e.g., PBDE-47, PBDE-99, and PBDE-153). There were significant correlations between PBDE-47 and PBDE-99 in both liver ($r^2 = 0.81$) and musculature ($r^2 = 0.82$), while much weaker correlations were observed between the total concentration of PBDE-47 and PBDE-99 ($\sum_{47/99}$) and the concentration of PBDE-153 ($r^2 = 0.67$ and $r^2 = 0.62$ for liver and musculature, respectively) (Fig. S1). Along with the distinct bioaccumulation and biotransformation properties of different PBDE congeners, such correlations could reflect the separate sources of these specific PBDEs, considering that PBDE-47 and PBDE-99 are two major components of the commercial "penta-BDE" formulation, while PBDE-153 is released to the environment from both "penta-BDE" and "octa-BDE" commercial formulations (La Guardia et al., 2006).

The HBCD diastereomer profile observed in all of the analyzed samples was similar to the pattern typically found in aquatic biota, including a pronounced domination of α-HBCDs over β- and γ-HBCDs (Covaci et al., 2006), with α-HBCDs contributing 85% of the $\sum_{HBCD}$ on average (Fig. 3). Considering that γ-HBCDs are the predominant contaminants in the majority of environmental matrices like soils and sediments, which tend to harbor HBCDs released from discarded polymers, as well as in technical HBCD mixtures, the observed diastereomer pattern probably arises by selective metabolism of the different enantiomers (Zegers et al., 2005). The occurrence of these processes in selected terrestrial biota is supported by the close correlation ($r^2 = 0.99$, Fig. 4B) between the mean concentrations of α- and γ-HBCDs observed in the analyzed samples (liver or musculature of red deer, wild boar, and moose), which was also consistent with a recent study on the distribution of HBCD diastereomers in fish and marine invertebrates (Son et al., 2015).

3.3. Lead and cadmium levels in the selected species

The overview of wet weight-based concentrations of lead and cadmium in the tissues of moose, red deer, and wild boar is shown in Fig. 2. Cadmium levels in the muscle tissues were relatively low. Twenty out of the twenty-four muscle samples contained cadmium at concentrations below the limit of detection (0.005 mg kg^{-1}, w.w.). One sample of moose muscle and two samples of red deer muscle contained levels of cadmium that ranged from the limit of quantification (0.02 mg kg^{-1}, w.w.) to 0.03 mg kg^{-1}, w.w. The determined levels of cadmium in red

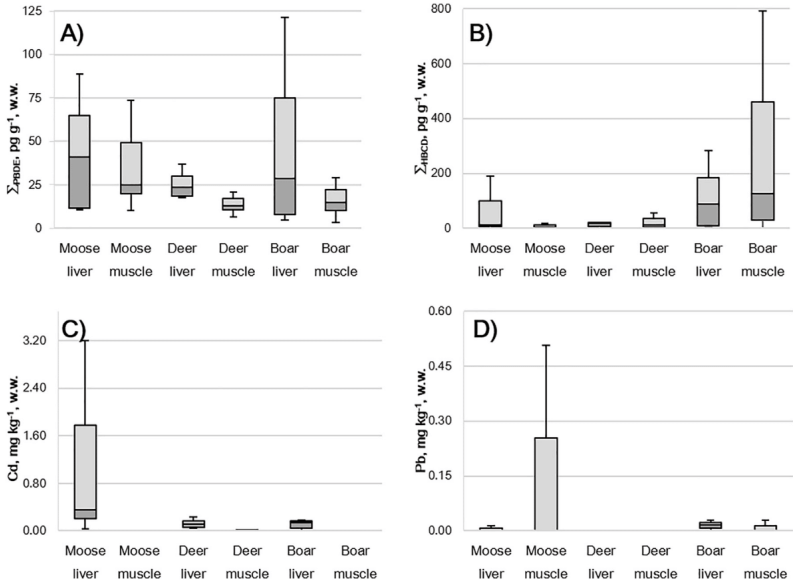

Fig. 2. Box plots of the concentrations of PBDEs (A), HBCDs (B), cadmium (C), and lead (D) in the liver and muscle tissues of the analyzed species.

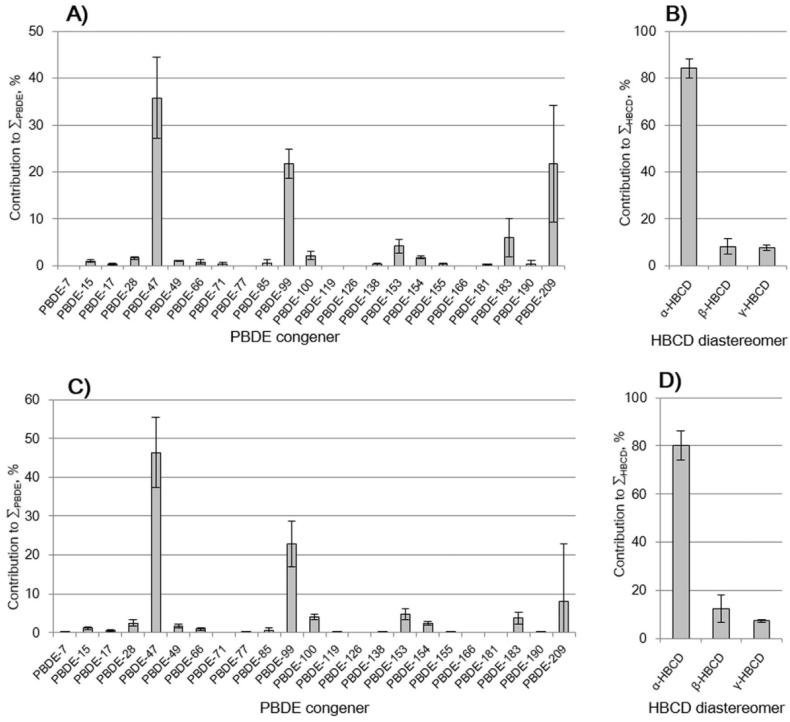

Fig. 3. The averaged congener profiles of PBDEs and diastereomer profiles of HBCD in the tissues of selected wild animals (liver – A and B; musculature – C and D).

deer muscle were similar to those observed in Spain, with the mean concentrations of 0.01 mg kg^{-1}, w.w. (Soler et al., 2016). None of the wild boar muscle samples contained cadmium at detectable levels. The highest cadmium levels were observed in liver samples, in a good agreement with the fact that cadmium does accumulate in liver and kidneys, but to a much lower degree in muscle tissue. Red deer and wild boar liver samples contained relatively low levels of cadmium, with the mean values of 0.18 and 0.15 mg kg^{-1} w.w., respectively. No correlations between the cadmium level in liver tissue and the age of animal were observed for any of the selected species. The levels found in red deer liver were similar to those in game meat from Croatia – 0.15 mg kg^{-1}, but the levels in wild boar were higher – 0.39 mg kg^{-1},

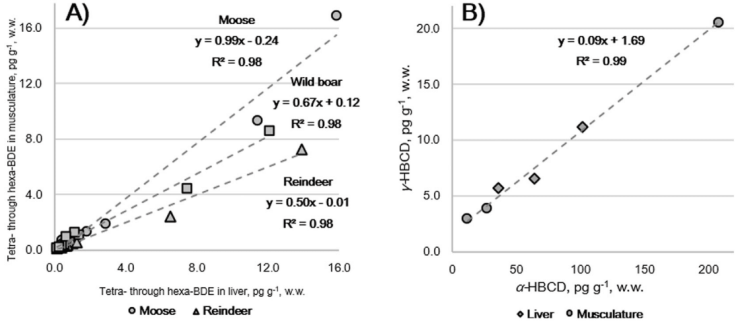

Fig. 4. Correlations between the concentrations of BFRs in the analyzed tissues (A for PBDEs and B for HBCD).

w.w. (Lazarus et al., 2014). The cadmium level detected in wild boar liver from Spain was 0.33 mg kg^{-1}, w.w. (Neila et al., 2017). The highest concentrations of cadmium (up to 2.32 mg kg^{-1}, w.w.) were observed in moose liver, and could be associated with the diet of moose, which consists mainly of aspen and willow. Studies by other authors have shown that these tree species are by far the most important cadmium contributors, while in the areas featuring extensive growth of aspen and willow the daily intake of cadmium by moose could be as high as 7 mg or even more (Brekken and Steinnes, 2004). Thus, much higher cadmium levels were observed in the Mackenzie Mountains region, Canada, with the mean concentrations of 48.3 mg kg^{-1}, w.w. (Larter et al., 2016). Considering that the cadmium levels in moose liver are likely to depend on the age of the animal (Danielsson and Frank, 2009), the very pronounced differences of cadmium concentration (ranging from 0.03 to 2.32 mg kg^{-1}, w.w.) in the analyzed moose samples could be attributed to the significant range of animal age. However, the number of analyzed samples was too small to ensure statistically reliable information and further studies should be carried out to confirm this trend. At the same time, it should be pointed out that no correlations or trends were observed between the animal age and cadmium levels in other species. In addition, our study demonstrated that the cadmium content in the analyzed animals was not dependent on the hunting region. The main source of cadmium in the environment is nonferrous mining and smelting. Since there are no smelters in Latvia, the only relevant anthropogenic sources of cadmium are phosphate fertilizers, fossil fuel combustion, and waste disposal, especially incineration (ATSDR, 2012). Considering that cadmium moves easily through soil layers and enters into the food chain through uptake by plants, root crops, and cereals, the contamination level of Latvian terrestrial environment with cadmium is likely to be evenly distributed, with no highly contaminated areas.

Overall, the concentrations of lead were lower than those of cadmium. Lead was found at quantifiable levels in thirteen (31%) out of the forty-two samples. The highest concentrations of lead (up to 1.21 mg kg^{-1}, w.w.) were found in moose muscle tissue samples. One of the main causes of lead contamination could be the use of lead-based rifle bullets in hunting. These bullets are designed to disintegrate on impact, resulting in widespread and significant fragment dispersion (Knott et al., 2010). The highest detection frequencies of 38% in muscle tissue and 78% in liver tissue were observed in wild boar samples. The concentrations ranged from 0.01 to 0.04 mg kg^{-1} (w.w.) in muscle tissue and from 0.02 to 0.03 mg kg^{-1} (w.w.) in liver tissue. The obtained concentrations were significantly lower compared to those observed in Croatia – 0.17 mg kg^{-1} in liver and 0.19 mg kg^{-1} in muscle tissue (Lazarus et al., 2014). The levels recently observed in Spain were even higher – up to 0.38 mg kg^{-1} in liver (Neila et al., 2017). No correlation between the concentration of lead and the age or origin of animals was observed for the selected species. Taking into account that wild boars are omnivore animals, heavy metals may be partly taken up by consumption of earthworms, which are known to accumulate considerable amounts of lead, as well as other heavy metals (Latif et al., 2013).

Documenting the contamination status of wild animals can provide a versatile tool for the characterization of terrestrial pollution and identification of potential health risks for wild game consumers. However, there is no systematic information available on the human consumption of wild game in Latvia, and therefore no assessment of contaminant intake can be provided. A comparison of the actual contaminant concentrations in wild game samples with the maximum levels (ML) established in the EU could support any risk assessment strategies. None of the analyzed samples exceeded the ML for cadmium in meat (0.05 mg kg^{-1}), as set by the European Commission, while three of the moose liver samples exceeded the ML for liver (0.50 mg kg^{-1}) (European Union, 2006). Considering the EU MLs for lead in meat (0.10 mg kg^{-1}, w.w.) and in offal (0.5 mg kg^{-1}, w.w.), none of the analyzed wild game samples exceeded the established maximum permissible concentrations. Therefore, it can be concluded that the consumption of wild game meat is unlikely to pose any risk due to the intake of high levels of cadmium or lead, while the consumption of moose liver might be less recommended.

4. Conclusions

Samples of moose, red deer, and wild boar tissues from different districts of Latvia were analyzed for the content of PBDEs, HBCD, TBBPA, cadmium, and lead in order to evaluate the contamination status of Latvian terrestrial biota. Among the analyzed species, the highest mean concentrations of PBDEs, cadmium, and lead were observed in the tissues of moose, while wild boar contained the highest levels of HBCDs. For all of the analyzed species, liver was found to contain higher concentrations of contaminants compared to the muscle tissue. Although the concentrations of the selected contaminants in the analyzed terrestrial biota samples were not dependent on the sampling site, significant differences in contaminant concentrations could reflect the heterogeneity of the pollution in the habitats of wild animals and probable presence of areas with elevated background contamination. The congener profile of PBDEs in the tissues of animals indicate a probable contamination from the recently used “penta-BDE” formulation, while the highly brominated components of the widely applied “octa-BDE” and “deca-BDE” formulations, as well as the components of technical mixtures of HBCDs may be prone to congener-specific or diastereomer-specific bioaccumulation or metabolism in terrestrial animals. Considering the data available in sparse reports from other regions, it can be concluded that the terrestrial biota in Latvia reflect a lower degree of environmental contamination.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2017.11.247.

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Emerging brominated flame retardants and dechlorane-related compounds in European eels (*Anguilla anguilla*) from Latvian lakes

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Emerging brominated flame retardants and dechlorane-related compounds in European eels (*Anguilla anguilla*) from Latvian lakes

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HIGHLIGHTS

- Occurrence of EBFRs and DRCs in eel samples from Latvian lakes was evaluated.
- Among the EBFRs, HBCD and DBDPE were found in eels in quantifiable concentrations.
- Dec 602 was the predominant component among selected DRCs.
- The increase of POP concentrations with fish age was observed for HBCD and DRCs.
- Levels of selected contaminants in eels from Latvia are lower than in other regions.

GRAPHICAL ABSTRACT

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ABSTRACT

Fifteen halogenated flame retardants (HFRs) including seven emerging brominated flame retardants (EBFRs) and eight dechlorane-related compounds (DRCs) were analyzed in eels (*Anguilla anguilla*) sampled from five Latvian lakes. Out of the seven EBFRs, hexabromocyclododecane (HBCD) and decabromodiphenyl ethane (DBDPE) were found in eels in quantifiable concentrations, up to 6.58 and 33.0 ng g^{-1} lipid weight (l.w.), respectively. The mean total concentration of DRCs ($\sum_{DRC}$) in the samples was 0.62 ng g^{-1} l.w. and the geographical distribution of DRC contamination was nearly uniform among the selected lakes. Dechlorane 602 (Dec 602) was the predominant component, whereas the composition of mixture containing *syn*- and *anti*-Dechlorane Plus (DP) stereoisomers showed a pronounced enrichment of the *anti*-DP isomer and was close to the composition of OxyChem® DP commercial product. The determined concentrations of HFRs were lower than in other studies of aquatic biota from Europe and Asia, and the obtained results reflect the acceptable environmental status of Latvian lakes with regard to the total content of HBCD ($\sum_{HBCD}$), considering the environmental quality standards (EQS) stated in the Directive 2013/39/EU. The highest $\sum_{HBCD}$ levels were observed in eels from lakes corresponding to the industrialization of those areas, while the results of principal component analysis (PCA) showed that the concentration of HBCD depended on the particular sampling lake, reflecting non-uniform contamination of the Latvian environment with this EBFR.

1. Introduction

Persistent organic pollutants (POPs) are of global concern because of their toxicity, bioaccumulation, and the potential for

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long-range transport. Due to the bioaccumulation in food webs, POPs pose significant risks to the environment and human health (WHO/UNEP, 2013). Considering the behavior and fate of POPs, marine and freshwater environments are highly affected by these compounds and the contamination status of aquatic environments could reflect the overall extent of pollution in the surrounding area (Wenning and Martello, 2014). While the high degree of pollution with POPs in the Baltic Sea environment has been known for several decades (Helkom, 2004; Koistinen et al., 2008), there is a notable knowledge gap on POP levels in the inland aquatic environments of the Baltic region. Only few references report the levels of such POPs as polychlorinated dibenzo-*p*-dioxins, dibenzofurans, and biphenyls (PCDD/Fs and PCBs), as well as polybrominated diphenyl ethers (PBDEs) in freshwater fish from the Baltic countries (Zacs et al., 2013, 2016). Considerable political and scientific interest over the past decades has been attracted by halogenated flame retardants (HFRs), with a special emphasis on emerging brominated flame retardants (EBFRs) (Iqbal et al., 2017). These compounds are used on a large scale in order to lower the flammability of materials used in electronic devices, plastics, polystyrene foams, textiles, paints, and other consumer materials (D'Silva et al., 2004). After restrictions imposed on the applications of PBDEs due to their adverse effects to the environment and human health (European Court of Justice, 2008) there has been a growing demand for alternate flame retardants, and therefore the usage of EBFRs has significantly increased. Nevertheless, these alternative BFRs also showed POP-like properties similar to those of PBDEs. The use and disposal of articles containing EBFRs resulted in their presence in various environmental samples, as confirmed by recent studies (Iqbal et al., 2017). Among the EBFRs, hexabromocyclododecane (HBCD) is one of the most widely used FRs in Europe (Covaci et al., 2006). However, due to its toxicity and the specific POP properties, legal and administrative measures were recently requested by the European Commission with regards to the production, use, import and export of this compound (Commission Regulation (EU) No 2016/293). A number of brominated chemicals have been recognized as EBFRs (e.g., 1,2-bis(2,4,6-tribromophenoxy)ethane (BTBPE) and decabromodiphenyl ethane (DBDPE)), although very scarce data is available about the occurrence of the majority of these compounds, and in order to implement effective strategies for the minimization of possible hazardous effects of these compounds, such information was requested by the relevant authorities (Commission Recommendation (EU) 2014/118/EU; EFSA, 2012). Other promising contenders expected to fill the vacant niche in the market of FRs after the prohibition of PBDEs were norbornene-based FRs, among which the most important representatives are Dechlorane Plus (DP), which is classified by the United States Environmental Protection Agency (US EPA) as a high production volume chemical (Ren et al., 2008), Dechlorane 602 (Dec 602), Dec 603, and Dec 604 (Shen et al., 2011a,b). However, compounds belonging to the family of dechlorane-related compounds (DRCs) were later found to be toxic and exhibiting the properties of POPs (Chen et al., 2017). Moreover, recent studies reflect a notable increase of DRC levels in the environment, therefore attracting the attention of scientists in the field of environmental protection. (Li et al., 2014; Liu et al., 2016).

Aquatic biota serves the role of a versatile indicator that can accumulate POPs and thus reflect the overall contamination status of the environment. The European eel (*Anguilla anguilla*) is a carnivorous, catadromous fish, which is widely distributed throughout Europe. Eel absorbs and concentrates the bioaccumulative organic pollutants that are present in low concentrations in its diet consisting of crustaceans, worms, snails, larvae, and small fish. For these reasons, eels have long been considered as a bioindicator species that can point to the contaminants present in local habitats (Malarvannan et al., 2014; Roosens et al., 2010; Santillo et al., 2005). The occurrence of FRs in eels has been documented in a number of publications (Bragigand et al., 2006; Malarvannan et al., 2014; McHugh et al., 2010; Roosens et al., 2010; Suhring et al., 2013, 2014, 2015; Ten Dam et al., 2012; Zacs et al., 2016). While the levels of legacy BFRs like PBDEs in eels from Latvian lakes have been recently reported (Zacs et al., 2016), there are no data on the occurrence of EBFRs and DRCs in inland aquatic biota in Latvia. Such information is of great importance for the assessment of contamination status of the Latvian environment, complementing the characterization of the overall situation regarding POPs in European environment and providing an insight into the bioaccumulation and persistence trends of these relatively new compounds in such bioindicator species as the European eel. Therefore, this study aims to investigate the concentration of EBFRs and DRCs in eels sourced from five Latvian freshwater lakes selected to representatively cover the geography of the region and to compare these results to the levels of the same pollutants in eels from other European countries.

2. Materials and methods

2.1. Sample collection and storage

Fifty eight eel (*Anguilla anguilla*) specimens of various length and weight were caught in Latvian freshwater lakes during the period from September 2013 to May 2014 at the 5 locations shown in Fig. 1. These locations were carefully selected to evenly cover all essential eel stocks in the Latvian territory and also to have a maximum variation in body weight and length. At least five eel specimens were collected to represent each sampling site. The samples were packed in polyethylene bags, uniquely coded, and stored with ice during delivery to the laboratory. The average length and weight of eels were 76 cm (ranging from 39 to 101 cm) and 1.0 kg (ranging from 0.10 to 1.87 kg). The age of eel specimens was determined at the National Marine Fisheries Research Institute (Gdynia, Poland) by applying otolith image analysis. The age was established only for 60% of the collected specimens and was found to be in the range from 11 to 30 years. The lipid content of samples was determined within the scope of a previous study (Zacs et al., 2016) and it was in the range from 20 to 41%. The specimens were dissected, musculature (including subcutaneous fat) was isolated and homogenized using a food blender (Kenwood FP101T, Kenwood Ltd, UK), and the homogenates were packed into polyethylene bags and stored at −18 °C until analysis.

2.2. Chemicals and materials

Standard solutions of individual $^{13}C_{12}$-labeled α-, β-, and γ-HBCD diastereomers, native Dec 602, *syn*-DP, *anti*-DP, and their isotopically labeled surrogates $^{13}C_{10}$-Dec 602, $^{13}C_{10}$-*syn*-DP, $^{13}C_{10}$-*anti*-DP, as well as $^{13}C_{12}$-PCB-194 were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Certified standards of target FRs, namely, α-HBCD, β-HBCD, γ-HBCD, (2,3-dibromopropyl) (2,4,6-tribromophenyl) ether (TBP-DBPE), hexabromobenzene (HBB), 2-ethylhexyl 2,3,4,5-tetrabromobenzoate (EH-TBB), 1,2-bis(2,4,6-tribromophenoxy)ethane (BTBPE), decabromodiphenyl ethane (DBDPE), hexachlorocyclopentadienyldibromocyclooctane (DBHCTD) and tetradecabromo-1,4-diphenoxybenzene (TDBDPB) were supplied by AccuStandard, Inc. (New Haven, CT, USA). The DP derivatives decachloropentacyclooctadecadiene (Cl10DP) and undecachloropentacyclooctadecadiene (Cl11DP) were supplied by Wellington Laboratories (Guelph, ON, Canada), while Mirex, Dec 603, and Dec 604 were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Stock solutions were prepared in toluene or in

Fig. 1. Distribution of sampling locations throughout Latvia.

toluene/acetonitrile mixture and were stored at −18 °C in amber colored glassware. High performance liquid chromatography (HPLC) grade methanol, pesticide grade *n*-hexane, dichloromethane (DCM), toluene, cyclohexane, ethyl acetate, and Florisil for column chromatography were purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland). Sulfuric acid and sodium sulfate were obtained from Acros Organics (Morris Plains, NJ, USA). Solid phase extraction (SPE) cartridges filled with 500 mg of acidic silica gel were prepared manually using 6 mL SPE cartridges from Phenomenex (Torrance, CA, USA), while Bio-Beads SX3 (200–400 mesh) sorbent was obtained from Bio-Rad (Philadelphia, PA, USA). Calibration solutions for GC-MS analysis of DRCs were prepared by serial dilution of stock solutions in toluene, whereas for the LC-MS analysis of EBFRs the respective solutions in toluene were evaporated to dryness under gentle nitrogen stream and reconstituted in methanol.

