WYDZIAŁ BIOLOGII
i OCHRONY ŚRODOWISKA
Uniwersytet Łódzki

**Anna Barańska**

# Ocena właściwości prooksydacyjnych, genotoksycznych i proapoptotycznych wybranych uniepalniaczy bromofenolowych w jednojądrzastych komórkach krwi obwodowej człowieka

Determination of prooxidative, genotoxic and proapoptotic properties of selected bromophenolic flame retardants in human peripheral blood mononuclear cells

Praca doktorska

wykonana w Katedrze Biofizyki Skażeń Środowiska
Instytutu Biofizyki UŁ

pod kierunkiem
prof. dr. hab. Jaromira Michałowicza

Łódź, 2022

# PODZIĘKOWANIA

Najserdeczniejsze podziękowania składam
mojemu promotorowi
**prof. dr hab. Jaromirowi Michałowiczowi,**
za poświęcony mi czas, cenne uwagi
i sugestie w trakcie realizacji badań
naukowych oraz nieocenioną pomoc
podczas pisania niniejszej pracy doktorskiej.

Dziękuję serdecznie
**pracownikom**
**Katedry Biofizyki Skażeń Środowiska**
za wielokrotnie okazaną pomoc, życzliwość,
cenne wskazówki oraz tworzenie wspaniałej
atmosfery.

**Mężowi i Rodzicom**
za wsparcie, zrozumienie i wiarę we mnie.

**BADANIA PROWADZONE W RAMACH NINIEJSZEJ PRACY DOKTORSKIEJ BYŁY FINANSOWANE Z:**

- Dotacji celowej dla młodych naukowców oraz uczestników studiów doktoranckich w **2018r**. (kod projektu: B1811000001801.02), pt. „*Ocena wpływu genotoksycznego wybranych uniepalniaczy bromofenolowych na jednojądrzaste komórki krwi obwodowej człowieka”*oraz w **2019 r.** (kod projektu: B1911000002126.02), pt. *„Ocena potencjału apoptotycznego wybranych bromofenoli w leukocytach człowieka”.*

WYDZIAŁ BIOLOGII
i OCHRONY ŚRODOWISKA
Uniwersytet Łódzki

- Projektu badawczego PRELUDIUM 17 w latach **2020-2022** (kod projektu: B2011000001034101), pt. *„Porównanie potencjału apoptotycznego wybranych uniepalniaczy bromofenolowych w jednojądrzastych komórkach krwi obwodowej człowieka”***.**

NARODOWE
CENTRUM
NAUKI

# SPIS TREŚCI

1. Spis publikacji wchodzących w zakres rozprawy doktorskiej……..……5
2. Streszczenie w języku polskim……..7
3. Streszczenie w języku angielskim……...10
4. Cel naukowy rozprawy doktorskiej oraz omówienie wyników……..12
5. Dorobek naukowy……...30
6. Kopie publikacji wchodzących w zakres rozprawy doktorskiej…….35
7. Oświadczenia współautorów prac ……..……..106

# SPIS PUBLIKACJI WCHODZĄCYCH W ZAKRES ROZPRAWY DOKTORSKIEJ

1. **Włuka A.,**Woźniak A., Woźniak E., Michałowicz J. (2020).T*etrabromobisphenol A, terabromobisphenol S and other bromophenolic flame retardants cause cytotoxic effects and induce oxidative stress in human peripheral blood mononuclear cells (in vitro study).* Chemosphere, 261, 127705.

   Praca oryginalna
   Punkty MEiN: 140
   Impact Factor: 7,086

2. **Barańska A.,**Woźniak A., Mokra K., Michałowicz J. (2022).*Genotoxic mechanism of action of TBBPA, TBBPS and selected bromophenols in human peripheral blood mononuclear cells*. Frontiers in Immunology, 13, 869741.

   Praca oryginalna
   Punkty MEiN: 140
   Impact Factor: 8,786

3. **Barańska A.**, Sicińska P., Michałowicz J. (2022).*Apoptosis-inducing potential of selected bromophenolic flameretardants 2,4,6-tribromophenol and pentabromophenol in human peripheralblood mononuclear cells*. Molecules, 27, 5056.

   Praca oryginalna
   Punkty MEiN: 140
   Impact Factor: 4,927

4. **Barańska A.,** Bukowska B., Michałowicz J. (2022).*Determination of apoptotic mechanism of action of tetrabromobisphenol A and tetrabromobisphenol S in human peripheral blood mononuclear cells*: *A comparative study*. Molecules, 27, 6052.

   Praca oryginalna
   Punkty MEiN: 140
   Impact Factor: 4,927

5. Michałowicz J., **Włuka A.,** Bukowska B. (2022).*A review on environmental occurrence, toxic effects and transformation of man-made bromophenols.* Science of the Total Environment, 811, 152289.

   Praca przeglądowa
   Punkty MEiN: 200
   Impact Factor: 10,754

Sumaryczna wartość **Impact Factor** publikacji wchodzących w zakres rozprawy doktorskiej wynosi **36,479** (**760 punktów MEiN**).

# STRESZCZENIE W JĘZYKU POLSKIM

Bromoorganiczne uniepalniacze należą do jednych z najefektywniejszych substancji ograniczających spalanie materiałów syntetytycznych. Związki te są szeroko wykorzystywane w wielu gałęziach przemysłu, w tym przy produkcji urządzeń elektrycznych, elektronicznych, mebli, tekstyliów oraz innych produktów codziennego użytku. Produkcja bromowanych uniepalniaczy stanowi 25% rynku antypirenów, a ich zawartość w produkcie gotowym wynosi od 5% do 30%. Najpowszechniej stosowanym antypirenem jest tetrabromobisfenol A (TBBPA), który stanowi 60% globalnej produkcji substancji uniepalniających. TBBPA jest produkowany głównie w Stanach Zjednoczonych, Izrealu, Japonii oraz Chinach, jednak znajduje zastosowanie na całym świecie. Do omawianej grupy związków należą również tetrabromobisfenol S (TBBPS), 2,4,6-tribromofenol (2,4,6-TBP) oraz pentabromofenol (PBP).

Europejski Urząd ds. Bezpieczeństwa Żywności w 2012 roku stwierdził, iż ze względu na brak wystarczających badań toksykologicznych oraz danych dotyczących obecności ww. substancji w żywności i łańcuchu pokarmowym, nie jest możliwe określenie zagrożenia, jakie stwarzają one dla zdrowia człowieka.

Celem badawczym niniejszej pracy doktorskiej było porównanie wpływu wybranych uniepalniaczy bromofenolowych, tj.: tetrabromobisfenolu A, tetrabromobisfenolu S, 2,4,6-tribromofenolu oraz pentabromofenolu na jednojądrzaste komórki krwi obwodowej człowieka. Prowadzone analizy miały na celu określenie właściwości prooksydacyjnych, genotoksycznych oraz proapoptotycznych analizowanych związków w badanym modelu komórkowym.

W niniejszej pracy doktorskiej wykorzystano metody analityczne z zakresu spektrofotometrii, spektrofluorymetrii, cytofluorymetrii oraz mikroskopii fluorescencyjnej.