2.3. Sample preparation and clean-up

The sample preparation procedures for the analyses of EBFRs and DRCs were performed separately. The eel homogenate samples (2 g each) were freeze-dried and spiked with 100 μL of ^{13}C-isotopically labeled surrogates (concentrations of the respective surrogates were 20 pg mL^{-1} for DRCs and 50–500 pg mL^{-1} for EBFRs) prior to a hot extraction procedure according to the method described by Zacs and Bartkevics (2015).

2.3.1. Clean-up procedure for the analytes of EBFR group

For the analysis of EBFR group that included HBCDs, TBP-DBPE, HBB, EH-TBB, BTBPE, DBDPE, and TDBDPB, the sample extract was treated according to the procedure outlined by Zacs et al. (2014a). In brief, the fatty residue obtained after hot extraction was reconstituted in *n*-hexane/DCM mixture, treated with sulfuric acid and acidic silica gel, and subjected to fractionation using a Florisil column. After washing of the column with *n*-hexane, the analytes of interest were eluted with *n*-hexane/DCM mixture, the organic solvents were evaporated, and the final extracts were reconstituted in methanol prior to LC-Orbitrap-MS analysis.

2.3.2. Clean-up procedure for the analytes DRC group

Analysis of the DRCs (Mirex, *syn*-DP, *anti*-DP, DBHCTD, Cl10DP, Cl11DP, Dec 602, Dec 603 and Dec 604) was performed using the method described by Rjabova et al. (2018), modified by including acidic silica gel in the clean-up protocol. After the hot extraction procedure, the bulk of high molecular mass compounds were removed by using gel permeation chromatography (GPC) on a column filled with Bio-Beads SX3 (Bio-Rad, Philadelphia, PA, USA) stationary phase and cyclohexane/ethyl acetate (1:1, v/v) mixture as eluent. The eluents obtained after GPC procedure were evaporated, reconstituted in of *n*-hexane (~0.5 mL), and applied on the top of the SPE cartridge filled with 500 mg of acidic silica gel. The DRCs were eluted from the column with 10 mL of *n*-hexane/DCM mixture (1:1, v/v), the solvents were evaporated under a gentle stream of nitrogen and the obtained residue was reconstituted in 50 μL of the recovery standard $^{13}C_{12}$-PCB-194 solution in toluene prior to the instrumental analysis.

2.4. Instrumental analysis

2.4.1. Instrumental analysis of EBFRs

The target analytes belonging to the EBFR group were determined using an HPLC-Orbitrap-MS system consisting of a Thermo Scientific UltiMate 3000 Rapid Separation system (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a Q Exactive Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a Thermo Scientific Ion Max atmospheric pressure chemical ionization/atmospheric pressure photo-ionization (APCI/APPI) interface. The Ion Max source was equipped with a Syagen PhotoMate vacuum UV light source (krypton discharge lamp, 10.0 eV) (Syagen Technology, Tustin, CA, USA). The APCI probe was used as a nebulizer-desolvation device without applying corona discharge. The detection of analytes was performed in negative

APPI mode by applying toluene as dopant, with the resolving power of Orbitrap MS equal to 17,500 (full width at half maximum (FWHM)). The HPLC separation of target compounds was carried out by using a Kinetex C18 analytical column (100 mm × 2.1 mm, 2.6 μm). The mobile phases consisted of methanol/water and methanol/toluene mixtures. Selected EBFRs were detected using the two most abundant fragments of the most intense ion cluster for each compound. Detailed information about the HPLC and Orbitrap MS conditions and characterization of the monitored ions can be found elsewhere (Zacs et al., 2016).

2.4.2. Instrumental analysis of DRCs

The analysis of DRCs was performed using a previously published gas chromatography – high resolution mass spectrometry (GC-HRMS) method (Rjabova et al., 2018). In brief, the measurements were performed on a Micromass AutoSpec HRMS system (Waters, Milford, MA, USA) coupled to an Agilent 6890 N gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with an Agilent DB-5MS capillary column (30 m × 0.25 mm, 0.1 μm). The split/splitless injector was operated in splitless mode, while helium was used as the carrier gas and electron ionization in positive mode (EI^+) with electron energy of 35 eV was applied for analyte ionization. The data acquisition was performed using selective ion monitoring (SIM) mode and the resolving power of HRMS was greater than 10,000 (at 10% valley definition). The analytes were detected using the two most abundant fragments of the most intense ion cluster for each compound.

2.5. Quality assurance/quality control

All stages of the analytical procedure, including sample extraction, purification, and handling of the final extracts were performed under conditions protected from UV light (i. e., using amber colored glassware or wrapping the glassware with aluminum foil). The quality control criteria for positive identification of analytes of interest included the retention time of the native compounds within a window of +3 to 0 s compared to the corresponding $^{13}C_{12}$-labeled surrogates for DRCs, and +5 to 0 s for EBFRs. The acceptable deviation of the isotopic ratio for two monitored ions (target/confirmation) was set as ±15% of the theoretical value for DRCs and ±25% of the value obtained at the calibration mid-point for EBFRs. Five-point calibration curves were used for the quantification of analyte concentrations in each sample run. Procedural blanks and quality control samples were included in the QC protocol on routine basis and were analyzed in each sample sequence. The concentrations of contaminants determined in real samples were corrected by taking into account the analyte concentrations found in procedural blanks. A list of average blanks is presented in the Supporting information. The quality control samples were in-house reference materials (naturally contaminated salmon tissue and fortified eel homogenate), and the results obtained for QC samples were in the range of 80–120% of the known fortification levels.

2.6. Statistics

Regression analyses, correlation analyses, principal component analysis (PCA), and graph construction were performed using Minitab 18 and Microsoft Excel 2013 software. Microsoft Excel 2013 was used to construct box plots for the data sets. The horizontal line within the box represents the median of the distribution. The ends of the box represent the 25th and 75th percentiles, while the interquartile range (IQR) is the difference between the 25th and 75th percentiles. The lines that extend from each end are whiskers, from the ends of the box to the outermost data point that falls within the distances computed as follows: 1st quartile − 1.5 × (IQR) 3rd quartile + 1.5 × (IQR).

3. Results and discussion

Detailed concentration data for the selected EBFRs and DRCs in the analyzed eel samples are provided in the Supporting Information. The current study deals with the actual levels of selected contaminants in eels from Latvian lakes, but does not provide human health risk assessment/dietary exposure estimation, where the worst case scenarios should be considered. Taking into account the significant differences between the lowerbound (LB) and upperbound (UB) values of the total contaminant concentrations ($\sum_{HBCD}$ and $\sum_{DRC}$), the LB concentrations were used as a more reliable indicator for the content of these compounds. The results were reported on a lipid weight (l.w.) basis. The length, weight, age (established only for 60% of the collected specimens), the lipid content, and the lake of origin were also included. The data obtained in this study is presented and compared to recent studies in Tables 1 and 2.

3.1. The levels of selected EBFRs

The current study reports data on the occurrence of seven EBFRs, namely, HBCD, DBDPE, TBP-DBPE, HBB, EH-TBB, BTBPE and TDBDP, which were prioritized considering the previous studies on the occurrence of EBFRs from other regions (Byer, 2006; Suhring et al., 2013, 2016) and the capacity of our analytical laboratory. Except for the scarce reports on HBCDs there is no information available about the occurrence of EBFRs in the environment of the Baltic states. Five out of the seven aforementioned EBFR analytes were not found in any of the eel samples above the LOQ (Supporting Information), with only HBCDs and DBDPE detected.

3.1.1. The levels and diastereomer profile of HBCDs

α-HBCD was found in all of the analyzed samples, confirming the ubiquitous distribution of HBCD in European aquatic environments (Law et al., 2014). The occurrence of HBCDs has been reported in Latvian biota before, however, the environmental data obtained were merely indicative since only a small number of samples and sampling places were considered (Zacs et al., 2014a,b). The sum of α-, β-, and γ-HBCD concentrations ($\sum_{HBCD}$) ranged from 0.05 to 6.58 ng g^{-1} l.w., with the average of 1.64 ng g^{-1} l.w. and the median value of 1.24 ng g^{-1} l.w. (Fig. 2), which was similar to those obtained in our previous study. As shown in Table 1, the determined levels of HBCD in European eel from other countries are significantly higher, with the highest average concentrations of 4500 ng g^{-1} l.w observed in eels from highly polluted areas in Belgium where a number of textile factories are located (Roosens et al., 2008). The pattern of HBCD diastereomers observed in the analyzed samples was typical for aquatic biota, with strongly pronounced predominance of α-HBCDs over β- and γ-HBCDs (Covaci et al., 2006). The main factors that may be responsible for this observation are: 1) enzymatic isomerization of β- and γ-HBCD to α-HBCD, as previously observed in fish (Lawet al., 2014); 2) α-HBCD has a higher water solubility (~49 μg L^{-1}) than β- and γ-HBCD (~2 μg L^{-1}), and thus is more readily available for uptake (Hunziker et al., 2004); 3) *in vitro* experiments with hepatic sub-cellular fractions obtained from rat and trout showed that the biotransformation of β- and γ-HBCD was approximately three times faster than that of α-HBCD (Abdallah et al., 2014); and 4) thermal isomerization of γ-HBCD incorporated in materials containing flame retardants to α-HBCD during the disposal of electronic waste, as well as photolytic conversion of γ- to α-HBCD (Harrad et al., 2009).

Table 1
Concentrations of EBFRs (ng g^{-1} l.w.) in eel from the current study and data from previous studies (expressed as minimum – maximum (mean) or mean value ± standard deviation).

Reference	Country	Fish	∑HBCD	HBB	DBDPE	EH-TBB	BTBPE	TBP-DBPE	TDBDPB
Present study	Latvia	European eel ($n = 58$)	0.05–6.58 (1.64) (0.01–1.92 (0.45))*	n.d.	n.d. – 33 (2.00) (n.d. – 9.06 (0.51))*	n.d.	n.d.	n.d.	n.d.
Zacs et al., 2014a	Latvia	European eel ($n = 8$)	0.22–2.49 (1.29)	–	–	–	–	–	–
Giulivo et al., 2017	Slovenia, Croatia, Bosnia and Herzegovina, Serbia	Freshwater fish ($n = 10$)	–	n.d. – 2.94	n.d.	–	–	–	–
Suhring et al., 2016	Germany	Dab ($n = 24$)	–	0.71	–	n.d.	–	11.8	–
		European eel ($n = 20$)	–	n.d.	–	n.d.	–	0.8	–
Suhring et al., 2013	Germany	European eel ($n = 45$)	–	–	–	–	–	0.67 ± 0.30	–
Santin et al., 2013	Spain	Freshwater fish ($n = 48$)	–	n.d.	n.d. - 130	–	–	–	–
Baron et al., 2013	Chile	Marine fish (Pooled samples)	–	n.d.	n.d.	–	–	–	–
Law et al., 2006	Canada	Walleye ($n = 5$)	–	–	n.d. – 2.71 (1.01)	–	n.d. – 0.84 (0.35)	–	–
		Whitefish ($n = 5$)	–	–	n.d.	–	0.026–0.22 (0.15)	–	–
		Emerald shiner ($n = 5$)	–	–	n.d. – 1.51 (0.30)	–	n.d. – 3.72 (0.95)	–	–
		Goldeye ($n = 3$)	–	–	n.d. – 1.63 (0.62)	–	0.19–0.45 (0.33)	–	–
		White Sucker ($n = 5$)	–	–	n.d. – 0.24 (0.08)	–	n.d. – 0.29 (0.13)	–	–
		Burbot ($n = 5$)	–	–	n.d. – 3.30 (0.66)	–	n.d. – 1.48 (0.79)	–	–
Ismail et al., 2009	Canada	Lake trout ($n = 29$)	–	–	–	–	1.90 ± 0.30	–	–
Houde et al., 2014	Canada	Yellow perch ($n = 29$)	–	n.d.	n.d.	–	–	–	–
		Muskellunge ($n = 10$)	–	1.4–3.9	n.d.	–	–	–	–
Byer, 2006	Canada, USA	American eel ($n = 60$)	–	0.004 ± 0.004	–	–	0.088 ± 0.071	–	–
Widelka et al., 2016	USA	Common carp (Pooled samples)	–	n.d. – 90 (5.8)	–	n.d. – 73 (1.2)	–	–	–
		Largemouth bass (Pooled samples)	–	n.d. – 286 (3.2)	–	n.d. – 225 (1.5)	–	–	–
He et al., 2012		Mud carp ($n = 9$)	–	–	n.d. – 64 (35)	–	–	–	–
		Nile tilapia ($n = 15$)	–	–	n.d. – 190 (37)	–	–	–	–
		Plecostomus ($n = 10$)	–	–	n.d. – 230 (68)	–	–	–	–
Wu et al., 2010	China	Mud carp ($n = 12$)	–	2451 ± 778	338 ± 171	–	518 ± 277	–	–
		Crucian carp ($n = 18$)	–	680 ± 158	14.0 ± 14.0	–	323 ± 315	–	–
		Snakehead ($n = 6$)	–	1153 ± 470	n.d.	–	1.71 ± 1.11	–	–
Shi et al., 2009	China	Carp ($n = 1$)	–	–	n.d.	–	0.150	–	–
		Bighead ($n = 2$)	–	–	n.d.	–	0.014–0.083	–	–
		Tilapia ($n = 2$)	–	–	n.d.	–	n.d. – 0.024	–	–
Roosens et al., 2010	Belgium	European eel ($n = 429$)	16–4397 (394)	–	–	–	–	–	–
Roosens et al., 2008	Belgium	European eel ($n = 10$)	390–12100 (4500)	–	–	–	–	–	–
Morris et al., 2004	Belgium	European eel ($n = 190$)	n.d. – 266 (43)	–	–	–	–	–	–
Santillo et al., 2005	Czech republic	European eel ($n = 4$)	40	–	–	–	–	–	–
Santillo et al., 2005	France	European eel ($n = 10$)	62	–	–	–	–	–	–
Santillo et al., 2005	Germany	European eel ($n = 20$)	93	–	–	–	–	–	–
Santillo et al., 2005	Ireland	European eel ($n = 10$)	10	–	–	–	–	–	–
Santillo et al., 2005	Italy	European eel ($n = 7$)	66	–	–	–	–	–	–
Santillo et al., 2005	The Netherlands	European eel ($n = 6$)	54	–	–	–	–	–	–
Santillo et al., 2005	Spain	European eel ($n = 9$)	84	–	–	–	–	–	–
Santillo et al., 2005	Belgium	European eel ($n = 4$)	24	–	–	–	–	–	–
Santillo et al., 2005	Poland	European eel ($n = 5$)	25	–	–	–	–	–	–
Van Leeuwen and De Boer, 2008	The Netherlands	European eel ($n = 14$)	n.d. – 5060 (1379)	–	–	–	–	–	–
Malarvannan et al., 2014	Belgium	European eel ($n = 443$)	7–9500 (510)	–	–	–	–	–	–
Ten Dam et al., 2012	The Netherlands	European eel ($n = 34$)	0.1–134	–	–	–	–	–	–
McHugh et al., 2010	Ireland	European eel ($n = 49$)	7.4–166	–	–	–	–	–	–

"*" - values below the limit of detection are labelled "not detected" (n.d.).
"–" – not included in the study.
()* – concentration data from this study recalculated on a fresh weight basis (ng g^{-1}).

Table 2
The concentrations of DRCs (ng g^{-1} lipid weight) in eel from the current study and data from other studies (data are expressed as minimum – maximum (mean) values).

Reference	Country	Fish	Mirex	Dec-602	DBHCTD	Dec-603	Cl10-DP	DP-syn	Cl11-DP	DP-anti	Dec-604
Present study	Latvia	European eel ($n = 58$)	n.d. – 0.22 (0.06) (n.d. – 0.05 (0.01))*	n.d. – 1.60 (0.25) (n.d. – 0.38 (0.06))*	n.d. – 0.27 (0.01) (n.d. – 0.06 (0.002))*	n.d. - 0.10 (0.01) (n.d. – 0.03 (0.003))*	n.d. – 0.06 (0.01) (n.d. – 0.02 (0.003))*	n.d. – 0.45 (0.06) (n.d. – 0.11 (0.02))*	n.d. – 0.14 (0.01) (n.d. – 0.04 (0.004))*	n.d. – 0.89 (0.20) (n.d. – 0.20 (0.05))*	n.d.
Zacs et al., 2016	Latvia	Baltic salmon ($n = 25$)	3.42–28.0 (11.1)	0.13–0.88 (0.37)	(0.89–3.83) 2.19	n.d. - 0.04 (0.04)	n.d.	n.d. – 0.59 (0.09)	n.d.	n.d. – 0.76 (0.16)	n.d.
Suhring et al., 2013	Germany	European eel ($n = 45$)	–	n.d. - 49.0 (1.17)	–	n.d. - 0.34 (0.01)	n.d.	n.d. – 27.0 (0.59)	n.d.	n.d. – 7.20 (0.18)	–
Suhring et al., 2015	Germany	European eel ($n = 16$)	–	–	–	–	n.d. - 0.04 (0.01)	0.01–0.38 (0.17)	n.d. - 0.10 (0.04)	n.d. – 0.97 (0.38)	–
Giulivo et al., 2017	Greece	Freshwater fish ($n = 4$)	–	n.d.	–	n.d.	–	n.d	–	n.d	n.d.
	Slovenia, Croatia, Bosnia and Herzegovina, Serbia	Freshwater fish ($n = 10$)	–	n.d.	–	n.d.	–	0.80–2.10 (0.51)	–	1.14–2.50 (0.77)	n.d.
	Italy	Freshwater fish ($n = 13$)	–	n.d. – 17.4 (2.60)	–	n.d.	–	n.d.	–	n.d.	n.d.-8.99 (2.07)
Kang et al., 2010	South Korea	Freshwater fish ($n = 22$)	–	–	–	–	–	0.17–30.0 (8.08)	–	0.44–97.0 (16.9)	–
Zhang et al., 2011	China	Northern snakehead ($n = 10$)	–	–	–	–	n.d.	(39.3)	(4.49)	(46.1)	–
		Mud carp ($n = 10$)	–	–	–	–	(0.85)	(15.4)	(0.85)	(18.8)	–
Suhring et al., 2016	Germany	Dab ($n = 24$)	–	n.d.	–	n.d.	n.d.	0.45–3.00 (1.71)	0.23–0.76 (0.57)	n.d.	n.d.
Strandberg et al., 1998	Sweden	Baltic herring ($n = 6$)	0.48–1.40	–	–	–	–	–	–	–	–
	Sweden	Perch ($n = 9$)	n.d. – 1.40	–	–	–	–	–	–	–	–
	Poland	Baltic herring ($n = 1$)	n.d.	–	–	–	–	–	–	–	–
	Poland	Perch ($n = 2$)	n.d.	–	–	–	–	–	–	–	–

*- values below the limit of detection are labeled "not detected" (n.d.).
"–" – not included in the study.
()*– concentration data from this study recalculated on a fresh weight basis (ng g^{-1}).

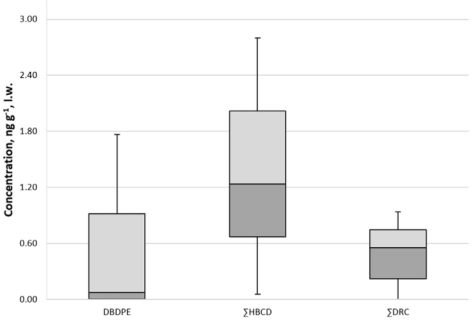

Fig. 2. Box plots of the concentrations (ng g^{-1} l.w.) of selected FRs in the analyzed eel samples.

3.1.2. The occurrence of TDB-DPE

No reports are available on the occurrence of TDB-DPE in aquatic biota and only limited information has been published on the usage volumes of TDB-DPE and its possible emissions. The non-detection of this chemical in the analyzed samples does not prove its absence in the environment, and probably could be explained with its high molecular weight resulting in low bioavailability. Nevertheless, the possible risks posed by the emissions of this compound into the environment remain, as some authors suggest that TDB-DPE undergoes chemical decomposition by photolysis to more bioavailable and persistent debromination products (Chen et al., 2013).

3.1.3. The occurrence of EH-TBB

A significant share of the EBFR market has been filled by EH-TBB, which was suggested as a "penta-BDE" replacement since its ban in Europe in 2004. The data on the occurrence of EH-TBB in Latvian lakes was in good agreement with the results from Germany, since in both studies (Suhring et al., 2016; Zacs et al., 2016) this chemical was not detected in eel samples. The lack of EH-TBB in eel muscle could either be an indication that this compound is not readily taken up by the fish, quickly metabolized, or that it is stored in other tissue types rather than muscle (Suhring et al., 2016). However, the presence of EH-TBB was reported in freshwater fish from Canada where the observed levels were up to 1.50 ng g^{-1} l.w. (Widelkaet al., 2016).

3.1.4. The occurrence of HBB

Regarding the occurrence of HBB in eels, our findings were in line with the German study in which HBB was not detected in eel tissue (Suhring et al., 2016), whereas the same study reported that the concentration of HBB in dabs was as high as 0.71 ng g^{-1} l.w. and the authors claimed that the concentration of HBB in dabs strongly correlated with the contamination level in sediments. Eels, however, despite being bottom-dwelling fish, are unsuitable as indicators for the POP pollution in sediments, because of the high intake of contaminants through food chain (Suhring et al., 2016). Currently no other studies are available regarding the levels of HBB in eels, while the detection of HBB in other fish species in Europe is not very common (see Table 1). This might be due to the fact that HBB was extensively used as a flame retardant additive in Asia, but no reports of production in Europe are available. It could be also supported by the high concentrations of HBB in fish from South China – up to 2451 ± 778 ng g^{-1} l.w., in samples collected from a natural pond near an e-waste recycling site (Wu et al., 2010) affirming the bioaccumulation potential of HBB.

3.1.5. The occurrence of BTBPE

Considering that BTBPE is classified as low production volume chemical in Europe (EFSA, 2012) and the generally low BFR background concentrations found in the Latvian environment (Zacs et al., 2016), the absence of this compound in our analyzed eels was not surprising. Previous studies reported relatively low BTBPE concentrations of 0.09 ± 0.07 ng g^{-1} l.w. in American eels (Byer, 2006), while the highest levels of this BFR were observed in fish from highly contaminated areas in South China, reflecting the bioaccumulation properties of this compound (Table 1).

3.1.6. The levels of DBDPE

In our study, DBDPE was found in 59% of the eel samples (34 out of 58 samples), showing a broad range of determined concentrations from 0.04 and 33 ng g^{-1} l.w., with an average of 2.00 ng g^{-1} l.w. and the median value of 0.07 ng g^{-1} l.w. (Fig. 2). According to the available references, DBDPE was not detected in various fish samples from St. Lawrence river, Canada and coastal waters of Chile (Baron et al., 2013; Houde et al., 2014), while the levels reported for samples originating from a highly polluted river in South China and various rivers in Spain showed concentrations up to 230 and 130 ng g^{-1} l.w., respectively (He et al., 2012; Santin et al., 2013). To our knowledge, there are no reports on the occurrence of DBDPE in eels until now. In a study by Santin et al. in 2013, eels were included in the scope of the analysis, although the results were presented for the pool of musculature from different fish species. DBDPE was introduced as a BFR in the mid-1980s and became commercially significant as an alternative to the BDE-209 based "Deca-BDE" formulations (Cruz et al., 2015; Kierkegaard et al., 2009). Despite being listed as a low production volume chemical (between 10 and 1000 tons per year) in the European Union, it has been the second most frequently used BFR in China (Shi et al., 2009). Due to the structural similarity of BDE-209 and DBDPE and the similar types of applications, some relationships between their concentrations in eel musculature could be expected. However, considering the concentrations of BDE-209 in our analyzed eel samples (Zacs et al., 2016), no significant correlation was observed between the levels of both compounds ($r_s = -0.011$; p-value = 0.933). The obtained data were in agreement with a recent study where the amounts of DBDPE were higher than those of BDE-209 in ambient air samples from southern Sweden, although the concentrations did not correlate, indicating different sources of these two compounds (Egeback et al., 2012). Taking into account the high heterogeneity of the observed concentrations, the main contamination pathways of DBDPE are still unknown. Contamination with DBDPE may originate both from point sources and long-range transport, since studies of BDE-209 have revealed that long-range transport in the air leads to contamination of distant regions. Due to their physicochemical similarity, this could be true for DBDPE as well (Cruz et al., 2015).

3.2. Levels of selected DRCs

3.2.1. Individual DRCs

Nine DRC representatives, including Mirex, *syn*-DP, *anti*-DP, DBHCTD, Cl10DP, Cl11DP, Dec 602, Dec 603, and Dec 604 were included in the scope of this study. Considering the whole data set, Dec-602 was the main contaminant among the individual DRCs, showing concentrations in the range of 0.05–1.60 ng g^{-1} l.w., with an average of 0.28 ng g^{-1} l.w. and approximately 40% contribution to the total DRC concentration ($\sum_{DRC}$). The concentrations of *syn*-DP and *anti*-DP in the analyzed samples ranged from n.d. to 0.45 ng g^{-1} l.w., and from n.d. to 0.89 ng g^{-1} l.w., respectively, with the

average concentration of 0.14 ng g^{-1} l.w., and 0.24 ng g^{-1} l.w., respectively. The contribution of *anti*-DP to the $\sum_{DRC}$ was approximately 33%, while *syn*-DP accounted for approximately 10% of the $\sum_{DRC}$ on average. Mirex was found in almost all of the samples, reaching 0.06 ng g^{-1} l.w. on average (ranging from n.d. to 0.22 ng g^{-1} l.w). Only few of the samples showed quantifiable concentrations of Dec-603 and DBHCTD, while Dec-604 was not found in any of the samples, which was in line with the previous studies, reflecting the fact that these chemicals are minor DRCs among the selected norbornene FRs in fish. Dec-602, Dec-603, and Dec-604 were produced during the last half-century (Shen et al., 2011a; Guerra et al., 2011; Wang et al., 2011), but no information is available on the producers or importers, as well as on the volumes of these chemicals used within the EU, therefore the evaluation of currently observed results in context of environmental hazards is very complicated. Despite the generally low concentrations of DRCs found in the analyzed eels in comparison with those obtained from other EU countries (Table 2), the observed fractional abundance of Dec-602 within the selected DRCs was consistent with previous studies (Suhring et al., 2013). The unexpected predominance of Dec-602 concentrations in eels, however, could be explained with the significantly higher bioaccumulation potential of this compound in comparison with other DRC representatives (the biota-sediment accumulation factor (BSAF) of Dec-602 is nearly 500 times higher than that of DP) (Shen et al., 2011b). The concentrations of DP found during this study were lower than those observed in eels and other fish species from EU countries, while the available data show that the DRC contamination in fish from Europe in general is significantly lower than in South Korea and China (Kang et al., 2010; Zhang et al., 2011).

3.2.2. Dechlorinated DP products

A notable observation was the occurrence of the dechlorinated DP analogs Cl10DP and Cl11DP in our analyzed eels, pointing to a probable transformation of DPs. However, the source of these DP derivatives in living organisms is still not entirely clear, as the possible presence of *anti*-Cl11DP in bottom-feeding fish is more closely associated with the bioaccumulation processes from the environment, rather than *in vivo* biotransformation from the parent compound (Li et al., 2014; Zhang et al., 2011). Such assumptions could be verified by exploring the distribution of DPs and its dechlorinated analogues in the tissues of snakehead, where a noticeably higher ratio of *anti*-Cl11DP to *anti*-DP was found in liver compared to the muscle, reflecting a possible species-specific hepatic dechlorination of anti-DP (Zhang et al., 2011).

3.2.3. The DP isomer profile

For characterization of the stereoselective distribution of DP isomers among various environments or types of species, the fractional abundance of one of the DP isomers expressed as a ratio of concentrations of the predominant isomer versus the sum of both isomers ($\sum_{DP}$) was proposed as a comparative criterion (Suhring et al., 2013; Sverko et al., 2010). Contrary to the previous studies on European and American eels, where *syn*-DP was found to be predominant in musculature over *anti*-DP isomer (Suhring et al., 2015, 2016), in almost all of the samples included in our study *anti*-DP predominated over *syn*-DP and therefore the f_{anti} values were calculated for the analyzed samples. Only for the samples in which both isomers were detected, f_{anti} values were calculated and subsequently considered in the calculations of mean f_{anti} values for the analysed samples. The f_{anti} values were found to be in the range of 0.48–0.88 with the average of 0.64, and these observed values agreed with the f_{anti} of 0.69 for five different fish specimens from South China reported by Kang et al. (2010), as well as with other studies (Kakimoto et al., 2012; Peng et al., 2014; Zhang et al., 2011). Similar results were obtained in a study on the isomeric distribution of DP in muscle, liver, and brain tissues of northern snakehead and mud carp, which showed the enrichment of *anti*-DP with the f_{anti} values ranging from 0.51 to 0.80, whereas higher affinity for *anti*-DP was determined in brain tissues (f_{anti} of 0.80 for northern snakehead and 0.71 for mud carp) (Zhang et al., 2011). Therefore, the observed distribution of DP isomers in eel tissue could reflect the probable contamination source from OxyChem® DP commercial product, in which the f_{anti} value was found to be in the range from 0.64 to 0.80 (Hoh et al., 2006; Tomy et al., 2007; Wang et al., 2010, 2011; Shen et al., 2011a).

3.3. Biological parameters vs FR concentrations

Similarly to our previous study on the content of POPs in eels (Zacs et al., 2016), no pronounced correlations were observed between the levels of HFRs and age, length, or weight of eels (Spearman rank correlation p-values > 0.05). The grouping of the samples according to length, age, or weight and the application of

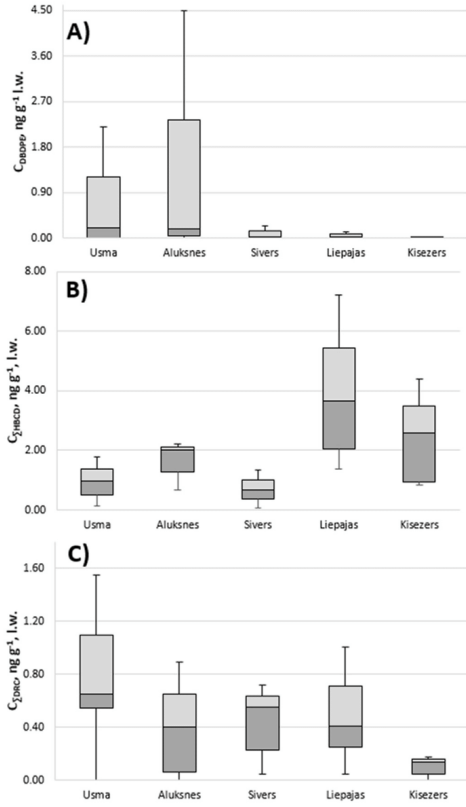

Fig. 3. Box plots of the concentrations (ng g^{-1} l.w.) of selected FRs in the analyzed eel samples from different Latvian lakes: (A) – DBDPE; (B) – $\sum_{HBCD}$; (C) – $\sum_{DRC}$.

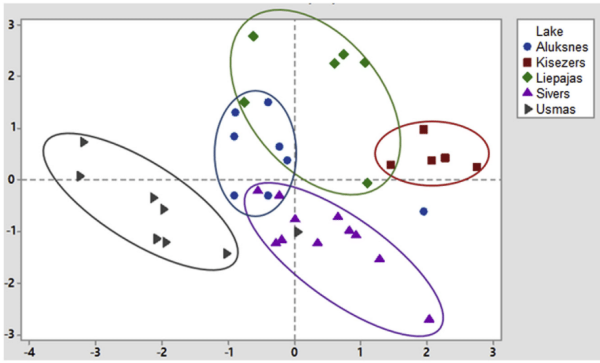

Fig. 4. Score plot of principal component analysis of HBCD concentrations and biological parameters.

T-test for data treatment did not reveal statistically significant differences between the sample groups (p-values > 0.05).

3.4. Geographical variation

Taking into account that the number of samples used in our study was relatively low for some of the sampling locations and certain variables are not known for several samples (e.g., the age of fish specimens), detailed statistical analysis of the whole dataset is problematic. According to the previously published geographical trends of POPs in Latvian lakes (Zacs et al., 2016), the highest PBDEs concentration range in eels were observed in Lake Kisezers and Lake Liepajas, since they are both located near industrialized areas. The box plot of concentrations of DBDPE, $\sum_{HBCD}$, and $\sum_{DRC}$ grouped by the sampling locations of the current study are shown in Fig. 3. The highest concentrations of HBCD were observed in eels from Lake Liepajas and Lake Kisezers corresponding to industrialization of the areas and reflecting similar geographical trend as for PBDEs (Zacs et al., 2016). However, relatively high levels were also observed in Lake Aluksnes, which is located outside the industrialized areas of Latvia, indicating unknown sources of contamination, probably historical wastewater influx into the lake or municipal waste disposal in the surrounding area. As discussed in the previous chapter, DBDPE and HBCD concentrations in our analyzed eels did not reveal significant correlations, showing the highest DBDPE concentrations in eels from Lake Usmas and Lake Aluksnes, while the specimens from Lake Kisezers, Lake Sivers, and Lake Liepajas contained significantly lower DBDPE concentrations. Therefore, the pattern of lower contamination in lakes near the Baltic Sea and higher contamination in inland lakes with poor water exchange has been observed for DBDPE. Considering the DRC data observed for the analyzed eel samples, there were no exceptional deviations in the levels of these contaminants within the selected lakes, reflecting nearly constant background concentrations for this group of contaminants.

In order to evaluate the effects of geographical distribution of selected HFR levels and to assess the possible data clustering related to the sampling locations, principal component analysis (PCA) was applied for the treatment of the raw data while considering the biological parameters and contaminant concentrations in the analyzed tissues of eels. The results of PCA showed that only the levels of HBCD depended on the sampling location (Fig. 4), whereas no data clustering was observed on the geostatic map for other contaminants, confirming the random distribution of DBDPE and DRC concentrations in samples from different locations (data not shown). It is well known that the use of HFR-containing materials is a major source for the influx of these compounds into the environment. While there are no electronic waste processing facilities and no large scale waste incinerators in Latvia that are known to be significant emitters of BFRs into the environment (Liagkouridis et al., 2014), it can be presumed that the main sources of pollution could be uncontrolled disposal of HFR-containing waste, atmospheric emissions through small scale incineration, atmospheric deposition of these compounds from more industrialized areas or pollution through the rivers, which are affected by industrial effluents from upstream factories beyond the Latvian territory.

Taking into account the environment quality standard (EQS) criteria developed for the assessment of environmental pollution (Directive (EU) No 2008/105/EC), the observed results indicate acceptable environmental status of Latvian lakes with regard to the content of HBCD, showing the actual concentrations of this chemical in the samples more than a hundred times lower than the EQS criteria for biota established by the Directive 2013/39/EU. At the same time, it should be pointed out that the EQS values established for biota are criticized in some studies and could not provide objective estimates in some cases (Jurgens et al., 2013). Despite the generally low concentrations of HFRs observed in this study as well as the absence of identified contamination hotspots and significant sources of HFR influx throughout the territory of Latvia, the observed clustering of HBCD data could indicate non-uniform contamination of the Latvian environment and, therefore, the possible presence of some areas with elevated levels of BFRs. Thus, it is advisable to continue the efforts to identify such areas where the protective measures and legislative initiatives on the reduction of the presence of HFRs in the environment should be focused.

4. Conclusions

In order to evaluate the contamination status of Latvian aquatic environment, samples of European eel from five Latvian lakes were analyzed for the content of EBFRs and DRCs. For the first time, data on these contaminants are presented for inland aquatic biota from the Baltic region. Among the selected EBFRs, only HBCD and DBDPE were found in the analyzed samples, whereas Dec-602 was the predominant compound in DRC group. The fractional abundance of

syn- and *anti*-DP stereoisomers showed pronounced enrichment of *anti*-DP, which is close to the composition of OxyChem® DP commercial product, reflecting a probable contamination source from releasing of DP from disposal of consumer products. The concentrations of HFRs observed in the current study were lower than in previous studies on aquatic biota from Europe and Asia. The environmental status of Latvian lakes with regards to the contamination with HBCD is acceptable according to the environment quality standard (EQS) criteria stated in the Directive 2013/39/EU. Considering the data available in reports from other regions, it can be concluded that the aquatic biota in Latvia reflect a lower degree of environmental contamination.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.chemosphere.2018.01.105.

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III

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Occurrence of polybrominated diphenyl ethers, perfluorinated compounds, and nonsteroidal anti-inflammatory drugs in freshwater mussels from Latvia

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Occurrence of polybrominated diphenyl ethers, perfluorinated compounds, and nonsteroidal anti-inflammatory drugs in freshwater mussels from Latvia

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HIGHLIGHTS

- Occurrence of PBDEs, PFCs, and NSAIDs in freshwater mussels from Latvia was evaluated.
- The levels of PBDEs in 83% of the samples exceeded the EQS set in the EU.
- The levels of PFCs in Latvian mussels were generally lower than observed elsewhere.
- Ibuprofen was the only NSAID detected in mussels.

GRAPHICAL ABSTRACT

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ABSTRACT

The occurrence of polybrominated diphenyl ethers (PBDE), perfluorinated compounds (PFC), and nonsteroidal anti-inflammatory drugs (NSAID) in Latvian freshwater ecosystems was evaluated by using filter-feeding mussels as bioindicators. Twenty four samples of mussels were collected from freshwater bodies throughout the territory of Latvia during the summer of 2017. PBDE contamination was ubiquitous, reaching the highest total concentration of 193.2 pg g^{-1} w.w. BDE-209 was the most abundant compound, followed by penta-BDE components BDE-49, -100, −99, −153, −154, and −47 in decreasing order. The levels of PFCs in Latvian mussels were generally lower than those reported from other regions. Perfluorooctanoic acid (PFOA) was more common in mussels than perfluorooctane sulfonate (PFOS). Ibuprofen was the only NSAID detected in mussels during this study (detection frequency 50%). The observed concentrations of this compound varied between 0.52 and 109 ng g^{-1} w.w., being noticeably higher than reported by other authors. Overall, the results indicate that among the three analysed groups of contaminants, ibuprofen is present in Latvian freshwater environment at relatively high levels and further monitoring should be carried out.

1. Introduction

Several characteristics of freshwater mussels make them suitable as an indicator organism for the occurrence of chemical contamination in the environment (Bedford et al., 1968). They are

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sessile, thus providing location-specific information. Furthermore, mussels are filter-feeders that mainly consume phytoplankton by pumping and filtering large volumes of water. This water filtration behaviour also enables them to effectively accumulate chemical pollutants from water, thereby providing an integrative measure of the concentration and bioavailability of water pollutants. As lower members of the aquatic food chain, they transfer anthropogenic pollutants from the abiotic phase and the primary production level to the higher trophic levels in the food chain, such as mussel-eating invertebrates, birds, and mammals (Beyer et al., 2017). Due to these qualities, bivalve organisms have been used as bioindicators for nearly 50 years (Farrington et al., 2016). The range of contaminants analysed in mussels ranges from "priority pollutants" like heavy metals and halogenated hydrocarbons to chemicals of emerging concern like flame retardants, surfactants, pharmaceuticals, and even drugs of abuse (Beyer et al., 2017; Cunha et al. 2005, 2017; de Solla et al., 2016). In this study, a wide range of contaminants was selected, including brominated flame retardants (PBDEs), surfactants (PFCs) and pharmaceuticals (NSAIDs). To our knowledge, no data on the contamination status of mussels from Baltic inland waters have been published before.

Flame retardants are a group of chemicals that are used to prevent fire incidents. PBDEs were one of the most frequently used groups of flame retardants until the mid-2000s when strict regulations were imposed on the production of these compounds (2003/11/EC, 2002/95/EC, and 2005/717/EC). Due to the high volumes of production, widespread use in the past, as well as their hydrophobicity (lipophilicity) and persistence, PBDEs have become ever-present contaminants in the environment and their occurrence has been confirmed in various environmental media including soil, air, water and sediment samples, as well as humans and biota (Akortia et al., 2016). As a result, their occurrence in various biotic matrices, including mussels, has been studied (Beyer et al., 2017; Vandermeersch et al., 2015). PBDE contamination status in Latvia has been previously reported for freshwater fish samples and significant concentrations were discovered (Zacs et al., 2016). PBDE concentrations in eel samples were above the environmental quality standard (EQS) stated in Directive 2013/39/EU and further studies were recommended.

PFCs are widely used as surfactants in industrial and consumer products and as additives for fluoropolymer production (Ahrens, 2011). PFCs are extremely persistent, bioaccumulative and toxic, and globally distributed in the environment. The best known PFCs are perfluorosulfonates (PFOS) and perfluorocarboxylic acids (PFOA). Both classes of substances are recognised as widespread in the environment (Ahrens, 2011). Despite phasing out the production of long-chain perfluorocarboxylic acids in the United States, Western Europe and Japan, many PFCs are still released into the environment due to continuous production and use in other countries (OECD, 2015). PFCs have been reported in bivalves worldwide (Houde et al., 2006).

Pharmaceuticals are referred to as emerging pollutants, because their occurrence in the environment has been investigated relatively recently and their impact has not yet been fully explored. NSAIDs are some of the most often investigated and detected pharmaceuticals in water samples all over the world (Nödler et al., 2014). They are present in raw and treated wastewater (Nikolaou et al., 2007; Pugajeva et al., 2017), surface water, groundwater (Santos et al., 2010), and in drinking water (Caban et al., 2015). Nevertheless, data on the occurrence of NSAIDs in bivalves are still scarce. Taking into account that the European Commission has recently included diclofenac in the watch list of substances to be monitored in aquatic environments, data on the occurrence of NSAIDs are particularly relevant (2015/495/EU).

This study aims to investigate the contamination levels and spatial distribution of PBDEs, PFCs, and NSAIDs in the Latvian freshwater environment using mussels as bioindicators. The study provides new results on the contamination of mussels in Latvia by compounds of various classes, thus filling the gaps in knowledge about the occurrence of these compounds on a national scale. It also improves the general understanding about the levels and temporal trends of contaminants in Europe.

2. Materials and methods

2.1. Sample collection and storage

Freshwater mussels were collected from July to August 2017 at twenty four sampling sites evenly distributed over the territory of Latvia, as shown in Fig. 1. Batches consisting of three to one hundred and eighty organisms of various species including swollen river mussel (*Unio tumidus*), painter's mussel (*Unio pictorum*), duck mussel (*Anodonta anatina*), swan mussel (*Anodonta cygnea*) and zebra mussel (*Dreissena polymorpha*) were collected at each sampling site. The amount of the specimens of each species is detailed in Table S1. The mussels were kept after collection in water from the respective sampling location for 24 h in order to eliminate particulate matter from the digestive tract. Each specimen was then wrapped individually in aluminium foil, except for zebra mussels that were wrapped in foil in batches because of their small size. Mussels were frozen and stored at −18 °C. Once delivered to the laboratory, shells of the specimens were removed, soft tissue was isolated and homogenised using a food blender (Kenwood FP101T, Kenwood Ltd., UK). The homogenates were packed into polyethylene bags and stored at −18 °C until analysis.

2.2. Chemicals and materials

All of the solvents used in this study were at least of pesticide or HPLC purity grade. Solvents, acids and sorbents were purchased from Sigma–Aldrich (Buchs, Switzerland). Solid phase extraction cartridges were obtained from Phenomenex (Torrance, CA, USA). Deionised water was prepared with a Milli-Q (Millipore, Billerica, MA, USA) water purification system.