Uzyskane wyniki wykazały, że analizowane związki, a szczególnie TBBPA i PBP spowodowały znaczący spadek żywotności jednojądrzastych komórek krwi obwodowej człowieka oraz obniżyły poziom wewnątrzkomórkowego ATP. Odnotowano, że badane substancje spowodowały powstawanie reaktywnych form tlenu (RFT) oraz wzmagały peroksydację lipidów i uszkodzenia białek w badanych komórkach. Stwierdzono większy potencjał cytotoksyczny i oksydacyjny PBP w porównaniu do 2,4,6-TBP, co wiązało się z większą liczbą atomów bromu w cząsteczce tej substancji. W odniesieniu do bromobisfenoli, można stwierdzić, że obecność grupy sulfonowej w TBBPS zmniejszała

toksyczność tego związku w porównaniu do TBBPA posiadającego w swojej cząsteczce grupy metylowe (obecność mostka propanowego).

Kolejny etap badań obejmował analizę potencjału genotoksycznego badanych uniepalniaczy bromofenolowych. Stwierdzono, że analizowane substancje spowodowały pęknięcia jednoniciowe, a w mniejszym stopniu pęknięcia dwuniciowe DNA oraz indukowały powstawanie oksydacyjnych uszkodzeń zasad pirymidynowych, a szczególnie zasad purynowych w DNA. Największe zmiany w ww. parametrach odnotowano pod wpływem TBBPA oraz PBP, najmniejsze w wyniku oddziaływania TBBPS. Zaobserwowano również, że jednojądrzaste komórki krwi obwodowej człowieka efektywnie naprawiały powstałe uszkodzenia DNA, jednak nie były w stanie (z wyjątkiem komórek inkubowanych z TBBPS) całkowicie usunąć powstałych zmian. Wykazano także, iż badane substancje nie tworzyły adduktów z DNA.W świetle uzyskanych wyników, można uznać, że badane uniepalniacze spowodowały uszkodzenia DNA pośrednio poprzez generowanie RFT, form rodnikowych i innych reaktywnych produktów, natomiast nie oddziaływały bezpośrednio na materiał genetyczny.

Analizowane związki wykazały również potencjał apoptotyczny w jednojądrzastych komórkach krwi obwodowej człowieka. Stwierdzono, że badane substancje zwiększały liczbę komórek apoptotycznych, podwyższały poziom jonów wapnia w cytozolu komórek, obniżały transbłonowy potencjał mitochondrialny, wzmagały aktywność kaspazy 8., 9. i 3., indukowały rozszczepienie PARP1 i fragmentację DNA oraz spowodowały zmiany w kondensacji chromatyny. Stwierdzono, większe zmiany w badanych parametrach apoptotycznych pod wpływem TBBPA i PBP w porównaniu do 2,4,6-TBP, a szczególnie TBBPS. Wykazano także, iż analizowane związki(z wyjątkiem TBBPS) w przebiegu procesu apoptotycznego w większym stopniu angażowały szlak mitochondrialny.

Podsumowując, analizowane uniepalniacze bromofenolowe wykazywały zróżnicowany potencjał prooksydacyjny, genotoksyczny i proapoptotyczny w jednojądrzastych komórkach krwi obwodowej człowieka. Wzrost poziomu RFT, uszkodzenia oksydacyjne lipidów, białek i DNA oraz zmiany w wewnątrzkomórkowym poziomie jonów wapnia i transbłonowym potencjale mitochondrialnym odnotowano w badanych komórkach pod wpływem TBBPA, PBP i 2,4,6-TBP w stężeniach, które oznaczane były w organizmie człowieka w warunkach narażenia środowiskowego oraz zawodowego na te substancje. Silniejsze zmiany w badanych parametrach spowodowane przez tetrabromobisfenol A w porównaniu do TBBPS wskazują

na zasadność zastępowania TBBPA przez ten analog w przemyśle, jako mniej toksyczny substytut.

# STRESZCZENIE W JĘZYKU ANGIELSKIM

## SUMMARY

Bromophenolic flame retardants (BRFs) belong to a group of the most effective substances reducing the combustion of synthetic materials. These compounds are widely employed in the industry, including the production of electric and electronic devices, as well as furniture, textiles and other everyday products. The production of BFRs includes 25% of antypirenes market, and their content in finished products is from 5% to 30%. The most commonly produced antypirene is tetrabromobisphenol A (TBBPA) that includes 60% of global production of BFRs. TBBPA is mainly produced in the United States, Israel, Japan and China, but it is used globally. Other compounds that belong to BRFs are tetrabromobisphenol S (TBBPS), 2,4,6-tribromophenol (2,4,6-TBP) and pentabromophenol (PBP).

In 2012 the European Food Safety Authority stated that due to insufficient toxicological studies on bromophenolic flame retardants, as well as in adequate data concerning the presence of BFRs in edibles and food chain, it is impossible to determine the threat that is posed by these substances to the human organism.

The purpose of this doctoral thesis was to compare the effect of selected BFRs, such as tetrabromobisphenol A, tetrabromobisphenol S, 2,4,6-tribromophenol and pentabromophenol on human peripheral blood mononuclear cells (PBMCs). The analysis aimed to determine prooxidative, genotoxic and proapoptotic properties of these substances in the investigated cellular model.

In this study, analytic methods that included spectrophotometry, spectrofluorimetry, cytofluorimetry and fluorescence microscopy were used.

The obtained results have shown that tested compounds, and particularly TBBPA and PBP caused significant decrease of PBMCs viability and depleted the level of intracellular ATP. It was also observed that studied substances induced reactive oxygen species (ROS) formation and caused damage to lipids and protein in incubated cells. Stronger cytotoxic and prooxidative potential was noted for PBP in comparison to 2,4,6-TBP, which was associated with higher number of bromine atoms in this molecule. It is also highly probable that the presence of sulfonyl group in TBBPS determined its lower cytotoxic and prooxidative potential in comparison to TBBPA having methyl groups in its molecule.

The next step of this study concerned evaluation of genotoxic potential of tested BFRs. It has been revealed that examined substances induced mainly single stranded

DNA breaks, and to a lesser extent, double stranded DNA breaks formation. Those compounds also caused oxidative damage to pyrimidines, and more strongly to purines. The greatest changes were noted in cells treated with TBBPA and PBP, while the smallest in PBMCs incubated with TBBPS. It was also observed that PBMCs efficiently repaired DNA damage caused by tested BFRs, but they were unable (except for the cells preincubated with TBBPS) to completely remove DNA lesions. Moreover, it was found that tested BFRs were unable to create adducts with DNA. In the light of obtained results, it may be concluded that studied compounds caused DNA damage indirectly by generation of ROS, organic radicals or/and other reactive products, but they did not influence directly genetic material of PBMCs.

The tested substances also exhibited significant apoptotic potential in human PBMCs. It has been shown that BFRs increased cytosolic calcium ion level, reduced transmembrane mitochondrial potential, activated caspase-8, -9, and -3, as well as induced PARP1 cleavage, DNA fragmentation and chromatin condensation. Stronger apoptotic changes were observed in PBMCs incubated with TBBPA and PBP when compared with cells treated with 2,4,6-TBP, and particularly TBBPS. It was also noticed that examined compounds (with exception for TBBPS) more strongly activated mitochondrial apoptotic pathway.