The following three groups of anthropogenic pollutants were analysed (the purity of the standards is given in parentheses; for compounds marked with asterisk (*), ^{13}C-labelled or deuterated surrogates were used as internal standards):

1) PFCs: PFOS*(98%) and PFOA*(95.5%)
2) Tri-through deca-PBDEs: IUPAC numbers 28*(99%), 47*(99%), 49 (99%), 99*(99%), 100*(100%), 138*(99%), 153*(99%), 154*(99%), 183*(99%), 209* (99%)
3) NSAIDs: tolfenamic acid*(99.7%), meloxicam*(98%), carprofen*(99.7%), flunixin*(99%), diclofenac*(99.5%), ibuprofen*(99.9%), phenylbutazone*(99%), ketoprofen (99.9%), mefenamic acid (98.5%).

The native standards for compounds listed above were obtained either from Dr. Ehrenstorfer (Augsburg, Germany), Sigma-Aldrich (St. Louis, MO, USA) or Cambridge Isotope Laboratories (Andover, MA, USA). Isotopically labelled standards were supplied either by Cambridge Isotope Laboratories, WITEGA Laboratorien (Berlin, Germany) or CDN Isotopes (Quebec, Canada).

2.3. Sample preparation and analysis

An aliquot of each sample was lyophilized in order to determine the water content. The results are presented in Table S1. Sample preparation and analysis was performed separately for each of the

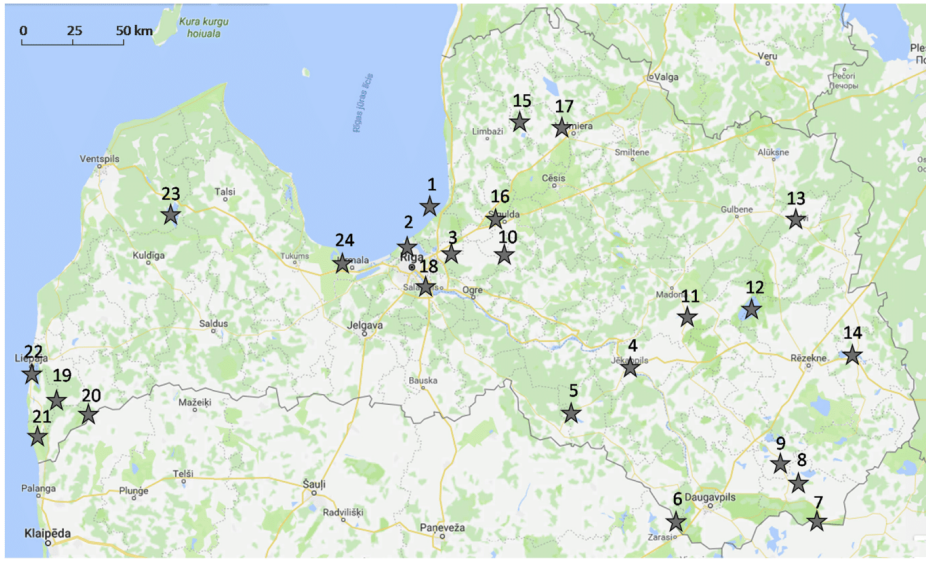

Fig. 1. The distribution of sampling locations throughout Latvia.

three contaminant groups. Detailed description of sample preparation and instrumental methods used in this study is provided in the Supplementary material.

2.3.1. Determination of the compounds of PFC group

The sample preparation procedure included ultrasound-assisted extraction with methanol and sodium hydroxide solution, followed by concentration of the analytes using solid phase extraction on a weak anion exchange cartridge. Instrumental analysis was based on high performance liquid chromatography coupled to Orbitrap high resolution mass spectrometry (HPLC-Orbitrap-HRMS) operated in selected reaction monitoring (SRM) mode, with detection of negative ions. The mass spectrometry conditions are presented in Table S2.

2.3.2. Determination of the compounds of PBDE group

The method for the determination of selected PBDEs was adopted from previously published studies (Zacs et al., 2016). Sample preparation consisted of Soxtec™ automatic extraction of freeze-dried samples with dichloromethane/*n*-hexane (1:1, v/v), cleanup procedure using various sorbents and, finally, treatment with sulfuric acid. Instrumental determination was performed using Agilent 6890N gas chromatograph (Santa Clara, CA, USA) coupled to a Micromass Autospec Premier high resolution mass spectrometer (Milford, MA, USA) by applying electron impact ionisation in positive ion mode (EI+) and selective ion monitoring (SIM) detection mode. Quantification was carried out using isotope dilution method by introducing $^{13}C_{12}$-labelled surrogates as internal standards. Detailed description of the instrumental analysis procedure for PBDEs can be found elsewhere (Zacs et al., 2016).

2.3.3. Determination of the compounds of NSAID group

For the analysis of NSAIDs, homogenised bivalve samples were extracted with acetonitrile and aqueous 0.02 M ascorbic acid solution. Concentration and purification of the analytes was achieved by solid phase extraction with Strata C18 cartridges. Instrumental separation and analysis of compounds was performed by LC-MS/MS using Acquity Ultra Performance liquid chromatography system (Waters, Milford, MA, USA) coupled to a QTrap 5500 (AB Sciex, MA, USA) equipped with an electrospray source operated in MRM mode with negative ionisation. Detailed MS parameters are shown in Table S3.

2.4. Quality assurance/quality control

In the analysis of PBDEs, all stages of the analytical procedure, including the sample extraction, purification, and handling of the final extracts were performed under conditions protected from ultraviolet (UV) radiation (i. e., using amber coloured glassware or wrapping the glassware with aluminium foil).

The quality control criteria for positive identification of the analytes of interest included the retention time of the native compounds within a window of +3 to 0 s compared to the corresponding $^{13}C_{12}$-labelled or deuterated surrogates. The acceptable deviation of the isotopic ratio for the two monitored ions (target/confirmation) was set as ±15% of the theoretical value. Five-point calibration curves were used for the quantification of analyte concentrations in each sample run. Procedural blanks and quality control samples were included in the routine quality control (QC) protocol and were analysed in each sample sequence. The concentrations of contaminants determined in real samples were corrected by taking into account the analyte concentrations found in procedural blanks. The QC samples were in-house reference materials (fortified bivalve homogenates) and the results obtained for QC samples were in a good agreement with the fortification levels (the recovery ranged from 75 to 123%).

2.5. Data analysis

Due to the small sample size and large variability among treatments, we treated observations below method the limit of detection to be zero. Open source software R (http://www.r-project.org/) was used to construct box plots for the data sets. Correlation analysis, principal component analysis (PCA), cluster analysis (CA), and graph construction were performed using Minitab 18 software. CA was applied to cluster observations together based upon similarity. Hierarchical agglomerative CA was performed on the normalised data set using squared Euclidean distances as a measure of similarity. The coordinates of the new cluster were calculated by complete linkage method. CA was performed on standardised experimental datasets in order to avoid misclassification due to wide differences in data dimensionality. PCA was used to interpret the relationship between the contamination of mussel samples and geographical locations. PCA calculates orthogonal linear combinations of the standardised variables of the correlation matrix according to the maximum variance criterion. Such linear combinations are called principal components and the coefficients of the linear combinations are called loadings. When the plot of object is projected onto the first two principal component axes, a linear projection of objects onto the two-dimensional subspace is obtained, saving most of the total variance (Jolliffe, 2002).

3. Results and discussion

Detailed concentration data for the selected compounds in the analysed mussel samples are provided in the Supplementary material, Table S4.

3.1. Levels and profiles of PFCs

In order to assess PFC contamination in the freshwater ecosystem of Latvia, two compounds (PFOS and PFOA) were analysed in mussels. PFOS was detected in 3 out of 24 (13%) samples, with the concentrations ranging from 10 to 21 pg g^{-1} w.w. PFOA, however, was detected more frequently, as 10 out of 24 (42%) samples were above the LOQ, ranging from 13 to 51 pg g^{-1} w.w. These values were two to three orders of magnitude lower than 9100 pg g^{-1} w.w., which is the EQS value for perfluorooctane sulfonic acid and its derivatives in biota stated in the European Commission Directive 2013/39/EC (2013/39/EC). As shown in Table 1, most of the values observed in other studies are significantly higher, with the highest average concentrations of PFOS of 72 000 pg g^{-1} w.w. in north-central Portuguese estuaries, where paper, textile, and leather factories discharge their effluents (Cunha et al., 2005). Similar levels of PFC contamination to ours were observed in coastal waters of Spain – up to 60 and 10 pg g^{-1} w.w for PFOS and PFOA, respectively (Domingo et al., 2012; Gomez et al., 2011). PFCs have already been monitored in Latvian freshwater biota during the summers of 2015 and 2016, when European perch (*Perca fluviatilis*) was used as the indicator organism. The ranges were 0.43–1.97 ng g^{-1} w.w. and n.d.–0.85 ng g^{-1} w.w., respectively (LEGMC, 2016; LEGMC, 2017). The difference in the levels observed in mussels and perch may be explained by accumulation differences. Some studies state that the low accumulation of PFCs in mussels is due to the increased efflux transport activity of proteins belonging to the adenosine triphosphate-binding cassette (ABC) superfamily, which act as a first line of defence of the organism, removing toxic chemicals from the cells (Epel et al., 2008).

Regarding the profiles of PFCs observed in our study, the most abundant compound in mussels was PFOA. PFOA is more water soluble than PFOS, demonstrating low bioconcentration factors (BCFs) at higher trophic levels, whereas the BCFs calculated for mussels are significantly higher (Quinete et al., 2009). It is in good agreement with the data obtained by other authors (Nakata et al., 2006; Quinete et al., 2009), although some studies have demonstrated the opposite trend (Kannan et al., 2005).

3.2. Levels and profiles of PBDEs

PBDEs were found in all of the analysed samples, confirming a wide distribution of these flame retardants in the freshwater environment. The total concentrations of Σ_{PBDE} ranged from 11.3 to 193.2 pg g^{-1} w.w., with the average of 42.1 pg g^{-1} w.w. These levels are lower than those observed in our previous study of European eels (*Anquilla anquilla*) from Latvian lakes, where the sum of PBDEs ranged from 280 to 26 700 pg g^{-1} w.w. (Zacs et al., 2016). Two sampling places overlapped in both studies. Higher contamination levels were observed in the Lake Kisezers – 14 900 pg g^{-1} in eels and 51.68 pg g^{-1} in mussels, whereas in Lake Liepaja the contamination levels were 9910 pg g^{-1} and 23.71 pg g^{-1}, respectively. The concentrations of PBDE observed in eels were more than one order of magnitude higher than those in mussels. This may be partly due to the great differences in lipid content of both organisms. Positive correlation between the lipid content and PBDE concentration has been observed before (Marriusen et al., 2008). Another possible explanation of the great concentration differences would be that eels are at a higher trophic level, and PBDEs are bioaccumulative, hence body burdens are proportional to trophic level. Furthermore, the overall lifespan of the analysed mussel species is shorter than that of eels, which may result in lower accumulation of contaminants in mussels. A comparison with other studies is provided in

Table 1
The concentrations of PFCs (pg g^{-1} w.w.) in mussels from the current study and the data from previous studies (expressed as minimum – maximum (mean)).

Reference	Region	PFOS	PFOA
Present study	Latvia	n.d.–21 (2)	n.d.–51 (9)
Domingo et al., 2012	Spain	n.d.	n.d.
Gomez et al., 2011	Spain	n.d.–60	n.d.–10
Kannan et al., 2005	USA, Great Lakes	n.d.–3100	n.d.
Munschy et al., 2013	France	5–900	–
Cunha et al., 2005	Portugal	36800–125900 (72000)	–
Nania et al., 2009	Mediterranean Sea	n.d.–3000	n.d.–2500
Nakata et al., 2006	Japan	n.d.	3400–8100 (6000)
Quinete et al., 2009	Brazil	n.d.–4700	n.d.–14900
Renzi et al., 2013	Italy	118–228	123–170
Zabaleta et al., 2015	Spain	n.d.–2400	–
So et al., 2006	Japan	109–586	n.d.–661

"n.d." – the value is below the limit of detection.
"-" – not included in the study.

Table 2.

Most of the concentrations observed elsewhere are significantly higher, with only those in mussels from Baiyangdian Lake, China being similar to our study (Hu et al., 2010). Despite the relatively low levels detected in Latvia, the concentration of brominated biphenyl ethers in 83% of the samples exceeded the EQS value in biota (8.5 pg g^{-1} w.w. for the sum of congeners 28, 47, 99, 100, 153, 154), as stated in the European Commission Directive 2013/39/EU. This fact is a source of concern regarding the water quality in Latvia with respect to this group of chemical pollutants.

The congener profile of PBDE (shown in Fig. 2) in most of the samples shows the predominance of tetra- and penta-brominated BDEs, including PBDEs No. 47, 49, 99, and 100. Similar observations have been reported by several other authors (Debruyn et al., 2009; Perez-Fuentetaja et al., 2015; Aznar-Alemany et al., 2017), with the exception of PBDE-49, which is rarely analysed. However, in our study PBDE-49 had the second highest average contribution of 18%. Although this congener was found in small quantities in penta- and octa-bromo technical BDE mixtures, significant amounts have been also observed upon reductive debromination of octa- and deca-brominated mixtures (Gaul et al., 2006). The highest levels were observed in the case of PBDE-209, which is the only deca-brominated BDE. The same results were obtained in the analysis of mussels from the Bohai Sea, China (Wang et al., 2009). This may be the result of several factors: 1) widespread usage of deca-BDE formulation; 2) mussels are known to act as natural water filters and possess limited metabolic capabilities, which leads to the accumulation of pollutants originating from suspended particulate matter, since highly brominated PBDE congeners tend to accumulate in sediments (De Boer et al., 2003); 3) ineffective depuration process. Therefore, the presence of PBDE-209 may result from the presence of particulate matter in the digestive tract of mussels. Some authors have not detected PBDE-209 in raw mussels, however Aznar-Alemany et al., 2017 have observed significant amounts of the congener in mussels after cooking – up to 1870.4 pg g^{-1} w.w. (Van Ael et al., 2012; Aznar-Alemany et al., 2017). The great variance of observed PBDE-209 concentrations could be a result of different usage of deca-BDE formulation across studied regions.

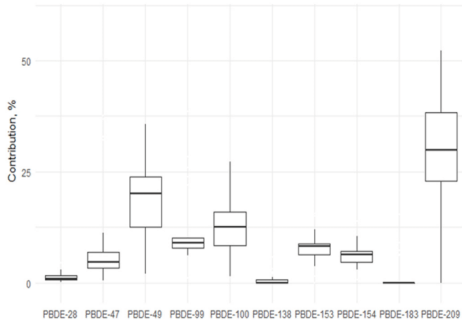

Fig. 2. Box plot of the contributions (%) of selected PBDEs in the analysed mussel samples.

In order to further evaluate the patterns of PBDE contamination and to identify likely sources of PBDE congeners, correlation analysis was performed. The obtained data revealed significant Pearson correlation between the presence of BDE-154 and the PBDE congeners No. 47, 49, 99, 100, and 153 (p-values < 0.01), which all are components of commercial penta-BDE mixture. This mixture was extensively used in the European Union until its phase out in 2004 (2003/11/EC). Strong correlation was observed between BDE-49 and BDE-209 by carrying out Spearman's correlation analysis (Spearman's rho 0.776, p-value < 0.001). This was in a good agreement with the aforementioned observation by other authors that BDE-49 is a degradation product of BDE-209 (Gaul et al., 2006).

3.3. Levels of NSAIDs

Eight of the nine analysed NSAIDs (tolfenamic acid, meloxicam,

Table 2
The concentrations of PBDEs (pg g^{-1} w.w. or pg g^{-1} d.w.) in mussels from the current study and data from previous studies (expressed as minimum – maximum (mean) or mean value ± standard deviation).

Reference	Region		PBDE-28	PBDE-47	PBDE-99	PBDE-100	PBDE-153	PBDE-154	PBDE-183	PBDE-209	Σ_{PBDE}
Present study	Latvia	pg g^{-1}, w.w.	0.1–1.4 (0.5)	0.5–21.5 (3.9)	1.1–29.0 (5.5)	0.9–46.8 (6.3)	0.8–13.4 (3.3)	0.8–5.1 (2.2)	0.9–4.6 (2.0)	3.5–179.0 (17.4)	11.3–193.2 (42.1)
Van Ael et al., 2012	The Netherlands		50–700 (270)	280–5160 (1430)	50–3350 (360)	720–2780 (1530)	50–450 (140)	200–580 (350)	40–270 (90)	n.d.	2090–12400 (3800)
Christensen et al., 2002	Greenland		–	(100)	(20)	–	n.d.	–	–	–	(120)
Perez-Fuentetaja et al., 2015	USA, Great Lakes		(15)	(910)	(1120)	100 ± 10	(80)	50 ± 10	–	(390)	–
Hu et al., 2010	China		2.0–4.1 (3.1)	6.5–9.2 (7.5)	3.9–5.1 (4.4)	1.5–2.4 (1.8)	2.5–5.4 (3.9)	1.8–3.1 (2.6)	0.3–11.6 (5.4)	–	25.4–58.9 (45.2)
Aznar-Alemany et al., 2017	Europe		n.d.-74.2 (27.5)[a]	n.d.-377.7 (123.9)[a]	n.d.-2066.8 (277.0)[a]	n.d.-275.7 (43.6)[a]	n.d.	n.d.	n.d.-2717.8 (292.7)[a]	n.d.	29.3–2989.2 (764.7)[a]
Present study	Latvia	pg g^{-1}, d.w.	1.1–13.2 (4.2)	3.7–183.3 (35.1)	10.0–255.5 (49.1)	7.9–425.4 (54.7)	6.9–121.6 (29.0)	6.9–46.2 (19.8)	7.3–42.2 (18.0)	29.2–1351.3 (143.3)	103.1–1560.1 (362.5)
Debruyn et al., 2009	Canada		–	–	–	–	–	–	–	–	(6710)
Oros et al., 2005	USA, CA		–	17000±700	8000±4000	4000±1000	–	–	–	–	29000±13000
Liu et al., 2005	China		320–25300 (7673)	1420–8560 (3859)	1410–21300 (8266)	n.d.–1270 (677)	n.d.–17400 (6870)	n.d.–1950 (782)	1580–20200 (8019)	n.d.–13900 (4097)	4730–109880 (40243)
Wang et al., 2009	China		20–390 (100)	140–1010 (280)	20–130 (80)	20–110 (30)	10–100 (30)	30–270 (50)	nd–100 (10)	1010 –1801000 (2430)	1250 –1803110 (3010)

"n.d." – the value is below the limit of detection.
"-" – not included in the study.
[a] Values recalculated from ng g^{-1} lipid weight to pg g^{-1} wet weight using data from Aznar-Alemany et al., (2017.

carprofen, flunixin, diclofenac, phenylbutazone, ketoprofen, and mefenamic acid) were not detected in any of the samples. Ibuprofen was present in 50% of the samples, with concentrations ranging from 0.52 to 109 ng g^{-1} w.w. or from 5.1 to 1363 ng g^{-1} d.w. A comparison with levels detected in other studies is shown in Table 3 (compounds that were not detected in this study and were not analysed by other authors are not included in the table). Relatively few studies have been reported so far on the occurrence of NSAIDs in mussels. Only ibuprofen and diclofenac have been detected in mussels and bivalves elsewhere. The levels of ibuprofen measured in Latvian mussels were up to ten times higher than in Taihu Lake, China, where the highest observed value was 93.7 ng g^{-1} d.w. (Xie et al., 2015). Similarly to our study, neither ketoprofen nor mefenamic acid were present in mussels from other regions (Caban et al., 2016; Nunez et al., 2015; Mezzelani et al., 2016; McEneff et al., 2014).

The detectable presence of ibuprofen in mussels corresponds to the statistics of pharmaceutical use in Latvia. The consumption of ibuprofen in 2017 was 23.46 defined daily doses per 1000 inhabitants per day (DID), making it the most consumed medication of NSAID group in Latvia. The consumption of diclofenac was similar to that of ibuprofen—19.73 DID (State Agency of Medicines, 2017). Nevertheless, diclofenac was not detected in any of the mussel samples. This fact can be explained by the rapid photodegradation of diclofenac in open basins (Buser et al., 1998). Furthermore, this trend is confirmed by data obtained in a study of Latvian and Norwegian surface waters, where ibuprofen was detected in 33 and 60% of the samples, whereas diclofenac in only 10 and 20% of samples, respectively (Reinholds et al., 2017). Ibuprofen was also the most common NSAID detected in untreated wastewaters from the Daugavgriva municipal wastewater treatment plant of the Riga city at up to 326 ng L^{-1} (Pugajeva et al., 2017). The primary route for the discharge of ibuprofen into surface waters is through the effluent of wastewater treatment plants. Ibuprofen and its metabolites are excreted into the sewage system along with unused drugs that may be disposed of via drains and toilets (Bound and Voulvoulis, 2006). Taking into account that only 1–8% of the consumed ibuprofen is excreted unchanged (Ternes, 1998), determination of ibuprofen metabolites in mussels should be considered in further studies. Besides that, other studies have shown that ibuprofen and its metabolites could pose ecotoxicological risks (Lienert et al., 2007).

3.4. Distribution patterns of the analysed contaminants

In order to interpret the data reported in Table S4, we focussed on the relationship between the levels of contaminants and sampling locations. The box plots for the total concentrations of each contaminant group are provided in Fig. 3. The samples were grouped by the drainage basin and types of the water bodies – lake, river, river delta or water reservoir. PBDEs were more common in the Daugava and Lielupe basins. The highest variations were observed in the case of ibuprofen, as it was present in the Lielupe and Venta basins at significantly higher concentrations than in the Gauja and Daugava basins. As for the different bodies of water, the only sample from Riga water reservoir contained remarkably high concentrations of PBDEs and ibuprofen. However, geographical variation may not be the only reason for great differences in the concentration of ibuprofen. Xie et al. (2015) have demonstrated drastic contrast in the levels of ibuprofen observed in mussels *Anodonta* and clams *Corbiculidae* – ibuprofen was not detected in mussels, while the concentration in clams reached 93.7 ng g^{-1} d.w. (Xie et al., 2015). This observation suggests that there are major differences in the ability of bivalve species to accumulate ibuprofen. Since the levels of ibuprofen observed in Latvian mussels varied greatly, correlation with mussel species was investigated. Significant correlation was found between the levels of ibuprofen and the contribution of zebra mussels *Dreissena polymorpha* in the sample batch (Pearson correlation 0.6, p-value 0.002 at confidence level 0.95). Nevertheless, no pronounced correlations were observed between the species and the levels of PFCs or PBDEs (p-values > 0.05). This observation should be further investigated in future studies by separately analysing the different mussel species.

We used CA and PCA to further evaluate the extent of contamination at the sampled locations and to identify its sources and distribution patterns. The CA method groups the objects into clusters on the basis of similarity. A dendrogram is presented in Fig. 4a. All 24 samples were grouped into six statistically significant clusters at a similarity level of 0.80. Since three of the clusters consisted of only a single sample each, they were considered as individual samples, rather than clusters. The majority of mussel samples belonged to the cluster 1, which included sampling locations showing low to moderate contamination with the analysed compounds. Cluster 2 (sites 21, 22, and 24) consisted of samples from the Liepaja region and was characterised by the presence of ibuprofen at a concentration of about one order of magnitude higher than those in the rest of the clusters. Cluster 2 samples also contained PFOS, which was not present in the other clusters. This trend may point to the significant anthropogenic impact in this region. Cluster 3 (sites 10, 11, 12, and 13) has the peculiar feature of BDE-47 and BDE-99 present at much higher concentrations than any other brominated compound, and BDE-209 is not present in samples of this cluster at all. The lack of BDE-209 may be due to the lower turbidity of water. The individual sample 8, which did not belong to any cluster, contained the highest concentration of BDE-209. The sampling location was Lake Dridzis – known as the deepest lake in Latvia. This suggests that there might be some relation between the depth of the water body and the

Table 3
The concentrations of NSAIDs (ng g^{-1} d.w.) in bivalves from the current study and previous studies (expressed as minimum – maximum (mean) or mean value ± standard deviation).

Reference	Region	Diclofenac	Ketoprofen	Ibuprofen	Mefenamic acid
Present study	Latvia	n.d.	n.d.	n.d.–1363 (111)	n.d.
Bayen et al., 2016	Singapore	n.d.	–	–	–
Caban et al., 2016	Poland	n.d.	n.d.	n.d.	–
Xie et al., 2015	China	1.41–5.42 (2.59)	–	n.d.–93.7 (41.6)	–
Klosterhaus et al., 2013	USA, CA	–	–	n.d.	–
Nunez et al., 2015	Spain	n.d.	n.d.	n.d.	–
Cunha et al., 2017	Portugal	n.d.–4.5	–	–	–
Mezzelani et al., 2016	Italy	16.11 ± 14.72	n.d.	9.39 ± 0.59	–
McEneff et al., 2014	Ireland	n.d.	–	–	n.d.

"n.d." – the value is below the limit of detection.
"-" – not included in the study.

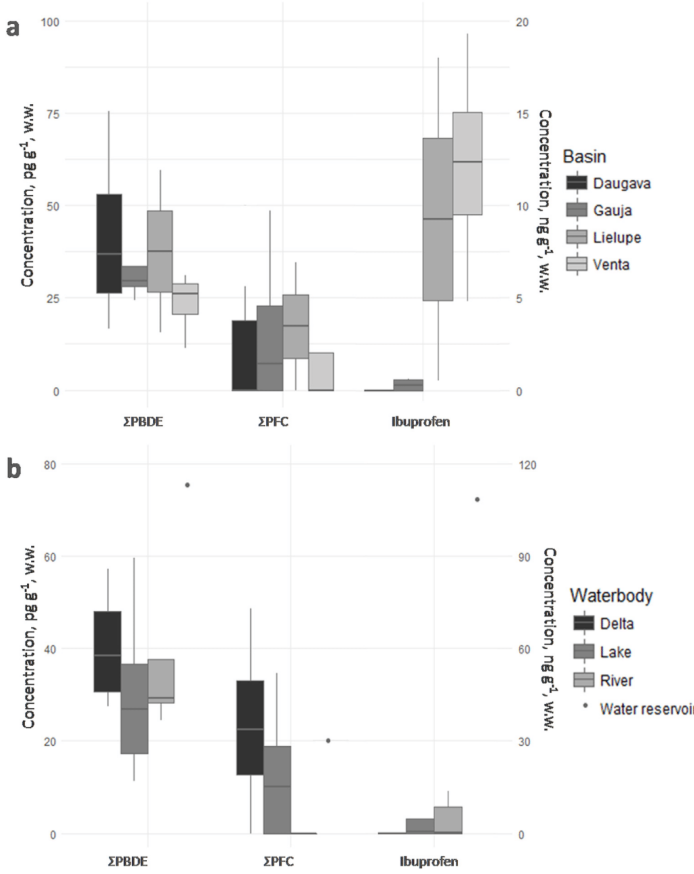

Fig. 3. Box plots of the concentrations of selected contaminants (pg g^{-1} w.w. (ΣPBDE and ΣPFC) or ng g^{-1} w.w. (ibuprofen)), grouped by the drainage basins (a) and types of water bodies (b).

concentration of PBDEs. Sample 18 from the Riga water reservoir had the highest level of ibuprofen. CA confirmed the previously mentioned observation that this sampling site was quite different. Sample 20, collected from the Barta river near the Latvia-Lithuania border had the highest concentrations of tetra-through hexa-BDEs. The source of contamination could be wastewater influx or municipal waste disposal in the surrounding area, since no known PBDE emission sources are located near this sampling site.

PCA was also applied to the data reported in Table S4. Each of the individual contaminant levels were included in the statistical analysis. First, three principal components (PC1, PC2, and PC3) with eigenvalues higher than 1 were extracted. The three principal components together accounted for 67% of the total variation. An exploratory view of the samples/scores plot on the principal components PC1 versus PC2, which accounts for more than 56% of the total variance, is illustrated in Fig. 4b. The principal component PC1 showed positive associations with most of the PBDEs, except for BDE-209, which indicated the possible role of a different source. The PC2 allowed to account for 22% of the total variance and had the highest negative association with BDE-183 and BDE-138. However, with the number of data sets available, it was not possible to identify significant correlation between the 24 sampling locations and the levels of contaminants. All samples in Fig. 4b are grouped in accordance with CA. Cluster 3, as well as the samples 18 and 20 were far different from the other samples, whereas Clusters 1 and 2, as well as the sample 8 were merged on the plot.

4. Conclusions

In order to evaluate the contamination status of Latvian aquatic environment, mussel samples from various freshwater bodies were analysed for the content of ten PBDEs, two PFCs, and nine NSAIDs.

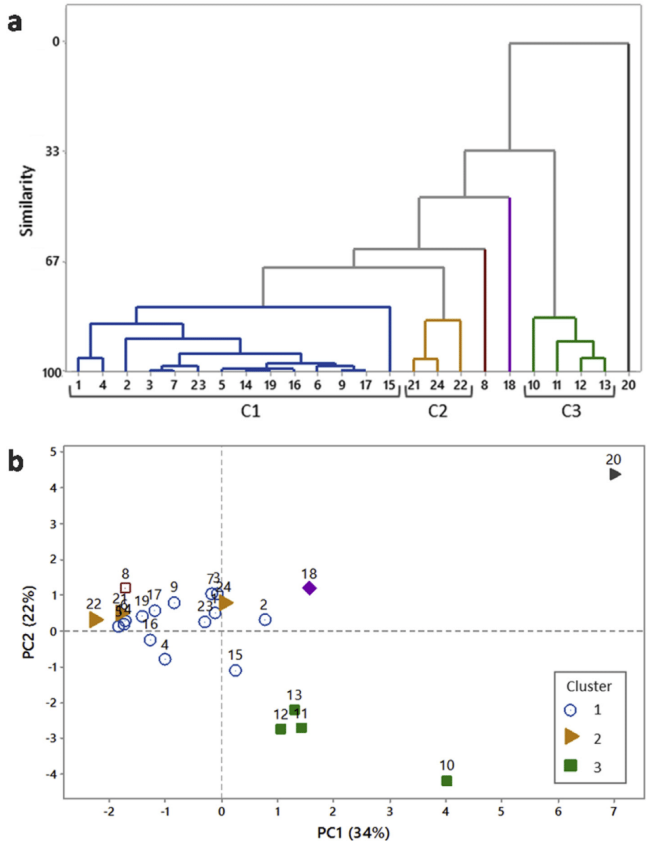

Fig. 4. Hierarchical dendogram and three distinguished clusters of mussel samples by the analysed compounds (a) and plot of the first two principal components after PCA of the analysed compounds in mussels collected from Latvian freshwater sites (b).

For the first time, data on the contamination status of mussels from Latvia are presented. PBDEs were detected in all of the mussel samples, but the concentrations were generally lower than those observed in mussels from other regions. Regardless, 83% of the samples exceeded the EQS threshold for the sum of six PBDEs stated in the Directive 2013/39/EU. The levels of PFCs in mussels were low throughout the territory of Latvia, compared to other regions and did not exceed the EQS criteria. The levels observed for ibuprofen, which was the only detected NSAID in Latvian mussels, were significantly higher than those detected by other authors. It is a source of concern that provides a reason for further monitoring and control of this emerging contaminant, as well as its metabolites.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.chemosphere.2018.09.036.

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IV

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Determination of residues and metabolites of more than 140 pharmacologically active substances in meat by liquid chromatography coupled to high resolution Orbitrap mass spectrometry

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Short communication

Determination of residues and metabolites of more than 140 pharmacologically active substances in meat by liquid chromatography coupled to high resolution Orbitrap mass spectrometry

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ABSTRACT

This study reports an analytical method for simultaneous identification, screening and quantification of 164 residues and metabolites of pharmacologically active substances belonging to such therapeutic classes as anti-infectious (antibiotics and chemotheurapeutics), anti-inflammatory and antiparasitic agents (against protozoa, endo- and ectoparasites), corticoids and agents acting on the nervous and reproductive systems, substances with hormonal and thyreostatic action, and beta agonists. Different sample preparation procedures were compared and optimised for the detection of selected veterinary drugs in chicken, porcine and bovine meat by ultra-high performance liquid chromatography coupled to high-resolution Orbitrap mass spectrometry. The optimised instrumental method and sample preparation procedures were validated by fortifying blank matrix at four levels (0.5, 1.0, 1.5, and 2 times the maximum residue limit (MRL)), or at concentrations as low as possible for substances without an MRL. The evaluated performance parameters were selectivity, matrix effect, method and instrument limits of quantification, accuracy, and repeatability. A total of 130 selected compounds in chicken meat, 127 compounds in bovine meat and 123 compounds in porcine meat samples could be quantified with accuracy ranging from 70 to 120% and RSDs less than 30%.

Finally, the method was successfully used to detect and quantify veterinary drug residues in real samples found to be suspect using the non-selective qualitative test for the detection of inhibitor substances. The results were confirmed using the relevant one-residue confirmatory methods revealing concentrations of residues higher than MRLs established for several samples.

1. Introduction

Veterinary drugs are an essential for modern animal husbandry and food production and are applied to maintain animal health, prevent infection, and to treat diseases. However, illegal use of prohibited veterinary drugs or improper use, such as ignoring the required withdrawal periods may result in the presence of drug residues in animal tissues and affect the food safety. Veterinary drug residues may include the parent compounds themselves, as well as metabolites and/or conjugates, and may have direct toxic effects on consumers, such as allergic reactions in hypersensitive individuals, hormonal effects by interfering with the balance of human hormones, or the development of antibiotic-resistant bacteria as a result of misusing antibiotics [1].

Considering the broad range of currently available veterinary drugs, the strict regulations and limits that have been established, it is clear that the development of multi-residue methods has become an essential task in food analysis. An emerging trend in drug residue analysis is the development of generic methods that are capable of monitoring a wide variety of compounds, belonging to different drug classes. This presents a considerable challenge since the different functional groups, often encountered amphoteric properties of analytes, and the wide range of polarity pose difficulties for extraction, clean-up, and analytical separation. Therefore, sample preparation is one of the most critical steps. The optimal sample preparation method should be as non-specific as possible to reduce the possible loss of information. The most frequently used sample pre-treatment methods for meat samples include solvent extraction (SE) with [2,3] or without [4] defatting step, solid-

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phase extraction (SPE) [5,6], and dispersive solid phase extraction (dSPE) method (QuEChERS approach) [7,8] for further purification or enrichment.

Liquid chromatography coupled to mass spectrometric detectors (LC–MS) provides an universal approach applicable to the broadest range of veterinary drugs [7]. The most common technique for veterinary drug analysis in foodstuffs has been LC coupled to tandem mass spectrometry (MS/MS) [2,3,8,9]. The current trend is focussed towards the use of powerful high-resolution mass spectrometric detectors (HRMS) like time-of-flight (TOF) [5,10] and Orbitrap [4,11,12] with modern chromatographic systems. This development is due to the availability of more rugged, sensitive, and selective instrumentation. The benefits provided by HRMS over classical unit-mass-resolution tandem mass spectrometry are considerable, for example, the collection of full-scan spectra, which provides greater insight into the composition of a sample and the freedom to measure compounds without previous compound-specific tuning. In the last years, the Orbitrap system has become more recognised because of its better dynamic range, higher resolving power, consequently better mass accuracy compared to the TOF system [13].

The aim of this work was the development, validation, and practical application of an analytical method for simultaneous identification and quantification of residues and metabolites of more than 140 pharmacologically active substances belonging to different therapeutic classes in meat samples.

2. Experimental

2.1. Chemicals and materials

All analytical standards used within this study were purchased from Sigma-Aldrich (St. Louis, MO, USA and Steinheim, Germany), Dr. Ehrenstorfer (Augsburg, Germany), Witega Laboratorien (Berlin, Germany), and Toronto Research Chemicals (Toronto, Canada).

HPLC grade methanol and acetonitrile were obtained from Merck (Darmstadt, Germany). Anhydrous magnesium sulphate and sodium chloride were supplied by Chempur (Piekary Śląskie, Poland). Formic acid (98%), ammonium formate, acetic acid (>99%), and octadecyl-functionalised silica gel were purchased from Sigma-Aldrich (St. Louis, MO, USA), whereas primary secondary amine (PSA) sorbent was purchased as a bulk sorbent from United Chemical Technologies (Brockville, ON, Canada). Phree™ Phospholipid Removal (1 mL) and Strata X (200 g/3 mL) solid phase extraction columns were obtained from Phenomenex (Torrance, CA, USA). Disposable Ultrafree® PVDF membrane filters (0.22 μm) were obtained from Merck Millipore. Deionised water was prepared with a Milli-Q (Millipore, Billerica, MA, USA) water purification system.

2.2. Selection of compounds

The selected list of compounds (Table 1) consists of the major classes of veterinary drugs that are commonly used in veterinary practice and also prohibited substances that are listed in Commission Regulation 37/2010/EC [14], as well as substances with hormonal and thyreostatic action and beta agonists, which are prohibited under Council Directive 96/22/EC [15].

2.3. Collection of samples

Within the framework of the National Residue Monitoring Plan, 40 bovine meat samples were collected from different slaughterhouses in Latvia identified to be suspected samples on-site by the microbial inhibition test (Explorer 2.0, Zeulab, S.L) for the screening of antimicrobial residues in food samples. Upon arrival at the laboratory, samples were homogenised and stored in freezer until analysis.

2.4. UHPLC-HRMS method

The chromatographic separation of the residues was carried out using a Dionex UltiMate 3000 UHPLC system (Thermo Fisher Scientific, San Jose, CA, USA) on a Phenomenex Luna Omega analytical column (100 × 2.1 mm, 1.6 μm). The mobile phase consisted of (A) 0.1% formic acid in water, (B) 0.1% formic acid in acetonitrile, and (C) 0.1% formic acid in methanol. The gradient program shown in Table S1 was used. A 10 μL aliquot of the extract was injected. The column and autosampler were maintained at 40 °C and 4 °C, respectively.

The UHPLC system was coupled to a Q-Orbitrap HRMS mass spectrometer (Thermo Fisher Scientific) equipped with a heated electrospray ionisation probe operating in the positive and negative ionisation modes. The following parameters were applied: electrospray voltage 4.0 kV in positive and 3.5 kV in negative ionisation modes, heater temperature 350 °C, capillary temperature 300 °C, sheath gas (N_2) 20 arbitrary units (arb), auxiliary gas (N_2) 6 arb, and S-lens RF level at 50 arb. The automatic gain control (AGC) was set to $3{\cdot}10^6$, the maximum injection time (IT) was set to 200 ms. Full scan data both in the positive and negative ionisation modes were acquired at a mass resolving power of 70,000 FWHM. The *m/z* scan range was 70–1050. The data processing was carried out with Xcalibur 2.2 software (Thermo Fisher Scientific).

2.5. Sample extraction methods

2.5.1. Solvent extraction (SE)

Homogenised meat sample (2 g) was transferred to a 50 mL polypropylene (PP) centrifuge tube. The mixture of internal standards and reference standards for quality control samples was added to the sample. A 0.1% solution of formic acid in acetonitrile (10 mL) was added and mixed over 20 min. After centrifugation at 4000 rpm for 10 min, 9 mL of the supernatant was transferred into a centrifuge tube and frozen for 30 min at −70 °C. The sample was centrifuged at 4000 rpm for 10 min and a 5 mL portion of the extract was evaporated under nitrogen stream at 45 °C. The residue was dissolved in 2:1 (v/v) water–acetonitrile solution (300 μL) containing 5 mM of ammonium formate and 0.01% of acetic acid, and filtered through a PVDF membrane centrifuge filter (0.22 μm) prior to the analysis.

2.5.2. Solid-phase extraction (SPE)

Homogenised meat sample (2 g) was transferred to a 50 mL PP centrifuge tube. The mixture of internal standards and reference standards for quality control samples was added to the sample. A 0.1% solution of formic acid in acetonitrile (10 mL) was added and the sample was vigorously shaken for 20 min and centrifuged at 4000 rpm for 10 min A 5 mL portion of the supernatant was loaded for the purification onto a Phree™ Phospholipid removal column or Strata X SPE column that was pre-conditioned with acetonitrile. The obtained extract (5 mL) was collected into clean sample tubes and evaporated to dryness under nitrogen stream at 45 °C. The residue was dissolved in 2:1 (v/v) mixture of water–acetonitrile solution (300 μL) containing 5 mM of ammonium formate and 0.01% of acetic acid, and filtered through a PVDF membrane centrifuge filter (0.22 μm) prior to the analysis.

2.5.3. Dispersive SPE method (dSPE)

Homogenized meat sample (2 g) was transferred to a 50 mL PP centrifuge tube. The mixture of internal standards and reference standards for quality control samples was added to the sample. A 0.1% solution of formic acid in acetonitrile (10 mL) was added and mixed during 20 min. After centrifugation at 4000 rpm for

Table 1
A summary of the analytes included in the study.