Summing up, it has been found that tested bromophenolic flame retardants exhibited different prooxidative, genotoxic and proapoptotic potential in human peripheral blood mononuclear cells. An increase in ROS level, damage to lipids, protein and DNA, as well as alterations in cytosolic calcium ion level and transmembrane mitochondrial potential were noted in PBMCs exposed to TBBPA, PBP and 2,4,6-TBP in the concentrations that were determined in humans environmentally or occupationally exposed to these compounds. Stronger cytotoxic and genotoxic potential of tetrabromobisphenol A in comparison to TBBPS indicates the validity of the substitution of TBBPA by this analog in the industry.

# CEL NAUKOWY ORAZ OMÓWIENIE WYNIKÓW

## WPROWADZENIE

Bromofenolowe związki ograniczające palność są substancjami chemicznymi powszechnie występujacymi w środowisku i bezposrednim otoczeniu człowieka. Związki te są stosowane w licznych gałęziach przemysłu w tym przy produkcji sprzętu elektrycznego i elektronicznego, polimerów, mebli oraz innych produktów codziennego użytku (Lai i wsp., 2015; Jarosiewicz i Bukowska 2017; Michałowicz i wsp., 2022). Najpowszechniej stosowanym uniepalniaczem bromofenolowym jest tetrabromobisfenol A (TBBPA), którego roczną produkcję w Stanach Zjednoczonych, Japonii oraz Izraelu oszacowano na poziomie ok. 170 tysięcy ton. Substancja ta stanowi 60% globalnego rynku wszystkich bromowanych związków uniepalniających (Jarosiewicz i Bukowska, 2017). Tetrabromobisfenol S (TBBPS) został wprowadzony na rynek jako substytutdla stosowanego TBBPA i jest wykorzystywany głównie jako dodatek do tworzyw systetycznych.Innymi powszechnie stosowanymi bromofenolowymi uniepalniaczami są 2,4,6-tribromofenol (2,4,6-TBP) oraz pentabromofenol (PBP) (Michałowicz i wsp. 2022). Produkcja 2,4,6-TBP ma miejsce głównie w Chinach, Japonii (3600 ton) i Stanach Zjednoczonych(od 4500 do 23000 ton) (Covaci i wsp., 2011;Jarosiewicz i wsp., 2019), natomiast dokładna skala produkcji PBP nie jest znana, przy czym substancja ta jest głównie produkowana w Chinach i Stanach Zjednoczonych (Michałowicz i wsp. 2022).

Ze względu na swoje zastosowanie, bromofenoloweuniepalniacze są obecne w produktach powszechnie używanych w gospodarstwach domowych, tj.: sprzęt elektryczny i elektroniczny, meble oraz środki ochrony zdrowia. Związki te oznaczono także w produktach spożywczych, wodzie pitnej oraz kurzu pomieszczeń użytkowych (Saito i wsp., 2007; Michałowicz i wsp., 2011; Michałowicz i wsp., 2022). Omawiane substancje wykryto w organizmie człowieka (nerki, płuca, wątroba, tkanka tłuszczowa) w populacjach Stanów Zjednoczonych, Kanady, Japonii, Tajwanu i krajów europejskich (Alaee i wsp., 2002; de Wit i wsp., 2002; Hale i wsp. 2003; Birnbaum i Staskal 2004; Remberger i wsp., 2004; Stapleton i wsp., 2005; Ni i wsp., 2008; Abdallah i wsp., 2008; Takigami i wsp., 2009; Wang i wsp., 2015).

Narażenie na TBBPA, TBBPS, 2,4,6-TBP oraz PBP jest wynikiem przenikania tych substancji przez skórę, układ pokarmowy oraz układ oddechowy (National Toxicology

Program 2013; Schauer i wsp., 2016; Jarosiewicz i Bukowska 2017; Michałowicz i wsp., 2022). TBBPA został wykryty w 44% próbek mleka ludzkiego w zakresie stężeń od 0,06 do 37,34 ng/g tłuszczu oraz w 30% próbek surowicy pochodzących zarówno od matek, jak ich płodów (Cariou i wsp., 2008). Ponadto TBBPA oraz 2,4,6-TBP oznaczono w próbkach mleka matek zamieszkałych Stany Zjednoczone, Czechy, Japonię, Chiny oraz Norwegię w zakresie stężeń od< 0,002 do 12,5 ng/g tłuszczu (Abdallah, 2016; Abdallah i Harrad, 2010; Lankova i wsp., 2013; Nakao i wsp., 2015; Ohta i wsp., 2004). Podobne badania przeprowadzono w Japonii, gdzie TBBPA wykryto w osoczu krwi mężczyzn w średnim stężeniu 950 pg/g świeżej masy ciała (Fujii i wsp., 2014). Znaczące stężenie 2,4,6-TBP (średnia 5,57 ± 4,05 μg/L)oznaczono w próbkach moczu mieszkańców Chin (Feng i wsp., 2016).Wykazano także akumulację wysokich stężeń tych substancji u osób narażonych zawodowona ich wpływ. Przykładowo, Gutierez i wsp. (2005) oznaczyli 2,4,6-TBP w średnim stężeniu 6 mg/L moczu pracowników tartakóww Chile.

TBBPA jestnajlepiej przebadanym związkiem pod kątem toksyczności. U szczurów rasy Sprague-Dawley, którym podawano TBBPA w dawkach 250 mg/kg i 500 mg/kg, zaobserwowano obniżenie poziomu tyroksyny w surowicy. Ponadto TBBPA w dawce 500 mg/kg zwiększał masę wątroby, indukował aktywność cytochromu CYP2B1 i powodował konstytutywną ekspresję receptorów androstanowych w wątrobie badanych zwierząt. TBBPA wykazywał także zdolność do indukowania reaktywnych form tlenu, które, jak stwierdzili Choi i wsp. (2011),były odpowiedzialne za hepatotoksyczne i nefrotoksyczne działanie tej substancji u gryzoni. Stwierdzonoponadto, że TBBPA charakteryzował się silnym działaniem prozapalnym w komórkach linii łożyska człowieka (HTR-8SVneo) poprzez zwiększenie uwalniania interleukiny 6. i 8. oraz prostaglandyny E2. W powyższym badaniu TBBPA zwiększał ekspresję genów zaangażowanych w szlaki zapalne i hamował uwalnianie transformującego czynnika wzrostu β (Park i wsp., 2014).

TBBPS jest sukcesywnie wprowadzany na rynek jako substytut tetrabromobisfenolu A ze względu na rosnącą liczbę danych wskazujących na toksyczne i potencjalnie rakotwórcze działanie TBBPA. TBBPS posiada grupę sulfonową, podczas gdy TBBPA grupy metylowe (mostek propanowy). Grupa sulfonowa wykazuje mniejszą toksyczność niż grupy metylowe, dlatego sugeruje się, że TBBPS będzie charakteryzował się mniejszą szkodliwościąod swego analogu. Niestety, brakuje danych literaturowych potwierdzających to założenie, aprzykładowo badania Liang i wsp. (2019) wykazały, że TBBPA i TBBPS charakteryzowały się podobną toksycznościąwzględem ludzkich embrionalnych komórek macierzystych.