No.	Compound	RT, min	VL, μg/kg	Bovine meat				Chicken meat				Porcine meat			
				CCβ, μg/kg	m-LOQ, ng/kg	Accuracy, %	RSD, %	CCβ, μg/kg	m-LOQ, ng/kg	Accuracy, %	RSD, %	CCβ, μg/kg	m-LOQ, ng/kg	Accuracy, %	RSD,%
Amphenicols															
1	Chloramphenicol	7.3	0.3	0.18	1.2	94	4.8	0.18	2.4	91	6.5	0.17	0.93	98	3.9
2	Florfenicol	6.9	200 (b), 100 (c), 300 (p)	123	4.0	112	9.9	67	5.3	108	6.3	179	1.8	112	3.6
3	Thiamphenicol	6.4	50	29	11	90	8.2	29	36	99	14	30	18	104	6.1
Anthelmintics															
4	2-Amino-flubendazole	7.3	50	31	22	90	5.3	30	40	97	7.0	31	19	95	7.3
5	5-Hydroxy-thiabendazole	6.3	100	41	53	91	8.1	36	87	96	6.9	38	39	91	7.9
6	5-Hydroxy-mebendazole	7.3	60	54	1.8	107	4.6	63	10	114	8.9	68	4.6	113	9.2
7	Albendazole	8.2	100	57	11	103	2.5	53	20	99	2.2	54	10	99	2.5
8	Albendazole sulphone	7.4	100	66	27	95	8.8	63	44	96	8.1	64	19	92	9.6
9	Albendazole sulphoxide	7.3	100	66	28	91	8.6	61	45	98	7.7	68	20	96	10
10	Albendazole-2-aminosulphone	6.1	100	67	65	88	9.1	61	138	99	7.7	65	58	96	9.3
11	Amino-mebendazole	7.2	60	38	23	96	5.5	35	39	97	8.1	38	19	97	8.2
12	Clorsulon	7.0	35	26	185	110	9.5	22	317	99	8.0	23	201	91	9.8
13	Febantel	9.4	50	34	4362	88	7.6	29	6987	96	7.1	34	2594	93	9.0
14	Fenbendazole	8.7	50	30	1.5	101	3.5	32	2.1	96	7.4	34	1.4	106	6.8
15	Flubendazole	8.3	50	31	19	93	5.8	28	29	94	5.2	30	13	94	5.9
16	Imidocarb	6.1	300	250	71	102	35	235	205	127	11	291	29	145	16
17	Ketotricla-bendazole	9.4	225	138	3.1	98	3.9	121	6.7	99	6.4	137	3.1	100	4.0
18	Levamisole	5.9	10	6.8	13	89	8.5	5.9	35.8	97	7.5	8.5	18.6	106	9.8
19	Mebendazole	8.1	60	38	4.1	101	6.3	32	6.3	95	6.0	35	2.9	93	6.5
20	Nitroxinil	7.8	400	288	6.1	110	9.3	277	10	107	14	285	5.3	96	9.7
21	Oxfendazole	7.7	50	47	748	127	19	32	3170	105	12	43	1892	109	10
22	Oxfendazole sulphone	7.8	50	45	4.8	94	7.5	29	8.2	94	6.6	30	3.3	90	7.8
23	Oxibendazole	7.6	100	62	3.6	92	4.9	54	6.1	100	4.1	60	2.8	96	5.6
24	Oxyclozanide	9.8	20	13	2.5	93	8.2	15	3.4	107	9.0	11	1.7	105	4.6
25	Piperazine	1.1	400	275	1414	118	25	262	326	117	13	297	280	90	19
26	Thiabendazole	6.6	100	68	24	90	8.0	61	46	108	7.9	67	19	105	8.7
27	Triclabendazole sulphoxide	9.7	225	135	17	99	3.6	123	34	99	5.7	135	16	94	6.0
28	Triclabendazole	10.2	225	129	11	102	2.4	124	20	98	2.7	120	9.4	98	2.4
29	Triclabendazole sulphone	9.6	225	144	3.8	102	4.2	135	6.8	103	6.8	146	3.3	99	7.3
Avermectins															
30	Abamectin B1a	14.8	20	12	162	97	13	15	480	137	23	13	281	122	7.7
31	Doramectin	16.0	40	27	132	135	11	32	365	145	31	26	216	148	11
32	Emamectin	10.3	100	61	12	95	6.2	65	24	120	26	65	17	105	6.4
33	Ivermectin B1a	8.5	100	82	2300	97	50	72	22769	101	31	77	8873	119	16
34	Ivermectin B1b	8.2	100	85	317	114	14	75	825	100	27	66	211	113	9.8

35	Moxidectin	16.4	50	39	20	79	19	49	53	89	34	47	8.8	113	33
Beta-agonists															
36	Brombuterol	7.1	0.05	n/a				0.038	31	93	25	0.06	6.0	70	41
37	Isoxsuprine	6.6	0.25	0.17	15	100	5.2	0.15	21	100	4.7	0.14	1.3	98	4.6
38	Ractopamine	7.1	0.5	1.2	2.6	69	13	1.0	4.2	94	9.5	0.92	2.9	77	5.6
39	Salbutamol	3.4	2.5	n/a				1.4	567	69	37	1.6	472	65	20
40	Zilpaterol	6.9	2.5	2.3	1168	62	37	2.1	2046	60	20	1.9	1246	50	29
41	Clenbuterol	8.5	0.1	0.090	3.0	92	19	0.083	5.2	110	26	0.058	6.6	88	9.1
Cephalosporins															
42	Cefacetrile	6.1	50	43	12262	105	27	41	12262	112	24	30	738	83	10
43	Cefalexin	6.9	200	135	1182	116	28	129	4703	89	8.1	123	2099	85	11
44	Cefoperazone	7.4	50	39	6299	49	43	46	23624	78	48	43	16370	52	58
45	Cefquinome	6.6	50	48	1508	119	13	47	18409	118	20	32	6725	90	7.4
46	Ceftiofur	7.4	1000	551	170	111	4.8	547	560	104	14	545	239	104	3.9
Coccidiostats															
47	Amprolium	6.6	100	84	5368	114	43	85	17533	101	19	87	8052	136	18
48	Clopidol	6.4	100	57	75	112	5.1	63	443	122	8.0	72	213	129	8.4
49	Decoquinate	13.0	100	78	1.3	89	15	81	1.4	84	30	91	2.9	109	25
50	Diclazuril	9.4	500	282	9.5	98	4.3	334	13	105	9.4	286	6.8	99	5.2
51	Dinitrocarbanilide (DNC)	9.0	100	58	1.5	112	3.6	54	1.6	108	2.9	62	0.9	104	4.1
52	Halofuginone	7.5	10	6.4	46	96	6.7	6.2	100	100	12	6.5	36	95	9.1
53	Maduramicin	18.3	30	36	1.5	173	21	22	5.7	152	32	28	4.1	169	18
54	Nequinate	10.0	100	56	9.1	108	8.3	69	20	99	9.1	58	14	92	6.9
55	Robenidine	7.8	200	111	157	106	6.1	109	383	98	4.6	114	196	103	4.1
56	Toltrazuril	9.7	100	56	8.5	91	5.6	67	14	98	10	55	6.9	94	5.1
57	Toltrazuril sulphone	8.8	100	58	11	91	4.1	60	16	96	8.3	63	8.7	91	8.8
58	Toltrazuril sulphoxide	8.4	100	59	11	93	4.4	59	15	103	12	67	8.6	90	11
Ionophores															
59	Lasalocid	16.2	10 (b,p), 20 (c)	6.7	2.3	101	18	9.1	8.4	116	24	7.8	2.7	121	24
60	Monensin	16.8	2	1.5	1.4	108	16	1.4	3.9	137	21	1.4	2.2	120	16
61	Narasin	18.4	50	43	0.35	123	20	42	1.0	128	33	46	0.48	135	27
62	Salinomycin	17.6	5	41	0.40	121	18	4.2	1.4	130	30	4.2	0.35	132	23
63	Semduramicin	9.9	2	1.3	158	102	11	1.3	1030	137	27	1.4	413	122	8.6
Lincosamides and macrolides															
64	Erythromycin A	7.9	200	123	24	93	17	194	22	118	28	152	11	88	20
65	Josamycin	8.2	100	108	17	121	14	77	41	100	26	65	17	104	9.0
66	Kitasamycin	7.9	100	91	94	140	35	70	214	98	26	74	202	145	13
67	Lincomycin	6.2	100	67	33	107	8.9	71	96	84	13	88	53	77	21
68	Neospiramycin	6.9	200	121	65	115	29	125	351	76	14	137	262	86	18
69	Pirlimycin	7.4	100	64	16	112	5.7	61	42	92	4.7	60	22	88	8.5
70	Spiramycin	7.0	200 (b,c), 250 (p)	117	10	115	17	116	19	70	12	164	17	67	15
71	Tiamulin	7.8	100	58	1.6	107	5.8	57	4.6	99	10	54	2.0	108	3.6
72	Tilmicosin	7.3	50 (b,p), 75 (c)	29	1.3	80	5.1	43	3.5	80	6.7	28	1.7	61	5.5
73	Tylosin A	7.9	100	83	20	180	21	70	32	117	32	70	41	143	11

Table 1 (*Continued*)

No.	Compound	RT, min	VL, μg/kg	Bovine meat				Chicken meat				Porcine meat			
				CCβ, μg/kg	m-LOQ, ng/kg	Accuracy, %	RSD, %	CCβ, μg/kg	m-LOQ, ng/kg	Accuracy, %	RSD, %	CCβ, μg/kg	m-LOQ, ng/kg	Accuracy, %	RSD,%
Nitroimidazoles															
74	Dimetridazole	5.1	1.5	1.1	156	129	24	1.3	352	112	16	1.2	254	147	16
75	HMMNI	3.8	1.5	1.1	69	99	13	1.1	499	118	13	1.1	406	110	9.1
76	Hydroxy-ipronidazole	6.9	1.5	0.9	15	108	6.0	0.92	42	109	7.3	1.0	23	128	7.5
77	Ipronidazole	7.3	1.5	1.0	13	125	31	1.0	35	111	15	1.1	22	124	17
78	Metronidazole	4.2	1.5	1.4	187	126	7.6	1.0	455	129	7.3	1.1	204	135	8.9
79	Ornidazole	6.8	1.5	1.0	18	100	5.8	0.80	48	109	5.9	1.0	23	116	6.1
80	Ronidazole	8.2	1.5	1.1	69	113	15	0.94	193	98	14	1.0	113	110	10
81	Tinidazole	6.1	1.5	1.0	7.3	95	7.2	0.86	24	111	7.2	1.0	11.5	114	5.7
NSAIDs															
82	Carprofen	9.3	500	289	13	103	3.9	300	25	94	4.0	297	12	99	5.8
83	Diclofenac	9.5	5	2.6	22	104	3.5	2.9	40.8	97	4.6	3.1	19.5	97	5.9
84	Flufenamic acid	10.2	10	5.6	1.9	108	2.8	6.4	3.1	93	11	6.8	1.5	103	10
85	Flunixin	9.1	20 (b,c), 50 (p)	11	2.2	102	1.9	11	4.3	97	1.8	27	2.0	98	2.6
86	Ibuprofen	6.3	10	5.8	119	106	7.6	7.0	776	103	14	7.2	452	109	11
87	Ketoprofen	8.5	10	6.6	192	93	6.8	6.0	636.3	90	11	5.4	194.1	82	7.3
88	Mefenamic acid	10.3	10	6.1	6.8	105	5.7	6.9	12.6	95	12	7.9	6.2	101	12
89	Meloxicam	8.5	20	12	15	109	4.3	12	28	108	8.1	11	11	95	5.9
90	Metamizole (4-methylaminoantipyrine)	4.9	100	79	191	109	23	62	947	107	19	89	474	61	46
91	Naproxen	7.7	10	8.5	1754	80	19	7.0	5583	92	9.4	7.0	2277	94	9.0
92	Niflumic acid	9.6	10	5.7	5.0	112	4.0	6.1	7.8	104	9.5	5.3	3.6	99	5.3
93	Oxyphenbutazone	8.3	5	3.2	18	111	7.8	3.0	54.8	96	13	3.1	18.4	78	18
94	Phenylbutazone	9.1	5	3.5	10	106	4.7	3.1	14.0	80	6.8	3.5	7.7	114	7.7
95	Tolfenamic acid	9.3	50	30	268	108	4.8	29	509	96	4.3	29	233	103	5.2
96	Vedaprofen	11.5	50	35	124	116	17	43	111	137	33	n/a			
Penicillins															
97	Amoxicillin	6.6	50	42	131	132	19	34	770	90	12	39	286	84	17
98	Ampicillin	6.7	50	47	4040	56	35	35	4542	84	22	45	3809	66	31
99	Benzylpenicillin	7.9	50	29	137	103	5.5	27	281	82	9.0	31	184	94	3.6
100	Cloxacillin	8.3	300	196	110	102	8.4	167	406	92	12	194	212	103	6.2
101	Dicloxacillin	8.5	300	178	1065	100	10	189	5517	102	15	209	2180	115	7.2
102	Nafcillin	8.5	300	180	139	104	5.5	169	370	88	11	191	187	87	5.1
103	Oxacillin	8.2	300	196	127	103	9.2	172	380	90	9.2	186	194	96	5.3
104	Phenoxymethyl-penicillin	8.1	25	16	4561	80	13	13	3277	78	6.0	17	3813	82	10

Quinolones															
105	Ciprofloxacin	6.7	100	62	8.9	106	6.7	56	27	97	2.9	56	14	101	3.4
106	Danofloxacin	6.7	200 (b,c), 100 (p)	116	10	101	3.7	105	25	101	5.8	56	11	102	3.7
107	Difloxacin	6.8	400 (b,p), 300 (c)	237	1.6	91	5.5	160	4.6	98	6.0	231	2.2	99	4.2
108	Enrofloxacin	6.7	100	58	1.9	101	3.9	53	4.6	95	2.9	57	2.2	100	3.8
109	Flumequine	8.0	200 (b,p), 400 (c)	115	186	109	4.3	225	469	107	7.8	119	226	123	4.5
110	Marbofloxacin	6.4	150	84	9.1	100	4.0	81	22	101	5.6	85	11	101	5.4
111	Nalidixic acid	8.0	100	57	10	110	4.9	58	22	108	7.1	64	11	130	5.0
112	Norfloxacin	6.6	100	59	9.0	105	6.0	56	27	96	3.5	55	14	99	3.5
113	Orbifloxacin	6.8	100	58	8.1	98	4.9	52	22	96	4.3	59	11	97	4.6
114	Oxolinic acid	7.6	100	56	12	103	4.4	60	23	110	8.0	62	12	125	4.8
115	Sarafloxacin	6.9	100	59	14	97	6.8	53	49	98	4.2	58	27	99	3.9
Sulphonamides															
116	Sulphachloro-pyridazine	6.8	100	62	7.5	101	6.7	57	23	112	8.0	63	12	117	7.8
117	Sulpha-dimethoxine	7.4	100	58	1.8	106	5.5	55	4.5	107	6.3	65	2.3	117	7.7
118	Sulphadimidine	6.6	100	67	9.2	103	7.1	56	23	112	5.9	62	11	117	5.6
119	Sulphamethiazole	6.6	100	67	8.1	109	7.4	56	25	113	6.3	59	11	117	6.9
120	Sulphathiazole	5.9	100	59	161	99	5.3	54	426	113	6.9	59	225	114	6.2
Tetracyclines															
121	Chlortetracycline	7.1	100	62	137	109	6.4	63	377	112	14	64	181	106	5.3
122	Doxycycline	7.5	100	65	191	123	5.4	59	554	110	11	74	444	87	12
123	Oxytetracycline	6.6	100	69	160	114	7.0	66	584	106	14	78	224	88	16
124	Tetracycline	6.6	100	54	127	108	4.6	68	378	102	14	61	192	93	7.5
Tranquilizers															
125	Acepromazine	7.8	50	36	4.7	92	8.4	29	8.8	97	6.1	32	3.3	95	6.4
126	Azaperol	6.5	100	60	5.3	113	6.0	64	24	107	8.1	69	11	107	7.4
127	Azaperone	6.8	100	60	3.9	110	7.4	57	20	97	6.7	54	8.8	99	6.1
128	Carazolol	7.2	5	3.0	0.78	111	6.0	3.8	4.1	108	11	3.5	2.3	108	8.6
129	Chlorpromazine	8.2	10	7.9	2.2	112	11	6.2	16	98	8.0	5.8	6.3	107	10
130	Promazine	7.8	10	6.4	36	93	6.8	5.8	83	99	5.7	6.1	29	102	3.0
131	Xylazine	6.8	100	60	4.7	113	5.5	67	23	114	9.2	72	11	121	8.1
Quinoxalines															
132	2-Quinoxaline-carboxylic acid	7.0	10	6.7	494	103	8.5	7.1	753	106	11	6.2	336	96	7.6
133	Carbadox	7.5	10	9.2	548	96	10	9.6	807	121	10	8.8	409	114	13
134	Desoxycarbadox	7.8	10	7.6	558	90	9.7	6.0	834	119	9.4	6.4	401	94	7.8
135	Mequindox	5.9	10	5.4	858	89	7.0	5.5	2823	99	6.6	9.0	2302	125	5.5
136	Olaquindox	4.2	10	6.7	68	110	10	11	126	88	37	6.2	145	47	43

Table 1 (*Continued*)

No.	Compound	RT, min	VL, μg/kg	Bovine meat				Chicken meat				Porcine meat			
				CCβ, μg/kg	m-LOQ, ng/kg	Accuracy, %	RSD, %	CCβ, μg/kg	m-LOQ, ng/kg	Accuracy, %	RSD, %	CCβ, μg/kg	m-LOQ, ng/kg	Accuracy, %	RSD,%
Glucocorticoids															
137	Methyl-prednisolone	8.4	10	7.7	30	122	16	7.3	82	102	14	6.2	44	91	7.9
138	Prednisolone	8.1	4	3.1	218	82	17	2.6	418	94	8.9	2.3	221	84	4.2
Progestins															
139	Chlormadinone acetate	9.8	0.5	0.30	1.3	86	18	0.31	53	107	9.1	0.35	21	100	12
140	Medroxy-progesterone acetate	9.9	1	0.94	22	100	22	0.71	42	113	8.5	0.93	25	97	11
141	Megestrol acetate	9.8	1	0.85	7.0	136	18	0.90	25	168	20	0.78	11	103	9.3
142	Melengestrol acetate	10.0	1	0.64	191	114	14	0.77	414	109	9.6	0.71	185	102	7.7
Insecticides															
143	Cyromazine	1.6	300	264	543	108	14	185	584	92	8.5	157	261	97	3.0
144	Diazinon	9.9	20	14	1.3	94	13	12	1.9	88	13	12	1.0	86	9.3
145	DPF (*N*-(2.4-dimethylphenyl)formamide)	7.7	200	127	546	108	8.3	127	765	105	12	137	383	116	7.9
146	DPMF (*N*-2.4-dimethylphenyl-*N*-methylformamide)	6.5	200	144	508	98	8.1	126	934	107	9.5	141	541	118	9.6
147	Phoxim	8.5	25	22	220	62	35	24	32	57	39	19	249	111	14
Estrogens															
148	Dienoestrol	6.8	1	0.88	196	109	31	0.66	378	74	26	0.71	183	82	61
149	Diethylstilbestrol	10.6	1	1.5	156	107	15	3.5	129	132	36	3.6	163	124	20
150	Taleranol	8.4	1	2.0	8.1	128	26	0.94	15	113	19	0.82	6.7	86	9.4
151	Zearalanone	9.0	1	1.0	4.4	82	11	0.63	10	83	6.2	0.62	5.4	86	5.6
152	Zeranol	8.8	1	2.2	2.2	129	29	0.61	7.5	84	11	0.56	3.7	85	6.6
Androgens															
153	17β-Testosterone	9.0	1	0.70	296	92	13	0.71	326	92	8.4	0.63	185	99	6.1
154	17α-Trenbolone	8.7	1	0.85	272	87	13	0.68	423	104	8.7	0.53	287	96	4.2
155	17β-Trenbolone	8.6	1	0.87	189	130	16	0.96	317	104	11	0.56	203	94	6.3
156	17α-19-Nortestosterone	9.1	1	0.91	224	105	19	0.94	354	125	13	0.78	187	103	8.0
157	17β-19-Nortestosterone	8.8	1	0.87	32	101	21	0.79	28	82	18	0.59	23	100	4.5
158	Methyl-testosterone	9.3	1	0.88	19	69	20	0.59	30	83	11	0.66	18	90	6.8
Other antibiotics															
159	Bacitracin A	7.5	100	82	260	72	28	77	293	93	23	81	91	54	61
160	Novobiocin	10.7	50	28	123	103	5.5	33	286	89	13	34	112	118	7.5
161	Rifaximin	9.3	100	56	216	100	8.2	68	590	129	23	65	346	99	7.2
162	Tylvalosin	8.5	50	42	364	137	20	46	594	154	40	47	324	126	16
163	Valnemulin	8.4	50	41	4.8	111	14	27	19	94	11	33	7.9	105	6.0
164	Trimethoprim	6.4	50	29	9.2	103	4.3	27	20	101	3.1	31	11	111	4.0

VL-validation level, b-bovine meat, p-porcine meat, c-chicken meat.

10 min, 9 mL of the supernatant was transferred into a centrifuge tube and frozen for 30 min at −70 °C. The sample was centrifuged at 4000 rpm for 10 min and the supernatants were decanted into 15 mL PP tubes containing $MgSO_4$ (2 g) and C18 sorbent (0.5 g) or PSA (0.5 g). After shaking, the sample was centrifuged at 4000 rpm for 10 min and a 5 mL portion of extract was evaporated under nitrogen stream at 45 °C. The residue was dissolved in 2:1 (v/v) mixture of water–acetonitrile (300 μL) containing 5 mM of ammonium formate and 0.01% of acetic acid, and filtered through a PVDF membrane centrifuge filter (0.22 μm) prior to the analysis.

2.6. Method validation

An in-house validation protocol was carried out taking into consideration the requirements outlined in Commission Decision 2002/657/EC [16] for a quantitative screening method, in order to establish the performance characteristics of the method, ensuring adequate identification and quantification of the target compounds. The performance of the method was evaluated by estimating its linearity, accuracy, and repeatability expressed as relative standard deviation (RSD), detection capability (CCβ), and specificity.

The instrumental limit of quantification (i-LOQ) was determined as the minimum detectable concentration of analyte on column with signal-to-noise (S/N) ratio exceeding 10 by sequential injection of decreasing concentrations of calibration standards.

The detection capability (CCβ) at the ½ validation level (VL) was determined from decision limit CCα that in turn was calculated from 1.64 times the standard deviation at the ½ VL level for allowed substances and 2.33 times the standard deviation at the ½ VL level for banned substances using the following equations: $CC\beta = CC\alpha + 1.64 \times SD_{0.5VL}$ and $CC\alpha = VL + 1.64\ (2.33) \times SD_{0.5VL}$

Matrix effects (ME) were assessed in porcine, chicken and bovine meat in order to evaluate the degree of ion suppression or enhancement. The matrix effects were calculated by dividing the slopes of the matrix-matched calibration curves after extraction ($slope_M$) and the slopes of the calibration curves obtained using standard solutions ($slope_{STD}$) according to the following equation: $ME\ (\%) = 100\% \times slope_M / slope_{STD}$. A value of 100% indicates that there is no matrix effect, while values higher than 100% point to ion enhancement and values lower than 100% indicate ion suppression.

2.7. Statistical analysis

A two-factor ANOVA was performed without replication for the determination of significant differences between the different extraction procedures tested for each therapeutic class.

3. Results and discussion

3.1. Instrumental method optimisation

Different mobile phases and additives were compared in order to optimise the chromatographic separation and selectivity. Acetonitrile, methanol, a mixture of both solvents with or without additives were tested as organic phase components while deionised water without additives, water buffered with 0.05 M ammonium formate and/or fortified with 0.1% formic acid were evaluated as the aqueous components of the mobile phase. Three different columns: Phenomenex Kinetex C18 (100 × 2.1 mm, 2.6 μm), Phenomenex Luna Omega (100 × 2.1 mm, 1.6 μm), and Thermo Fisher Scientific Hypersil GOLD (50 × 2.1 mm, 1.9 μm) analytical columns were examined. All columns provided satisfactory peak shapes and responses. The Luna Omega column was chosen for all further experiments due to the optimal distribution of analyte retention times from 1.6 min for cyromazine to 18.4 min for narasin.

Ion source parameters like temperature and nitrogen gas flow were optimised for improved efficiency of the ionisation process. Accurate masses of the analytes were calculated using isotope simulation calculator provided by Xcalibur 2.2 software (Table S2). During the method development, unsatisfactory instrumental method reproducibility was observed that can be related to such parameters as AGC (ion count transferred to the analyser) settings and ion accumulation time in the C-trap prior to the HRMS analysis. Optimisation of these parameters using spiked samples with 164 compounds does not really reflect a real sample analysis where only few analytes at the time might be detected. To improve reproducibility of the instrumental method several internal standards representing different classes of drugs were included in the methods for accurate quantitation and internal quality control purposes. These few internal standards were assigned to analytes by practical checking which one better covers a shift of the peak intensities during the instrumental analysis. Information on the method performance in terms of repeatability and accuracy not corrected by internal standards is presented in Supplementary data as Table S2.