Oba związki zaburzały rozwój ektodermy neuronalnej, wpływały na wzrost aksonów i transmisję neuronalną, a także zaburzały szlaki sygnałowe WNT i AHR. Ponadto stwierdzono, że TBBPS zmieniał rytm okołodobowy we wczesnych stadiach życia ryb (danio pręgowany) i spowodował opóźnienia rozwojowe w embrionach badanego gatunku (Ding i wsp., 2022).

Lee i wsp. (2016) przeprowadzili badanie w którym myszom podawano wysokie dawki 2,4,6-TBP (40 mg/kg i 250 mg/kg). Zaobserwowali oni, że 2,4,6-TBP obniżył poziom dejodynazy 1 oraz indukował limfostazę, a takżezmiany w aktywności receptora tarczycowego (TRBβ 2). W innym badaniu, ekspozycja embrionów danio pręgowanego na 2,4,6-TBP w stężeniu 0,3 µg/L i 3 µg/L przez 120 dni skutkowała zmianami w poziomie estradiolu i testosteronu u osbników obu płci badanych zwierząt. Ponadto odnotowano wzrost transkrypcji genów steroidogennych w mózgu i jądrach osobników płci męskiej oraz spadek tych parametrów w jajnikach osbników płci żeńskiejbadanego gatunku ryby (Deng i wsp., 2010).

Liczba badań toksykologicznych dotyczących PBP jest bardzo ograniczona. Meerts i wsp. (2010) zaobserwowali, że substancja ta wiąże się z ludzką transtyretyną, a następnie wypiera tyroksynę, co w konsekwencji prowadzi do obniżenia jej poziomu w surowicy krwi, imoże skutkować niedoczynnością tarczycy.

Należy zaznaczyć, że wyniki badań przeprowadzone na bezjądrzastych komórkach krwi(dojrzałych erytrocytach) człowieka wykazały, że TBBPA, TBBPS, 2,4,6-TBP i PBP indukowały proces hemolizy i apoptozy tych komórek, utleniały hemoglobinę, zmieniały aktywność systemu antyoksydacyjnego oraz spowodowały zmiany strukturalne i funkcjonalne w błonie krwinek czerwonych (Jarosiewicz i wsp., 2017; 2019a; 2019b; 2021).

Jednojądrzaste komórki krwi obwodowej człowieka odgrywają kluczową rolę w układzie immunologicznym organizmu. Są one odpowiedzialne za produkcję przeciwciał, usuwanie komórek zakażonych wirusami i komórek nowotworowych, a także za regulację odpowiedzi układu odpornościowego (LaRosa i Orange, 2008). Udowodniono, że uszkodzenie tych komórek, a w szczególności limfocytów (np. przez ksenobiotyki) może przyczynić się do dysfunkcji układu odpornościowego, co może prowadzić do rozwojuchorób autoimmunologicznychi nowotworów (Ratomski i wsp., 2007; Hait i wsp., 2017).

Pomimo, że TBBPA jest najlepiej przebadanym uniepalniaczem bromofenolowym, obecne prace badawcze nadal nie pozwalają na udzielenie odpowiedzi, czy związek ten jest toksyczny dla ludzi, natomiastliczba badań toksykologicznych dotyczących wpływu TBBPS,

2,4,6-TBP oraz PBP jest zdecydownie niewystarczająca. Z tego powodu podjęcie badań w zakresie tej tematyki i określenie mechanizmu działania TBBPA, TBBPS, 2,4,6-TBP i PBP w jednojądrzastych komórkach krwi obwodowej człowieka było w pełni zasadne.

## CEL PRACY

Celem badawczym niniejszej pracy doktorskiej było określenie mechanizmu działania wybranych uniepalniaczy bromofenolowych, tj.: tetrabromobisfenolu A, tetrabromobisfenolu S, 2,4,6-tribromofenolu oraz pentabromofenolu w jednojądrzastych komórkach krwi obwodowej człowieka.

Prowadzone badania miały na celu ocenę właściwości prooksydacyjnych, genotoksycznych oraz proapoptotycznych analizowanych związków w badanym modelu komórkowym.

## MATERIAŁ BADAWCZY I METODY

### Materiał badawczy

W badaniach wykorzystano jednojądrzaste komórki krwi obwodowej człowieka, które wyizolowano z kożuszka leukocytarno-płytkowego zakupionego w Regionalnym Centrum Krwiodawstwa i Krwiolecznictwa w Łodzi. Krew, która posłużyła do izolacji kożuszka pochodziła od zdrowych dawców. Badania uzyskały zgodę Komisji do spraw bioetyki badań naukowych UŁ (Nr. 7/KBBN-UŁ/II/2015).

### Badane związki

- Tetrabromobisfenol A o czystości 99% został zakupiony w LGC Standards (Niemcy).
- Tetrabromobisfenol S o czystości 98,8% został zsyntetyzowany w Instytucie Przemysłu Organicznego w Warszawie.
- 2,4,6-Tribromofenol o czystości ≤ 100% zakupiono w firmie Sigma-Aldrich (USA).
- Pentabromofenol o czystości 99% został zakupiony w LGC Standards (Niemcy).

### Przygotowanie prób

Do zawiesiny komórek (gęstość 1 x $10^6$ – 4 x $10^6$ w zależności od zastosowanej metody) dodawano przygotowane roztwory związków (o odpowiednich stężeniach wyjściowych) rozpuszczonych w dimetylosulfotlenku (DMSO), uzyskując końcowe stężenia substancji od 0,0001 do 100 µg/ml. Próby kontrolne stanowiły komórki inkubowane z DMSO. Końcowe stężenie DMSO w próbach kontrolnych i badanych wyniosło 0,2%.

**Metody**

- Oznaczenie liczby (odsetka) żywych oraz martwych (nekrotycznych) komórek za pomocą podwójnego barwienia kalceiną-AM i jodkiem propidyny przy zastosowaniu cytometrii przepływowej.
- Analiza lumenometryczna poziomu wewnątrzkomórkowego ATP.
- Określenie poziomu reaktywnych form tlenu za pomocą sondy fluorescencyjnej dioctanu 2',7'- dichlorodihydrofluoresceiny ($DCFH_2$-DA) i cytometrii przepływowej.
- Spektrofluorometryczne oznaczenie poziomu peroksydacji lipidów przy użyciu kwasu cis-parynarowego.
- Oznaczenie poziomu utleniania białek poprzez pomiar fluorescencji tryptofanu.
- Oznaczenie poziomu uszkodzeń jedno- i dwuniciowych DNA przy użyciu metody kometowej w wersji alkalicznej i mikroskopii fluorescencyjnej.
- Oznaczanie poziomu uszkodzeń dwuniciowych DNA przy użyciu metody kometowej w wersji neutralnej i mikroskopii fluorescencyjnej.
- Oznaczanie z użyciem metody kometowej w wersji alkalicznej i mikroskopii fluorescencyjnej uszkodzeń oksydacyjnych puryn i pirymidyn w DNA z wykorzystaniem endonukleazy III oraz glikozylazy 8-oksoguaniny.
- Ocena poziomu naprawy DNA z wykorzystaniem metody kometowej w wersji alkalicznej i mikroskopii fluoroscencyjnej.
- Ocena zdolności tworzenia adduktów z DNA przy użyciu plazmidu *E. coli* (pUC 19) oraz elektroforezy w żelu agarozowym.
- Ilościowa ocena zmian apoptotycznych z zastosowaniem podwójnego barwienia aneksyną V wyznakowaną izotiocyjanianem fluoresceiny i jodkiem propidyny orazdetekcją z wykorzystaniem cytometrii przepływowej.
- Analiza poziomu jonów wapnia w komórce z użyciem znacznika Fluo 3-AM i cytometrii przepływowej.
- Spektrofotometryczne oznaczenie poziomu mitochondrialnego potencjału transbłonowego przy użyciu sondy fluoroscencyjnej MitoLiteCMXRos.
- Fluorymetryczna ocena aktywności kaspazy3. przy zastosowaniu substratu peptydowego acetylo-Asp-Glu-Val-ASP-7-amino-4-metylokumaryny.
- Fluorymetryczna ocena aktywności kaspazy 8. przy zastosowaniu substratu peptydowego acetylo-Ile-GluThr-Asp-7-amino-4-metylokumaryny.

- Spektrofotometryczna ocena aktywności kaspazy9. przy zastosowaniu substratu peptydowego acetylo-Leu-Glu-His-Asp-p-nitroaniliny.
- Analiza poziomu fragmentów o masie 85 kDa powstających w wyniku rozszczepienia PARP-1 z zastosowaniem przeciwciała monoklonalnego HLNC4 skoniugowanego ze znacznikiem Alexa Fluor 488 i pomiarem techniką cytometrii przepływowej.
- Ocena poziomu pęknięć DNA z wykorzystaniem metody TUNEL poprzez wyznakowanie końców 3'-OH jedno- i dwuniciowych fragmentów DNA znakowanych bromowanymi nukleotydami trifosforanudeoksyurydyny (Br-dUTP).
- Ocena zmian w kondensacji chromatyny z wykorzystaniem podwójnego barwienia znacznikami Hoechst33342 i jodkiem propidyny oraz mikroskopii fluorescencyjnej.

## OMÓWIENIE WYNIKÓW

Cykl artykułów wchodzących w skład niniejszej rozprawy doktorskiej otwiera publikacja pt. „T*etrabromobisphenol A, terabromobisphenol S and other bromophenolic flame retardants cause cytotoxic effects and induce oxidative stress in human peripheral blood mononuclear cells (in vitro study)”.* **Praca oryginalna obejmuje pierwszy etap badań, których celem była ocena zmian w żywotności oraz zmian w poziomie stresu oksydacyjnegopod wpływem wybranych uniepalniaczy bromofenolowych, tj.: tetrabromobisfenolu A (TBBPA), tetrabromobisfenolu S (TBBPS), 2,4,6-tribromofenolu (2,4,6-TBP) i pentabromofenolu (PBP) w jednojądrzastych komórkach krwi obwodowej człowieka.**

Ocena żywotności analizowana była po 1-godzinnej oraz 24-godzinnej inkubacji komórek ze związkami i pozwoliła dobrać odpowiednie stężenia badanych substancji do prowadzenia dalszych analiz. Znacząco większy spadek żywotności komórek (wzrost liczby komórek nekrotycznych) odnotowano pod wpływem wszystkich badanych związków (od stężenia 25 μg/ml, TBBPS od 50 μg/ml)po 24-godzinnej inkubacji. Zaobserwowano, że TBBPA i PBP indukowały silniejsze zmiany w omawianym parametrze, natomiast najsłabsze zmiany stwierdzono w komórkach narażonych na TBBPS. Wykazano także, iż PBP spowodował silniejszy spadek żywotności komórek w porównaniu z 2,4,6-TBP, co prawdopodobnie wiązało się z większą liczbą atomów bromów w cząsteczce tej substancji. Ponadto silniejszy spadek żywotności komórek pod wpływem TBBPA w porównaniu do TBBPS mógł wynikać z obecności grupy sulfonowej w cząsteczce tego związku, która w mniejszym stopniu niż obecność grup metylowych (mostka propanowego) w cząsteczce TBBPA decydowała o potencjale cytotoksycznym tej substancji.

Stwierdzono, że wszystkie analizowane związki, a w szczególności TBBPA i PBP (od stężenia 25 μg/ml) spowodowały znaczący (szczególnie po 24-godzinnej inkubacji) spadek poziomu wewnątrzkomórkowego ATP. Najsłabsze zmiany odnotowano w wyniku oddziaływania TBBPS, który wyłącznie w stężeniu 100 μg/ml obniżył poziom badanego parametru. Uzyskane wyniki wykazały, że wyczerpywanie się puli ATP w jednojądrzastych komórkach krwi obwodowej człowieka prowadziło do spadku ich żywotności, a różnice w zdolności obniżania poziomu ATP przez badane substancje odzwierciedlały ich zróżnicowany potencjał w indukowaniu śmierci nekrotycznej badanych komórek.

Analizowane związki w niskich stężeniach (1-godzinna inkubacja), wykazywały zdolność generowania reaktywnych form tlenu (RFT) w badanych komórkach. Największy wzrost poziomu RFT odnotowano pod wpływem TBBPA (już od stężenia 0,0001 µg/ml). Ponadto TBBPA w wyższych stężeniach (od 1µg/ml) spowodował najsilniejszy wzrost poziomu silnie reaktywnych form tlenu (1-godzinna inkubacja).

Wzmożona produkcja RFT prowadzi do uszkodzeń oksydacyjnych makromolekuł komórkowych. Dlatego kolejnym etapem badań była ocena wpływu badanych uniepalniaczy bromofenolowych na indukcję uszkodzeń oksydacyjnych białek i lipidów. Odnotowano, że badane związki wzmagają peroksydację lipidów, przy czym najsilniejszy wpływ na zmiany w badanym parametrze wykazano w komórkach inkubowanych z TBBPA (od 1 µg/ml), szczególnie po dłuższym (24-godzinnym) czasie inkubacji. Wykazano także, że TBBPS (wyłącznie w stężeniu 20 µg/ml) spowodował najsłabsze zmiany w peroksydacji lipidów. Badane substancje powodowały również uszkodzenia białek po 1-godzinnej, a szczególnie 24-godzinnej inkubacji. Dłuższy czas inkubacji spowodował, że TBBPA, TBBPS oraz PBP (1 – 20 µg/ml) indukowały znaczne zmiany w badanym parametrze, natomiast słabszy efekt odnotowano pod wpływem 2,4,6-TBP.

Druga praca oryginalna pt. „*Genotoxic mechanism of action of TBBPA, TBBPS and selected bromophenols in human peripheral blood mononuclear cells*” obejmuje **zakres badań dotyczących określenia mechanizmu genotoksycznego działania TBBPA, TBBPS, 2,4,6-TBP oraz PBP w jednojądrzastych komórkach krwi obwodowej człowieka.**

Wzrost poziomu reaktywnych form tlenu (obserwowany w niniejszej pracy) oraz form reaktywnych cząsteczek przyczynia się do uszkadzania struktury DNA przez ksenobiotyki. Tym samym w kolejnym etapie badań dokonano oceny potencjału genotoksycznego badanych uniepalniaczy bromofenolowych w jednojądrzastych komórkach krwi obwodowej człowieka.