The resolving power was set at 70 000 FWHM that was found sufficient by many authors [4,7] in order to distinguish the possible interfering endogenous matrix components from the analytes of interest using full scan mode. Mass resolution of 140 000 FWHM was also examined and showed satisfactory results, providing sufficient data points for all analytes at 1 V L. However, higher mass resolving power usually requires longer scanning speed times, and thus fewer data points are acquired across the target peak that can cause difficulties for quantification. For that reason all subsequent experiments were performed with the resolving power of 70 000 FWHM.

3.2. Sample preparation

Meat is a complex and challenging matrix due to the high protein and lipid content. Considering the large number of target compounds belonging to various classes and having different chemical properties, the most challenging part of the multi-residue method is the sample preparation in terms of both the extraction and clean-up. The most frequently used pre-treatment methods for meat samples (SE, SPE, and dSPE) were evaluated with some modifications. The procedures were evaluated by fortifying blank meat samples at 0.5, 1, 1.5, and 2 V L. The relative extraction recoveries were compared based on recoveries at the concentration of 1 V L, assuming that extraction recovery applying the SE method (see 2.5.1) was equal to 100%. The linearity of calibration curves and the number of the extracted analytes were also investigated.

3.2.1. The evaluation of a solvent extraction method

SE can be used as an effective, simple and rapid procedure for non-targeted screening. However, there is a significant risk of MS source contamination due to injecting extracts without any clean-up. The methodology evaluated in the current study is based on extraction with acetonitrile. High concentrations of acetonitrile precipitated proteins and caused denaturation of enzymes, which was considered an advantage, as enzymes might otherwise degrade drug residues during the extraction and clean-up steps. The effect of formic acid in acetonitrile on the extraction efficiency was also tested and was shown to be superior for all analytes except amphenicols, were an average recovery of 214% was obtained by extraction with pure acetonitrile.

A salting-out step with the addition of NaCl and $MgSO_4$ during the extraction process was also evaluated, where NaCl was added to promote the separation of aqueous and acetonitrile phases, while $MgSO_4$ was added to remove water from the acetonitrile supernatant for quicker evaporation. It was noticed that the salting-out step caused decreased recoveries for such chemical classes

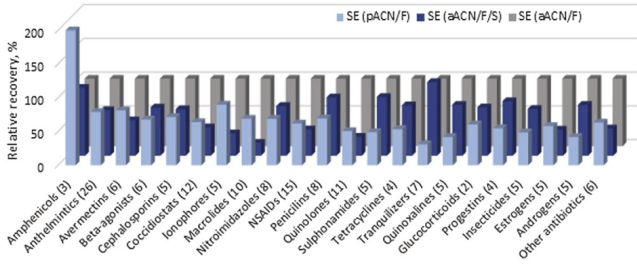

Fig. 1. Summary of relative recoveries: pACN/F – SE with pure acetonitrile and freezing out; aACN/F/S – SE with acidic acetonitrile, salting out and freezing out; aACN/F – SE with acidic acetonitrile and freezing out (assumed as 100% of recovery).

as quinolones, NSAIDs, macrolides, anthelmintics, tranquilisers, ionophores, and coccidiostats, resulting in recoveries below 50%. A summary of the relative recoveries for different classes of compounds is shown in Fig. 1.

The solutions obtained without any clean-up steps contained large amounts of matrix components, therefore we evaluated a defatting step with hexane and freezing out at −70 °C for 30 min. The freezing out procedure was found to be more suitable as a clean-up step for the multi-residue method, yielding higher recoveries of analytes in comparison to the defatting with hexane. The additional clean-up step is very important for the removal of interfering matrix peaks and lowering the noise level in the case of analytes that must be quantitated at low levels, such as estrogens and androgens, progestins, nitroimidazoles, beta-agonists, and chloramphenicol.

In the case of analytes that preferably form sodium adducts during the ionisation step, such as cocciodiostats and avermectins, the most critical step was reconstitution of the evaporated samples before analysis. Reconstitution in only acidic water/acetonitrile solution yielded decreased analyte signals due to insufficient ionisation of molecules.

3.2.2. Evaluation of the SPE method

SPE is useful for sample clean-up and effective concentration of analytes belonging to particular classes. There are certain limitations to using SPE for multi-residue screening of components with various physicochemical properties, because SPE sorbents have limited interaction mechanisms. In the current study, two SPE columns were evaluated for retaining such matrix components as phospholipids, proteins and particulates on the stationary phase, while the analytes of interest passed through the stationary phase: Strata X (a mixed-mode sorbent) column and Phree™ Phospholipid removal column.

The SPE method generally provided relative recoveries in the range from 80 to 140%, except some cases when the relative recoveries were lower than 20% (Fig. 2A and B). Purification of the extracts with SPE resulted in fewer interfering peaks on chromatograms. Good results were obtained by using Strata X columns for avermectins, sulphonamides and quinolones, with the average recovery for all analytes within those groups being 114%, 120%, and 118%, respectively. When a Phree™ Phospholipid removal column was used for the clean-up, highly variable relative recoveries were observed for analytes within one group, for example, the relative recoveries of NSAIDs ranged from 10% for naproxen to 109% for carprofen.

3.2.3. Evaluation of the dSPE method

Another common pre-treatment method for multi-residue screening is dispersive solid phase extraction (dSPE) according to the QuEChERS approach, which is commonly used for contaminant analysis in various matrices. Two different sorbents were evaluated for dSPE during this study: C18 bulk sorbent and PSA. The clean-up experiments with C18 did not indicate any significant improvements, while the use of PSA sorbent significantly improved the recoveries of some compounds, for example, glucocorticoids and estrogens. At the same time, losing tetracycline, quinolone, and some analytes of the penicillin group (Fig. 2C and D).

3.2.4. Comparison of the proposed sample preparation procedures

There is no decisive recommendation in relation to the most effective sample preparation procedure for multi-residue method. In the procedures involving clean-up steps, better recoveries were obtained for some analytes, while other analytes could be lost completely. At the same time the matrix components and a high background noise level do not present a significant problem for substances with higher MRL values, while this issue is very critical for banned substances that must be detected at very low concentrations. Statistical analysis showed significant difference for the variance between the means of populations for the sample preparation procedures for all chemical classes listed in Table 1, except for nitroimidazoles, where $F < F_{crit}$, indicating relatively uniform recovery among the different methods.

In summary, it can be concluded that only the dSPE method using PSA for clean-up was not appropriate for the analysis of a wide range of substances, due to the losses of a number of analytes. The best results overall were observed with two sample preparation procedures - SE combined with a freezing out step and SPE using Phree™ Phospholipid removal column or a Strata X column. In our study we preferred the first option, as it was more convenient and affordable. This procedure was evaluated in terms of selectivity, matrix effect, method limits of quantification, accuracy, and repeatability and used for the screening the real samples.

3.3. Performance of the method

The analytical method validation for the determination of veterinary drugs in bovine, chicken and pork meat was carried out at four concentration levels at 0.5, 1, 1.5, and 2 times of VL, which was equal to the maximum residue level (MRL), the minimum required performance limit (MRPL), or to a specific "level of interest", if no MRL or MRPL levels were defined. All concentration levels used in this validation study are listed in Table 1. The compounds for which the MRL or MRPL levels could not be achieved due to the limited sensitivity of the detector (carnidazole, hexestrol and firocoxib) or losses during the sample preparation procedure (closantel, rafoxamide, bethametasone, dexamethasone and amitraz), were excluded from the study. The validation experiments for each type of meat were performed on separate days.

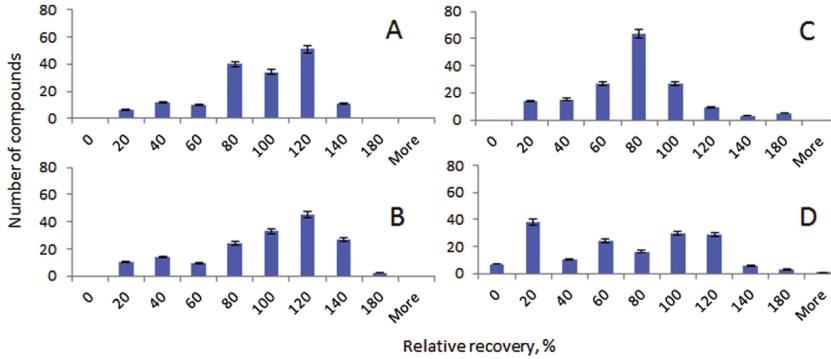

Fig. 2. Relative recovery histograms for different sample preparation methods: A – SPE with Phree™ Phospholipid column, B – SPE with Strata X column, C – dSPE with C18, D – dSPE with PSA. The recoveries obtained by SE with acidic acetonitrile and freezing out were assumed as 100%.

The method sensitivity was evaluated by measuring the instrumental limit of quantification (i-LOQ) that was experimentally determined as the lowest amount of the analyte injected on-column for which the S/N ratio exceeded 10. The LOQ values varied from 0.033 to 67 pg of absolute amount (Table S2). The m-LOQ values were calculated by taking into account the sample preparation procedure (such as sample weight taken for the analysis, the amount of extraction solvent used, the final volume of the sample after reconstitution and the injection volume), as well as the matrix effect obtained during the method validation. The m-LOQs were expressed as ng kg^{-1} of sample fresh weight (Table 1). The obtained m-LOQs were always lower than the CCβ value obtained during validation of the method.

The CCβ value is defined as the lowest concentration of the substance that may be detected, identified and/or quantified in a sample with an error probability of β (in this study, β = 5%). The calculated CCβ values are presented in the Table 1 and are lower than the validation levels, except for estrogens in bovine muscle where the established MRPL is 1 μg kg^{-1}, but the obtained CCβ values were as high as 2.2 μg kg^{-1}. In the case of diethylstilbestrol, the CCβ values were even higher for all meat types, varying from 1.5 to 3.6 μg kg^{-1}.

The specificity of the method was checked by analysing 20 blank samples for each type of matrix. The chromatograms were monitored for peaks that can potentially interfere with the analytes of interest. No interfering peaks were observed within the mass tolerance of 5 ppm. Higher mass deviations resulted in false positive cases for some analytes in particular types of meat.

The mean repeatability and accuracy were calculated from the data obtained by analysing blank samples fortified before extraction at 0.5, 1.0, 1.5, and 2.0 times the VL in 4 replicates each level. A total of 130 selected compounds could be quantified in chicken meat samples with accuracy ranging from 70 to 120% and RSDs less than 30%. Analogously, 127 compounds could be quantified in bovine meat and 123 compounds in porcine meat samples. Detailed information on the RSD values and accuracy for each analyte in different matrices is listed in the Table 1.

For the evaluation of ME, blank samples fortified before extraction at 0.5, 1.0, 1.5, and 2.0 times the VL were analysed and standard calibration was performed in solvent at the same levels. The results showed a strong matrix effect, especially in the case of chicken meat where 145 analytes were affected by ion enhancement effect, while only 10 analytes showed negligible ME and 9 analytes – ion suppression effect (Table S2). A summary of the observed matrix effects

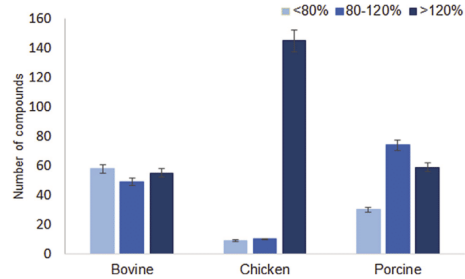

Fig. 3. A summary of matrix effects observed in our study in different types of meat.

is provided in Fig. 3. Especially high matrix effects occurred in the cases of piperazine, cefacetrile, and vedaprofen in bovine meat samples, while diethylstilbestrol showed the highest ME in all meat types. A dramatic ion suppression effect was observed in the case of brombuterol and salbutamol in bovine meat and vedaprofene in porcine meat resulting in non-detection of these analytes. Taking into account that ME in LC–MS with electrospray ionization source can severely compromise quantitative analysis of compounds at trace levels, as well as greatly affect the method reproducibility and accuracy, standard calibration curve could not be used for quantification. The results demonstrated that a calibration curve made up in the matrix with addition of isotopically labelled standards must be used to minimise the matrix interference and to avoid any under or over estimation during quantification. At the same time, the unacceptable accuracy and repeatability results could be improved by selection or introduction in the method of more appropriate internal standards for particular components.

Our study presents simultaneous within run screening of chicken, bovine and pork meat for 164 veterinary drugs belonging to 22 different classes that are either commonly used or prohibited in veterinary practice. Many previous multi-residue methods have been suitable for the detection of various types of antibiotic classes, such as amphenicols, beta-lactams, lincosamides, macrolides, pleuromutilins, quinolones, sulphonamides, tetracyclines, and others [4,6]. In some cases the scope of the analytical methods has been extended beyond antibiotics. For example, 13 classes of veterinary drugs, including 76 compounds [3] and 131

Table 2
Concentrations of pharmacologically active substances found in meat samples.

Sample No.	Compound	Result obtained by LC-HRMS, μg/kg	Confirmed result by LC-MS/MS, μg/kg	MRL (bovine muscle), μg/kg
1	Oxytetracycline	6045	5679	100
	Tylosin A	340	301	100
	Metamizole (4-methylamino-antipyrin)	9.6	11	100
	Benzylpenicillin	410300	407700	50
2	Metamizole (4-methylamino-antipyrin)	42	56	100
	Ketoprofen	1770	1602	No MRL
3	Metamizole (4-methylamino-antipyrin)	54	11	100
	Ketoprofen	13	14	No MRL
4	Meloxicam	502	734	20
5	Meloxicam	1.4	2.9	20
6	Trimethoprim	1.9	1.7	50
7	Tetracycline	157	151	100
8	Ketoprofen	7.4	4.3	No MRL

compounds [17], were determined in bovine meat by LC–MS/MS analysis. Even more types of veterinary drugs were detected by employing high-resolution mass spectrometry, including antibiotics, benzimidazoles, anthelmintics, nitroimidazoles, ionophores, tranquilizers, and NSAIDs in chicken muscle [5]. Other screening methods of animal origin products include column switching using different mobile phase compositions [18]. Screening of 90 veterinary drugs belonging to 14 therapeutic classes in royal jelly samples was presented by Zhang et al. [10], while Zhao et al. reported the determination of 80 veterinary drugs belonging to 12 different classes in *Oplegnathus punctatus* [11] and Jia at al. –137 drug residues and metabolites from 16 classes in tilapia [19], and the screening of more than 200 pharmaceutical and other residues in aquatic foods was developed by Kong et al. [12]. At the present time, only Yin at al [2]. have reported multi-residue determination of 210 drugs in pork by LC–MS/MS and Dasenaki et al. [9] presented an LC–MS/MS method for the determination of 115 drugs belonging to 20 different classes in milk powder and butter, but these studies include drugs used for humans, such as beta-blockers, analgetics, diuretics, statins, antiepileptic drugs, and fibrates.

3.4. Application to real samples

In order to evaluate the applicability of the proposed method, 40 bovine meat samples found suspected on farms were analysed. Inspection of the animals prior to slaughter showed clearly visible injection sites without any records of the treatment for the particular animal, and the analysed samples showed positive results in non-selective qualitative test for inhibitor substances.

In 8 out of the 40 analysed samples, pharmacologically active substances of various therapeutic classes, namely, NSAIDs and antibiotics of tetracycline, macrolide, and penicillin groups were found (Table 2). In most of the cases, the detected concentrations of pharmaceutical residues were higher than the MRL established by the EU, indicating non-compliance with the veterinary regulations.

The residue concentrations determined using the proposed method were quantified with matrix-matched calibration over the relevant concentration ranges (the R^2 values for all calibration curves were higher than 0.99) and the calculated concentrations of residues were confirmed with a validated confirmatory LC–MS/MS method used in the laboratory for the analysis of the particular group of drugs. Oxytetracycline, tylosin A, trimethoprim, and benzyl penicillin were analysed by a method designed for the analysis of antibiotics (excluding aminoglycosides) [6], while ketoprofen, meloxicam and marker residue of metamizole (4-methylaminoantipyrin) were analysed with specific method designed for confirmation of NSAIDs [20]. These methods were validated as confirmatory methods in compliance with the Commission Decision 2002/657/EC.

The concentrations of substances obtained by both methods (screening and confirmatory) were in a good agreement.

4. Conclusions

The analytical method for the simultaneous identification and quantification of 164 residues and metabolites of pharmacologically active substances were evaluated and validated in terms of selectivity, matrix effect, method and instrument limits of quantification, accuracy, and repeatability in chicken, porcine and bovine muscle by ultra-high performance liquid chromatography coupled to high-resolution Orbitrap mass spectrometry. The elaborated multi-class residues method provides simultaneous screening of drugs belonging to 22 different classes commonly used or prohibited in veterinary practise. The performance of the method was good for the detection of compounds above CCβ levels, proving the effectiveness of this methodology for fast routine analysis of these compounds, followed by confirmation according to the requirements of Commission Decision 2002/657/EC in case of positive findings.

Finally, the method was successfully used to detect and quantify veterinary drug residues in real samples found to be suspected using the non-selective qualitative test for inhibitor substances. The results were confirmed using the relevant confirmatory one-residue class methods that revealed concentrations of residues above the established MRLs.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2019.01.024.

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Direct injection Fourier transform ion cyclotron resonance mass spectrometric method for high throughput quantification of quinolones in poultry

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ABSTRACT

Many recent studies have shown high detection frequencies of quinolone antibiotics in poultry, as well as an increasing incidence of antimicrobial resistance. The main purpose of this project was to develop a fast and reliable analytical method for the detection of quinolones in poultry meat. In order to develop a rapid quantitative confirmation method, ion cyclotron resonance mass spectrometer was used. First, the sample preparation procedure was simplified by reducing the procedure to extraction and freezing out steps. Second, the chromatographic separation step was excluded and mass spectrometric parameters were optimised. Third, the method was validated by fortifying a blank matrix at four levels (0.5, 1, 1.5, and 2 times the maximum residue limit (MRL) or level of interest in those cases when no MRL was established). As a result, the overall analysis time was reduced to less than an hour. The validation study revealed that the method is capable of detection and confirmation of ten quinolone compounds in poultry above the detection capability (CCβ) of the procedure. Finally, the developed method was applied to 19 commercially available chicken meat samples. None of the samples contained quinolones above the limit of quantification (LOQ) of the method. Analysis of treated chickens revealed that the developed method is suitable for the determination of ciprofloxacin and enrofloxacin. The developed method could be one of the fastest quantitative confirmatory methods for the analysis of quinolones available so far.

1. Introduction

Quinolone antibiotics are a group of widely used antimicrobial agents. Quinolones (Qs) are often employed as first-choice drugs for the treatment of acute gastrointestinal infections in humans. Since Qs possess activity against the full range of pathogens that may cause bacterial gastroenteritis (most commonly, *Campylobacter*, *Salmonella*, *Shigella* or *E. coli*), they are often used for empirical treatment in human medicine when the pathogen is yet unknown [1]. Because of their broad-spectrum activity, Qs have been used successfully in veterinary medicine as well. However, it has been observed that the introduction of Qs in veterinary practice has led to higher incidence of antimicrobial resistance [2,3]. Concerned about the growing trend of antimicrobial resistance, USA has banned the use of Qs for poultry since 2005. Nevertheless, no action has been taken in the European Union and Qs are still extensively used for the medication of poultry. In order to reduce the risk associated with the presence of these compounds in animal tissues, the maximum residue limits (MRLs) for several Qs in foodstuffs of animal origin have been established by the Commission Regulation (EU) No 37/ 2010 [4].

Numerous papers have been published regarding the analysis of Qs residues in animal products [5]. Liquid chromatography (LC) is the most commonly used analytical separation technique, but such methods as gas chromatography [4,6] and capillary electrophoresis [7–9] have also been employed. Regarding the detection, tandem mass spectrometry (MS/MS) is the favoured confirmatory method [10]. LC–MS and LC–MS/MS methods are typically characterised by superior selectivity and sensitivity, for instance, the limit of detection (LOD) can be as low as 1 ng kg^{-1} for enrofloxacin [11]. Nevertheless, in order to achieve such method performance, excessive sample clean-up procedures and at least a ten minute chromatographic run must be performed. The analytical procedure could be shortened either by simplification of sample preparation

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or by reducing the time of instrumental analysis. To date, only a few reports of non-chromatographic methods for the determination of Qs are available. These include immunoassay and luminescence techniques. The latter usually lacks the required selectivity in the case of complex mixtures, as it has been applied to the determination of individual Qs [12,13]. Immunochemical methods have shown great potential as a screening tool, although most of them only allow semi-quantitative analysis [14]. Regarding the sample preparation, procedures consisting only of extraction step without any clean-up have been reported. UHPLC-Orbitrap-HRMS, operated at a resolution of 70 000 FWHM, was used by Cepurnieks [15]. HRMS methods such as Orbitrap and ion cyclotron resonance are capable of achieving a resolution of up to 500 000 FWHM. Such a high resolving power enables the separation of very close *m/z* values of analytes, as well as provides a better resolution of analyte peaks from the matrix signals. Consequently, there is no need for a thorough sample clean-up. In our present study, we utilize Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR MS). This allows simplification of the sample preparation procedure, as well as elimination of chromatographic separation. The benefits of HRMS over the classical unitmass resolution tandem mass spectrometry are considerable, for instance, the collection of full-scan spectra, which enables both non-targeted and retrospective analysis [16].

The objective of this work was to develop a rapid, sensitive, and reliable method for the determination of residues of ten Qs in poultry by direct injection FT-ICR MS. The sample preparation procedure involved a simple extraction step and clean-up by freezing out at 70 °C. Combined with ultra-fast detection, the achieved overall analysis time was less than an hour. The method was comprehensively validated in accordance to the Guidelines for the validation of quantitative methods for residues of veterinary medicines [17]. To the best of our knowledge, this is the first time that a complete quantification and confirmation of quinolone residues in poultry has been achieved in less than an hour.

2. Materials and methods

2.1. Chemicals and materials

Analytical standards of ciprofloxacin (CIP), danofloxacin (DAN), difloxacin (DIF), enrofloxacin (ENR), flumequine (FLU), and norfloxacin (NOR) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Analytical standards of nalidixic acid (NAL), orbifloxacin (ORB), oxolinic acid (OXO), and sarafloxacin (SAR), as well as formic acid (98 %) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Gradient grade acetonitrile was obtained from Merck (Darmstadt, Germany). Deionised water was obtained using a MilliQ water purification system from Millipore (Billerica, MA, USA).