Wykazano, że badane związki (24-godzinna inkubacja) spowodowały powstanie zarówno pojedynczych, jak i podwójnych pęknięć nici DNA. Najsilniejsze uszkodzenia odnotowano pod wpływem PBP i TBBPA, które od stężenia 0,01 µg/ml wzmagały zmiany w badanym parametrze. Słabsze zmiany odnotowano pod wpływem 2,4,6-TBP, a szczególnie w wyniku oddziaływania TBBPS, który indukował wzrost poziomu uszkodzeń DNA wyłącznie w stężeniu 10 µg/ml. Stwierdzono ponadto, że badane związki indukowały uszkodzenia dwuniciowe DNA, jednak wyłącznie w stężeniu 10 µg/ml.

Kolejnym badanym parametrem była ocena oksydacyjnych uszkodzeń puryn i pirymidyn w DNA jednojądrzastych komórek krwi obwodowej człowieka. Spośród analizowanych substancji wyłącznie TBBPA (24-godzinna inkubacja) w niższym stężeniu (0,1 µg/ml) spowodował niewielki wzrost poziomu utlenionych pirymidyn, natomiast wszystkie badane związki spowodowały istotne zmiany w badanym parametrze wyłącznie w najwyższym stężeniu 1 µg/ml. Wykazano, że badane uniepalniacze bromofenolowe w większym stopniu uszkadzały puryny niż pirymidyny w jednojądrzastych komórkach krwi obwodowej człowieka. Największe zmiany odnotowano pod wpływem TBBPA, natomiast najmniejsze w wyniku działania TBBPS. Należy nadmienić, iż zarówno TBBPA, jak i 2,4,6-TBP już w niskich stężeniach (0,01 µg/ml) spowodowały oksydacyjne uszkodzenia zasad purynowych.

Zaobserwowano również, że jednojądrzaste komórki krwi obwodowej człowieka efektywnie naprawiały uszkodzenia DNA spowodowane działaniem badanych związków, jednak nie były w stanie całkowicie usunąć (z wyjątkiem komórek traktowanych TBBPS) powstałych zmian.

W pracach badawczych wykazano, że uszkodzenia DNA mogą powstawać w wyniku bezpośredniego oddziaływania ksenobiotyków na jego strukturę. Tym samym dokonano analizy tworzenia ad duktów przez badane związki z DNA przy użyciu plazmidu pUC19. Wykonane oznaczenia wykazały, że analizowane substancje nie były zdolne do tworzenia adduktów z DNA. W świetle uzyskanych wyników można stwierdzić, że oznaczone zmiany w strukturze DNA były indukowane pośrednio przez czynniki uszkadzające, takie jak RFT oraz inne formy reaktywne generowane przez badane uniepalniacze bromofenolowe.

Należy nadmienić, iż utlenianie puryn było spowodowane przez TBBPA oraz 2,4,6-TBP w stężeniach, które wykryto u ludzi narażonych środowiskowo na te substancje, natomiast powstawanie uszkodzeń jednoniciowych oraz oksydacyjnych uszkodzeń zasad DNA stwierdzono pod wpływem ww. związków w stężeniach oznaczonych u ludzi narażonych zawodowo na te uniepalniacze.

**Ostatnim etapem rozprawy doktorskiej była ocena potencjału apoptotycznego TBBPA, TBBPS, 2,4,6-TBP oraz PBP w jednojądrzastych komórkach krwi obwodowej człowieka** na opis której składają się dwie prace oryginalne pt. (1) „*Determination of apoptotic mechanism of action of tetrabromobisphenol A and tetrabromobisphenol S in human peripheral blood mononuclear cells: A comparative study*" oraz (2)*„Apoptosis-*

*inducing potential of selected bromophenolic flame retardants 2,4,6-tribromophenol and pentabromophenol in human peripheral blood mononuclear cells”.*

Pierwszym z badanych parametrów było ilościowe określenie zmian apoptotycznych w jednojądrzastych komórkach krwi obwodowej człowieka. Wykazano, że proces apoptozy najsilniej wzmagany był przez TBBPA oraz PBP (począwszy od stężenia 5µg/ml), natomiast najsłabiej w wyniku oddziaływania TBBPS, który indukował apoptozę wyłącznie w stężeniu 50µg/ml.

Istotnym czynnikiem inicjującym apoptozę jest wzrost poziomu jonów wapnia w komórce. Zaobserwowano, że badane uniepalniacze już w niskim stężeniu (0,1 µg/ml) spowodowały wzrost poziomu analizowanego parametru. Wzrost poziomu jonów wapnia w cytozolu komórki może przyczyniać się do zmian funkcjonalnych w mitochondrium skutkujących spadkiem transbłonowego potencjału tego organellum. W niniejszej pracy, spadek wartość transbłonowego potencjału mitochondrialnego odnotowano w jednojądrzastych komórkach krwi obwodowej człowieka głównie pod wpływem TBBPA i PBP (od stężenia 1µg/ml), natomiast w znacznie mniejszym stopniu w wyniku oddziaływania TBBPS.

Spadek transbłonowego potencjału mitochondrialnego prowadzi do uwolnienia z mitochondrium licznych czynników inicjujących proces apoptozy. Cząsteczkami uczestniczącymi aktywnie w procesie apoptozy są kaspazy, w tym prokaspaza 9. uwalniana z przestrzeni mitochondrialnej, a uczestnicząca w tzw. wewnętrznym szlaku apoptotycznym. Inną proteazą uczestniczącą w tzw. zewnętrznym szlaku apoptotycznym jest kaspaza 8. Obydwie kaspazy zdolne są do aktywacji efektorowej kaspazy 3., która następnie degraduje liczne cząsteczki prowadząc pośrednio do procesu kontrolowanej śmierci komórki.

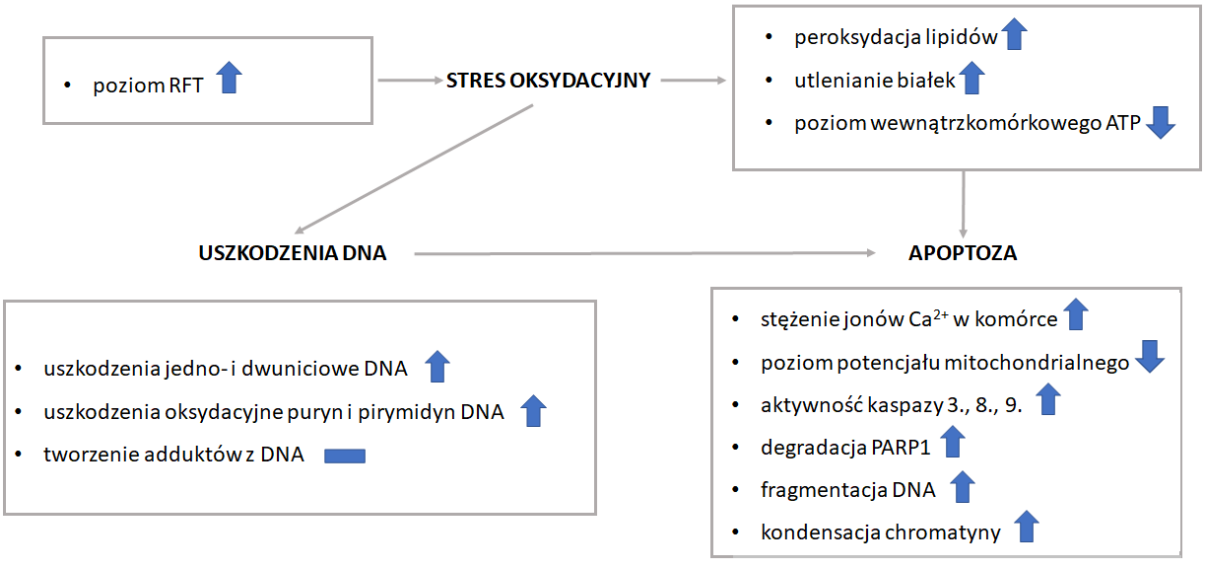
Odnotowano, że wszystkie badane związki od stężenia 5 µg/ml aktywowały kaspazę 9. (najsilniej TBBPA i 2,4,6-TBP) raz w mniejszym stopniu kaspazę 8. (najsilniej TBBPS). Ponadto badane uniepalniacze bromofenolowe (najsilniej TBBPA) znacząco wzmagały aktywność kaspazy 3. Uzyskane wyniki wskazują, że badane związki indukowały proces apoptozy głównie poprzez angażowanie szlaku wewnętrznego, przy czym TBBPS silniej niż pozostałe uniepalniacze angażował zewnętrzny szlak apoptotyczny.

Ludzka polimeraza poli (ADP-rybozy) (PARP1) jest enzymem jądrowym uczestniczącym między innymi w naprawie DNA. Podczas procesu apoptozy kaspaza 3 rozszczepia PARP1, co prowadzi do inaktywacji polimerazy ułatwiając przebieg procesu apoptotycznego. Zmiany w powyższym parametrze analizowano pod wpływem uniepalniaczy

bromofenolowych w stężeniu 25 µg/ml, które spowodowało największy wzrost aktywności kaspazy 3.Uzyskanewyniki wykazały, że wszystkie badane substancje, a najsilniej TBBPA i PBP indukowały rozszczepienie PARP-1.

Podczas procesu apoptozy dochodzi również do fragmentacji DNA na równe odcinki o długości 180-200 par zasad. W celu oceny wpływu badanych związków (stężenie 25 µg/ml) na powyższy proces wykorzystano metodę TUNEL, która wykrywa proces apoptozy poprzez znakowanie końców 3'-OH jedno- i dwuniciowych fragmentów DNA znakowanym Br-dUTP (bromowane nukleotydy trifosforanudeoksyurydyny). Stwierdzono, że badane substancje, a szczególnie PBP i TBBPA spowodowały wzrost liczby (odsetka) komórek TUNEL-pozytywnych.

Badanie procesu apoptotycznego obejmowało także ocenę kondensacji chromatyny przy użyciu mikroskopii fluorescencyjnej i podwójnego barwienia komórek znacznikami Hoechst3342 i jodek propidyny (PI). Hoechst 33342 jest wrażliwy na konformację DNA i stan chromatyny w komórce, dlatego jest stosowany do wykrywania komórek żywych i apoptotycznych, natomiast PI znakuje komórki nekrotyczne. Wykonane analizy potwierdziły, że badane związki, głównie TBBPA i PBP (w mniejszym stopniu 2,4,6-TBP) zwiększały liczbę komórek apoptotycznych i nekrotycznych, natomiast TBBPS zwiększał głównie liczbę komórek ulegających apoptozie.




**Schemat 1.** Proponowany mechanizm działania bromoorganicznych uniepalniaczy w jednojądrzastych komórkach krwi obwodowej człowieka.

## WNIOSKI

- Badane związki wykazują zróżnicowany potencjał cytotoksyczny i genotoksyczny w jednojądrzastych komórkach krwi obwodowej człowieka; większe zmiany w badanych parametrach wykazano pod wpływem tetrabromobisfenolu A i pentabromofenolu.
- Analizowane substancje wzmagając produkcję reaktywnych form tlenu (RFT) w tym rodnika hydroksylowego, powodują uszkodzenia oksydacyjne lipidów, białek oraz zasad purynowych i pirymidynowych w DNA.
- Badane uniepalniacze indukują jedno- i dwuniciowe uszkodzenia DNA w sposób pośredni, prawdopodobnie poprzez generowanie RFT, form rodnikowych i innych reaktywnych produktów, natomiast nie oddziałują bezpośrednio na materiał genetyczny.
- Badane substancje indukują apoptozę poprzez podwyższanie poziomu jonów wapnia w cytozolu komórek, obniżanie potencjału mitochondrialnego, aktywację kaspazy 8., 9. i 3., rozszczepienie PARP1 i fragmentację DNA; w procesie apoptozy angażują głównie szlak mitochondrialny.
- Podwyższenie poziomu RFT, uszkodzenia oksydacyjne lipidów, białek i DNA oraz zmiany w wewnątrzkomórkowym poziomie jonów wapnia i transbłonowym potencjale mitochondrialnym odnotowano w badanych komórkach pod wpływem uniepalniaczy bromofenolowych (z wyjątkiem tetrabromobisfenolu S) w stężeniach, które oznaczane były w organizmie człowieka w warunkach narażenia środowiskowego oraz zawodowego na te substancje.
- Największe zmiany w analizowanych parametrach zaobserwowane pod wpływem tetrabromobisfenolu A oraz najmniejsze odnotowane w wyniku oddziaływania tetrabromobisfenolu S, sugerują zasadność zastępowania TBBPA przez TBBPS w przemyśle, jako mniej toksyczną substancję.

## LITERATURA UZUPEŁNIAJĄCA


1. Abdallah M.A.E., Harrad S., Covaci A. 2008. Hexabromocyclododecanes and tetrabromobisphenol-A in indoor air and dust in Birmingham, UK: Implications for human exposure. Environmental Science and Technology, 42, 6855–6861.
2. Abdallah M.A.E. 2016. Environmental occurrence, analysis and human exposure to the flame retardant tetrabromobisphenol-A (TBBP-A) – a review. Environment International, 94, 235–250.
3. Alaee M, Arias P, Sjodin A, Bergman A. 2003. An overview of commercially used brominated flame retardants, their applications, their use patterns in different countries/regions and possible modes of release. Environment International. 29, 683-689.
4. Birnbaum L.S., Staskal D.F. 2004. Brominated flame retardants: cause for concern?. Environmental Health Perspectives, 112, 9-17.
5. Cariou R., Antignac J.P., Zalko D., Berrebi A., Cravedi J.P., Maume D. et.al. 2008. Exposure assessment of French women and their newborns to tetrabromobisphenol-A: Occurrence measurements in maternal adipose tissue, serum, breast milk and cord serum. Chemosphere, 73, 1036–1041.
6. Choi J.S., Lee Y.J., Kim T.H., Lim H.J., Ahn M.Y., Kwack S.J. et.al. 2011. Molecular mechanism of tetrabromobisphenol A (TBBPA)-induced target organ toxicity in Sprague-Dawley male rats. Toxicology Research, 27, 61–70.
7. Covaci A., Harrad S., Abdallah M.A-E., Ali N., Law R.J., Herzke D., de Wit C.A. 2011. Novel brominated flame retardants: A review of their analysis, environmental fate and behavior. Environment International, 37, 532-556.
8. DeWit C.A. 2002. An overview of brominated flame retardants in the environment. Chemosphere, 46, 583-624.
9. Deng J., Liu C., Yu L., Zhou B. 2010. Chronic exposure to environmental levels of tribromophenol impairs zebrafish reproduction. Toxicology and Applied Pharmacology, 243, 87–95.
10. Ding Y., Dong X., Feng W., Mao G., Chen Y., Oiu X., et al. 2022.Tetrabromobisphenol S alters the circadian rhythm network in the early life stages of Zebrafish. Science of the Total Environment, 806, 150543.
11. Feng C., Xu Q., Jin Y., Lin Y., Qui X., Lu D., Wang G. 2016. Determination of urinary bromophenols (BrPs) as potential biomarkers for human exposure to polybrominated

diphenyl ethers (PBDEs) using gas chromatography-tandem mass spectrometry (GC– MS/MS). Journal of Chromatography B, 1022, 70–74.