2.2. Chicken meat samples

2.2.1. Commercial samples

A total of 19 chicken meat samples were purchased from different hypermarkets and farmers' markets in Latvia, representing the range of chicken meat types available on the markets of Latvia. The origin countries of the obtained meat samples were Latvia (n = 3), Lithuania (n = 5), Estonia (n = 1), Poland (n = 9), and France (n = 1).

2.2.2. Treated chicken samples

Six chickens (age: 10 days) treated with a commercially available veterinary medication containing ENR were obtained. The antibiotic was administered to the chickens *via* drinking water. The water containing 0.01 % of ENR was offered to the animals in the morning. After the treatment, fresh water was available *ad libitum*. The treatment was performed for five days. The chickens were slaughtered on the next day after the last treatment.

Meat of each sample was minced separately, homogenised, and stored at −20 °C until sample preparation for the analysis.

2.3. Sample preparation procedure

An aliquot of muscle sample (2 g) was extracted by adding acetonitrile (10 mL) and shaking for 10 min. After centrifugation at 4000 rpm for 5 min, the supernatant was transferred into another centrifuge tube and frozen for 15 min at -70 °C. The sample was then centrifuged at 4000 rpm for 10 min and a 1 mL portion of the supernatant was diluted with 1 mL of 0.1 % formic acid solution in water. Finally, the sample was filtered through a PVDF membrane centrifuge filter (0.22 μm).

2.4. DI ESI FT-ICR MS method

Prior to the first analysis, a sodium formate solution was used to calibrate the FT-ICR MS instrument equipped with a 7.0 T superconducting magnet (Bruker Daltonics, Bremen, Germany). The sample was directly introduced at a flow rate of 250 μL h^{-1} into the ESI source. The mass spectrometer was set to operate over the mass range of *m/z*100–1000 in the positive ion mode. The ESI source conditions were as follows: the nebuliser gas pressure was 0.3 bar, the dry gas flow rate was 9.0 L min^{-1}, capillary voltage 4.0 kV, ion flight time 0.65 ms, ion accumulation time 0.1 s, and the transfer capillary temperature was 250 °C. Each spectrum was acquired by accumulating 32 scans of time-domain transient signals in 2 mega-point timedomain data sets. The resolving power $m/\Delta m(50\ \%)$ = 140,000 and mass accuracy of <3 ppm provided for unambiguous molecular formula assignments of singly charged molecular ions. In the MS/MS experiments, collision-induced dissociation (CID) mode was selected, argon was used as the collision gas, the laboratory collision energy was adjustable from 5 eV to 25 eV, the isolation windows were set at *m/z*5 and the collision RF amplitude was set at 1500 Vpp. Bruker Compass HyStar 4.1 SR.1 and FTMS Control 2.2.0 software were used to control the FT-ICR MS system, while Bruker Compass DataAnalysis 5.0 SR.1 software suite was used for raw data interpretation.

2.5. Data processing

Open access software R (www.r-project.org) was used for further processing of the obtained full-scan spectra. The complete mass list was subjected to analysis using an algorithm that can be found in the Supplementary material file. The output of the algorithm contained the observed *m/z* values of the first and the second most abundant isotopic ion signals of ten quinolones targeted in this study. For each compound, six types of adducts were investigated, including $[M+H]^+$, $[M+NH_4]^+$, $[M+Na]^+$, $[M+K]^+$, $[M+CH_3OH+H]^+$, $[2M+H]^+$, and $[M+2H]^+$. A compound was considered present in the sample if the *m/z* error was below 2 ppm and the ion ratio error was less than 30 %. The final MS parameters used in the study are presented in Table 1. Quantification was achieved by a procedural five-point calibration.

2.6. Method validation

An in-house validation protocol was carried out, taking into consideration the requirements outlined in the Commission Decision 2002/657/EC [17] for establishing the performance characteristics of quantitative confirmation methods, ensuring adequate identification, quantification, and confirmation. The performance of the method was evaluated by estimating its linearity, accuracy, and

Table 1
MS parameters for the determination of Qs included in the study.

Compound	Molecular formula	Accurate mass, *m/z*		Isotopic ratio, %	MS/MS fragments		
		Q1	Q2		Species	Accurate mass, *m/z*	Collision energy, V
CIP	$C_{17}H_{18}FN_3O_3$	332.140496	333.143857	18.6	$[M\text{-}CO_2+H]^+$	288.150667	10
DAN	$C_{19}H_{20}FN_3O_3$	358.156146	359.159507	20.7	$[M\text{-}H_2O+H]^+$	340.145581	15
DIF	$C_{21}H_{19}F_2N_3O_3$	400.146724	401.150088	22.9	$[M\text{-}CO_2+H]^+$	356.156895	10
ENR	$C_{19}H_{22}FN_3O_3$	360.171796	361.175159	20.7	$[M\text{-}CO_2+H]^+$	316.181967	10
FLU	$C_{14}H_{12}FNO_3$	262.087398	263.090759	15.3	$[M\text{-}H_2O+H]^+$	244.076833	5
NAL	$C_{12}H_{12}N_2O_3$	233.092069	234.095431	13.1	$[M\text{-}H_2O+H]^+$	215.081504	5
NOR	$C_{16}H_{18}FN_3O_3$	320.140496	321.143857	17.5	$[M\text{-}CO_2+H]^+$	276.150667	10
ORB	$C_{19}H_{20}F_3N_3O_3$	396.152953	397.156318	20.8	$[M\text{-}CO_2+H]^+$	352.163123	10
OXO	$C_{13}H_{11}NO_5$	262.070999	263.074365	14.3	$[M\text{-}H_2O+H]^+$	244.060434	10
SAR	$C_{20}H_{17}F_2N_3O_3$	386.131074	387.134436	21.8	$[M\text{-}CO_2+H]^+$	342.141245	10

repeatability expressed as the relative standard deviation (RSD), detection capability (CCβ), and specificity.

LOQ was determined as the minimum concentration of analytes with the signal-to-noise (S/N) ratio exceeding 10 by sequential injection of decreasing concentrations of matrix-matched standards.

CCβ at the ½ validation level (VL) was determined from the decision limit CCα, which in turn was calculated from 1.64 times the standard deviation at the ½ VL level using the following equation: $CC\beta = CC\alpha + 1.64 \times SD_{0.5VL}$.

Matrix effects (ME) were assessed in order to evaluate the degree of ion suppression or enhancement. ME was calculated by dividing the slope of the matrix-matched calibration curve after extraction (slopeM) and the slope of the calibration curve obtained using standard solution (slopeSTD) according to the following equation: ME(%) = 100 % × slopeM/slopeSTD. A value of 100 % indicated that there was no matrix effect, while values higher than 100 % pointed to ion enhancement, and values lower than 100 % indicated ion suppression.

Extraction recovery (ER) was calculated by dividing the slope of the matrix-matched calibration curves prior to extraction (slopeP) with the slopes of the matrix-matched calibration curves after extraction (slopeM) according to the following equation: ER(%) = 100 % × slopeP/slopeM.

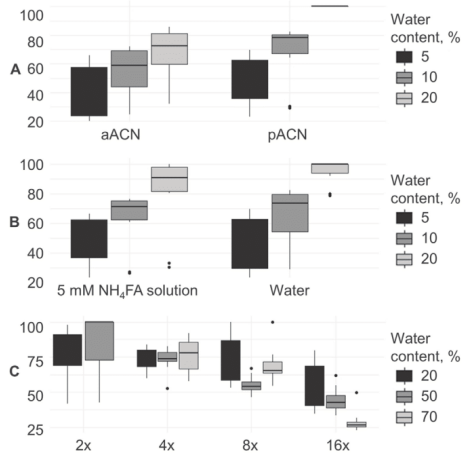

Fig. 1. The relative signal heights of ten quinolones during sample preparation optimisation: A) the effect of extraction solvent and water content; B) the effect of ammonium formate and water; C) the effect of dilution and water content.

3. Results and discussion

3.1. Sample preparation and clean-up

The objective of the study was to develop a fast and generic sample preparation procedure to ensure high sample throughput. It was found from our previous study that extraction with acidified acetonitrile followed by clean-up by freezing out was the most suitable procedure for the analysis of a broad range of veterinary drugs [18].

Minor optimisation of sample preparation was carried out. First, the effect of formic acid (FA) addition to the extraction solvent was evaluated, since lesser extraction of interfering matrix components was observed by Pugajeva [11]. The effect of water content in the final extract was examined simultaneously. It should be noted that the FA content in the final extract was kept constant at 0.1 % during all experiments. As shown in Fig. 1a, the extraction with pure acetonitrile gave the most intense signals for all compounds if 20 % of water was added. The introduction of 5 mM aqueous ammonium formate had no significant effect (Fig. 1b). Finally, the effect of dilution was tested, including by adding up to 70 % of water. As shown in Fig. 1c, the best results for most Qs were observed if the extract was diluted with an equal volume of pure water.

It was found that the $[M+K]^+$ adducts, instead of $[M+H]^+$, gave overall higher peaks for NAL, OXO and FLU. However, poor linearity was observed during the validation study, therefore $[M+H]^+$ adducts were selected for the quantification of all Qs.

The optimised sample preparation procedure consisted of extraction with pure acetonitrile, followed by freezing out as a clean-up step. The obtained extract was diluted to one half of the concentration with 0.1 % FA solution in water.

3.2. Instrumental method optimisation

Instrumental parameters were optimised consecutively. A matrix-matched standard containing concentrations corresponding to 100 μg kg^{-1} of each Q was used for the experiments. The obtained curves are shown in Fig. 2. The initial parameters were as follows: flow rate 150 μL h^{-1}, scan count 32, capillary voltage 4500 V, end-plate offset -500 V, nebuliser pressure 0.7 bar, dry gas flow rate 6.0 L min^{-1}, and drying temperature 220 °C. The parameters were optimised in the aforementioned order. Regarding scan count, a linear relationship was observed, as expected, and 32 scans were chosen because the intensities of all ions were sufficient and the analysis time was less than one minute. The optimised values of all the parameters were chosen by compromise between all of the compounds. The final parameters were as follows: flow rate 250 μL h^{-1}, scan count 32, capillary voltage 4000 V, end plate offset -100 V,

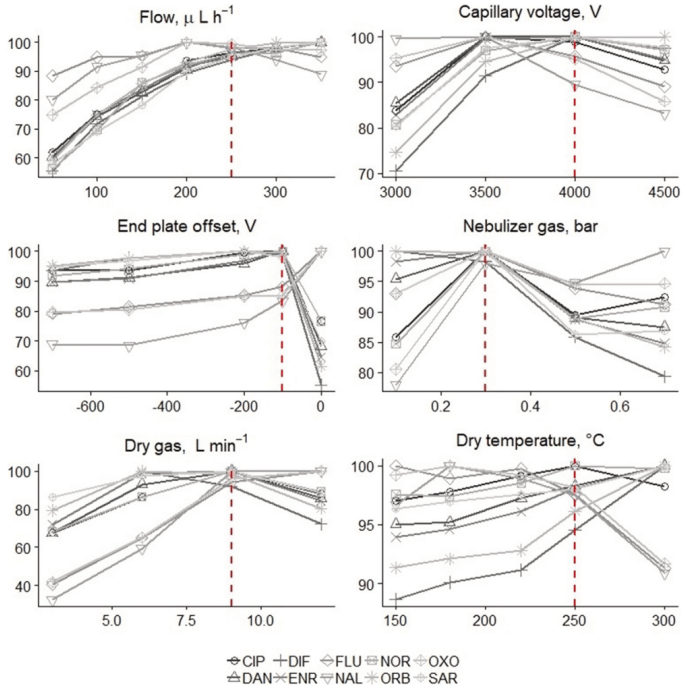

Fig. 2. Optimisation of instrumental parameters for ten quinolone type analytes.

nebuliser pressure 0.3 bar, dry gas flow rate 9.0 L min^{-1}, and drying temperature 250 °C.

3.3. Performance of the method

The analytical method validation for the determination of Qs in chicken meat was carried out at four concentration levels: 0.5, 1, 1.5, and 2 times of VL. The VLs were equal to the MRLs for CIP, DAN, DIF, ENR, FLU, and OXO or to the specific "levels of interest" for NAL, NOR, and ORB, as no MRL levels were defined for the latter three compounds. The VL of 30 μg kg^{-1} (MRL for *Salmonidae*) was chosen for SAR in accordance with the "cascade" principle laid down in Commission Regulation 2018/470 [19]. All of the concentration levels used in this validation study are listed in Table 2.

The sensitivity of the method was evaluated by measuring the LOQ. Matrix extracts spiked prior the analysis at the levels of 1, 5, 10, 25, and 50 μg kg^{-1} were used for the experiments. Two parallel samples were prepared and each was analysed in triplicate. The concentration levels for which the S/N ratios of quantitation ion signals were at least 10 were recorded as the LOQs. The LOQ values varied from 5 to 10 μg kg^{-1}. The differences of back-calculated values were in the range of 0.6–10.6% (Table 2). The obtained LOQs were always lower than the CCβ values obtained during the method validation.

An additional series of MS/MS fragmentation experiments was performed in order to achieve unambiguous confirmation and to prevent any false positive results. First, a solution containing all Qs was analysed by isolating the corresponding parent ions and applying increasing collision energies. Next, the most intense fragment signals and the corresponding collision energies were selected. The fragments were either $[M+H-H_2O]^+$ or $[M+H-CO_2]^+$ (Table 1). Finally, the matrix extracts fortified with 5, 10, and 25 μg kg^{-1} of each compound were analysed correspondingly. As can be seen from Table 1, the parent ions of FLU and OXO had very similar *m/z* values. Unambiguous distinction was possible due to the high mass accuracy (Fig. 3). The confirmatory fragments were detectable at the concentration level of 5 μg kg^{-1} for all of the compounds, hence providing confirmation at the LOQ level.

The CCβ value is defined as the lowest concentration of the substance that may be detected, identified, and/or quantified in a sample with an error probability of β (in this study, β = 5%). The calculated CCβ values are presented in Table 2, and they are lower than the validation levels for all compounds.

The specificity of the method was checked by analysing 20 blank samples. The mass spectra were monitored for peaks that can potentially interfere with the analytes of interest. Matrix interference was observed in the mass spectra of SAR. The effect of resolution was investigated by analysing spiked blank samples (10 μg kg^{-1}) using various time domain data sets. The acquisition time was directly proportional to the resolution, reaching 5 min at R = 500 000. As can be seen from Fig. 4, a minimum resolution of 187 032 (2 M data set) was necessary for distinguishing the SAR signal from interference. This value was chosen for the method since it provided a sufficient resolution and an acquisition time shorter than one minute. The interfering signal (*m/z* = 386.12922) was dis-

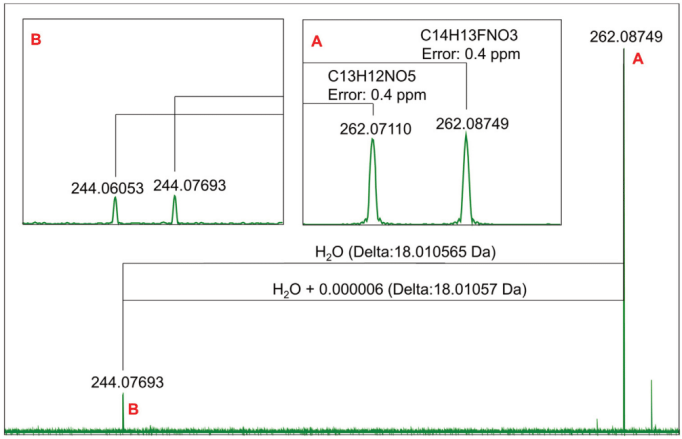

Fig. 3. MS/MS spectra of oxolinic acid (m/z = 262.07110) and flumequine (m/z = 262.08749): a) parent ions; b) fragment ions ([M-H_2O+H]$^+$).

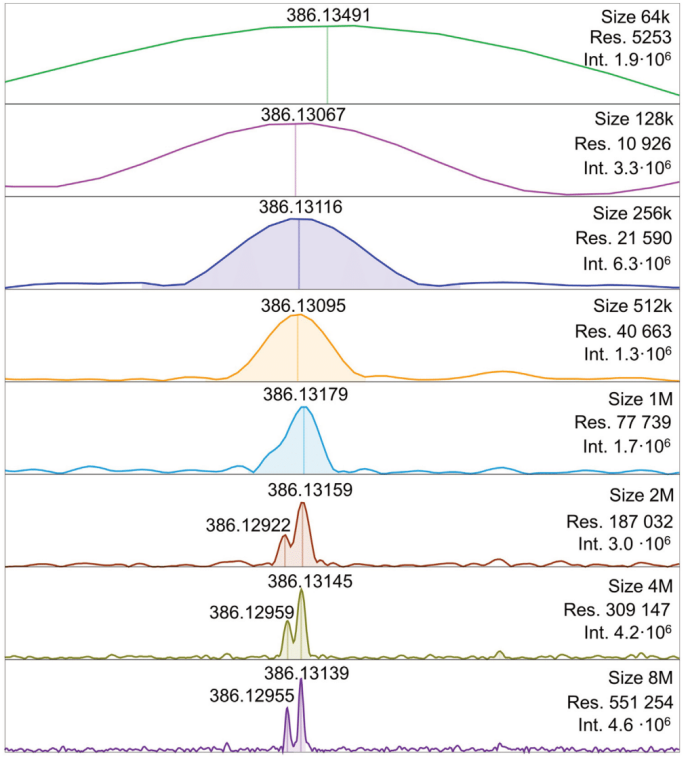

Fig. 4. Mass spectra of SAR and the interfering signal obtained using different resolutions.

Table 2
A summary of method performance.

	Compound	CIP	DAN	DIF	ENR	FLU	NAL	NOR	ORB	OXO	SAR
Validation study	VL, μg kg^{-1}	50	200	300	50	400	50	50	50	100	30
	Linearity	0.992	0.994	0.990	0.993	0.992	0.98	0.97	0.991	0.996	0.996
	CCα, μg kg^{-1}	25.6	103	161	25.6	205	25.7	26.6	25.2	51.1	15.1
	CCβ, μg kg^{-1}	26.2	106	172	26.2	210	26.4	28.2	25.5	52.2	15.2
	Precision, % (n = 24)	4.8	3.1	6.4	2.6	3.6	2.8	3.5	2.5	3.5	4.4
	Recovery, % (n = 24)	49	66	65	82	93	95	51	78	92	34
	Ion ratio range, %	17–19	19–29	22–23	20–22	15	12–15	17–20	19–20	14–15	21–23
	ME, %	36	38	42	39	6.4	8.4	36	40	14	25
	ER, %	47	66	99	78	90	80	43	73	92	73
LOQ study	LOQ, μg kg^{-1}	10	5	5	5	10	10	10	5	5	10
	Back-calculated value, μg kg^{-1}	9.6	4.8	4.7	5.4	9.3	9.2	10.1	4.7	5.5	8.9
	Difference, %	−4.3	−3.1	−5.9	8.6	−6.9	−7.6	0.6	−6.9	9.3	−10.6
	S/N ratio at the LOQ level	14	12	24	17	14	11	13	45	10	13

tinguishable from the analyte signal (m/z = 386.13159). Hence the method meets the specificity criterion.

The mean repeatability and accuracy were calculated from the data obtained by analysing blank samples fortified before extraction at 0.5, 1.0, 1.5, and 2.0 times the VL in 6 replicates for each level. Detailed information on the RSD values and the accuracy for each analyte is listed in Table 2. For the evaluation of ME, standard solutions and blank sample extracts fortified prior to the analysis at the aforementioned levels were analysed. The results showed a strong ion suppression effect for all Qs, especially NAL, OXO, and FLU. For the evaluation of ER, blank sample aliquots fortified before extraction and blank extracts fortified prior to the analysis at the same levels were analysed. The results are shown in Table 2. ER varied from 43 % for NOR to 99 % for DIF. The obtained results clearly indicate, that standard calibration cannot be used. Instead, procedural calibration must be performed.

3.4. Application to real samples

In order to evaluate the applicability of the proposed method, 19 chicken meat samples from different hypermarkets and farmers' markets in Latvia were analysed. ORB was the only Q detected during the initial full-scan analysis. A second injection for MS/MS analysis was performed to confirm the obtained results. The characteristic fragment was not observed, indicating a lack of ORB in the samples. Example of suspicious sample spectra and fragmentation pattern is presented in Fig.S2. The results were confirmed by a validated confirmatory LC–MS/MS multi-residue method used in the laboratory for the analysis of antibiotics.

Six samples of chickens treated with ENR were analysed in order to evaluate the method applicability, since commercial chicken samples did not contain any residues of Qs above the method LOQ. The results were compared with the previously described ultrasensitive UHPLC-MS/MS method for the determination of ENR and CIP in poultry [11]. The results obtained both by ICR-HRMS and UHPLC-QqQ-MS/MS methods are compiled in Table S1. The total concentration of CIP and ENR determined by ICR-HRMS method ranged between 102 and 1064 μg kg^{-1}. Obtained results were in good agreement with those obtained by the LC–MS/MS method, namely 148-1164 μg kg^{-1}.

4. Conclusions

An analytical method for the simultaneous detection, quantification and confirmation of ten quinolones in chicken meat by direct injection Fourier transform ion cyclotron resonance mass spectrometry was evaluated and validated. The elaborated analytical method provides a high throughput determination of quinolones in chicken, which is a major source of concern regarding the development of antibiotic resistance. The performance of the method was good for the detection of compounds above CCβ levels, proving the effectiveness of this methodology for fast routine analysis of these compounds. Finally, the method was successfully applied to real samples. Quinolones were not detected in any of the commercial samples. The obtained results were confirmed using the relevant confirmatory HPLC-MS/MS method. Analysis of treated chickens revealed that the developed method is suitable for the determination of total ciprofloxacin and enrofloxacin amount.

CRediT authorship contribution statement

L.E. Ikkere: Writing - original draft, Methodology, Investigation, Validation, Visualization. **I. Perkons:** Data curation, Software, Formal analysis, Methodology, Writing - review & editing. **I. Pugajeva:** Methodology, Writing - review & editing. **R. Gruzauskas:** Resources, Methodology. **E. Bartkiene:** Resources, Methodology. **V. Bartkevics:** Conceptualization, Supervision, Writing - review & editing.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2020.113389.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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