12. Gutierrez M., Becerra J., Godoy J., Barra R. 2005. Occupational and environmental exposure to tribromophenol used for wood surface protection in sawmills. International Journal of Environmental Health Research, 15, 171–179.
13. Hale R., Alaee M., Manchester-Neesvig J., Stapleton H.M., Ikonomou M.G. 2003. Polybrominated diphenyl ether flame retardants in the North American environment. Environment International, 29, 771-779.
14. Halit S, Salameh P. 2017. Exposure to toxins during pregnancy and childhood and asthma in children: A pilot study. Journal of Epidemiology and Global Health, 7, 147–154.
15. Jarosiewicz M., Bukowska B. 2017.Tetrabromobisphenol A - toxicity, environmental and occupational exposure. Occupational Medicine, 68, 121–134.
16. Jarosiewicz M., Duchnowicz P., Włuka A., Bukowska B. 2017. Evaluation of the effect of brominated flame retardants on hemoglobin oxidation and hemolysis in human erythrocytes. Food and Chemical Toxicology, 109, 264-271.
17. Jarosiewicz M., Krokosz A., Marczak A., Bukowska B. 2017a. Changes in the activities of antioxidant enzymes and reduced glutathione level in human erythrocytes exposed to selected brominated flame retardants. Chemosphere, 227, 93-99.
18. Jarosiewicz M., Michałowicz J., Bukowska B. 2019b.*In vitro*assessment of eryptotic potential of tetrabromobisphenol A and other bromophenolic flame retardants. Chemosphere, 215, 404–412.
19. Jarosiewicz M., Duchnowicz P., Jarosiewicz P., Huras B., Bukowska B. 2021. An *in vitro*comparative study of the effect of tetrabromobisphenol A and tetrabromobisphenol S on human erythrocyte membranes – changes in ATP level, perturbations in membrane fluidity, alterations in conformational state and damage to proteins. International Journal of Molecular Sciences, 22, 9443.
20. Lai Y.D., Kacew S., Dekant W. 2015. Tetrabromobisphenol A (TBBPA): Possible modes of action of toxicity and carcinogenicity in rodents. Food and Chemical Toxicology, 80, 206–214.
21. Lankova D., Lacina O., Pulkrabova J., Hajslova J. 2013. The determination of perfluoroalkyl substances, brominated flame retardants and their metabolites in human breast milk and infant formula. Talanta, 117, 318-25.

22. LaRosa F.D., Orange S.J. 2008. Lymphocytes. Journal of Allergy and Clinical Immunology, 121,364–369.
23. Lee D., Ahn C., Hong E., An B., Hyun S., Choi K., Jeung, E. 2016. 2,4,6-Tribromophenolinterferes with the thyroid hormone system by regulating thyroid hormones and the responsible genes in mice. International Journal of Environmental Research and Public Health, 13, 697.
24. Liang S, Liang S, Yin N, Hu B, Faiola F. 2019.Toxicogenomic analyses of the effects of BDE-47/209, TBBPA/S and TCBPA on early neural development with a human embryonic stem cell *in vitro*differentiationsystem. Toxicology and Applied Pharmacology, 379, 114685.
25. Meerts I.A.T.M., van Zanden J.J., Luijks E.A.C., van Leeuwen-Bol I., Marsh G., JakobssonE., Bergman A., Brouwer A., 2000. Potent competitive interactions of some brominatedflame retardants and related compounds with human transthyretin *in vitro*. Toxicological Sciences, 56, 95–104.
26. Michałowicz J., Stufka-Olczyk J., Milczarek A., Michniewicz M. 2011. Analysis of annual fluctuations in the content of phenol, chlorophenols and their derivatives in chlorinated drinking waters. Environmental Science and Pollution Research, 18, 1174-1183.
27. Michałowicz J., Włuka A., Bukowska B. 2022. A review on environmental occurrence, toxic effects and transformation of man-made bromophenols. Science of the Total Environment, 811, 152289.
28. Nakao T., Akiyama E., Kakutani H., Mizuno A., Aozasa O., Akai Y., Ohta S. 2015. Levels of tetrabromobisphenol A, tribromobisphenol A, dibromobisphenol A, monobromobisphenol A, and bisphenol A in Japanese Breast Milk. Chemical Research in Toxicology, 4, 722-728.
29. Ni H.G., Zeng H., Tao S., Zeng E.Y. 2010. Environmental and human exposure to persistent halogenated compounds derived from e-waste in China. Environmental Toxicology and Chemistry, 29, 1237–1247.
30. NTP. 2014. Technical Report on the toxicology studies of tetrabromobisphenol A (CAS NO. 79–94-7) in F344/NTac rats and B6C3F1/N mice and toxicology and carcinogenesis study of tetrabromobisphenol A in Wistar Han [Crl:WI(Han)] rats and B6C3F1/N mice (gavage studies). TP TR 587. National Toxicology Program, Research Triangle Park, NC.

31. Ohta S., Okumura T., Nishimura H., Nakao T., Shimizu Y., Ochiai F., Aozasa O., Miyata, H. 2004. Levels of PBDEs, TBBPA, TBPs, PCDDs/DFs, PXDDs/DFs and PBDDs/DFs in human milk of nursing women and dairy milk products in Japan. Organohalogen Compounds, 66, 2857–2862.
32. Park H.R., Kamau P.W., Korte C., Loch-Caruso R. 2014. Tetrabromobisphenol A activates inflammatory pathways in human first trimester extravillous trophoblasts *in vitro*. Reproductive Toxicology, 50, 154–162.
33. Ratomski K., Skotnicka B., Kasprzycka E., Żelazowska-Rutkowska B., Wysocka J., Anisimowicz S. 2007. Evaluation of percentage of the CD19+ CD5+ lymphocytes in hypertrophied adenoids at children with otitis media with effusion. Otolaryngologia Polska, 61, 962–966.
34. Remberger M., Sternbeck J., Palm A., Kaj L., Stromberg K., Brorstrom-Lunden E. 2004. The environmental occurrence of hexabromocyclododecane in Sweden. Chemosphere, 54, 9-21.
35. Saito I., Onuki A., Seto H. 2007. Indoor organophosphate and polybrominated flame retardants in Tokyo. Indoor Air, 17, 28-36.
36. Schauer U.M., Völkel W., Dekant W. 2006. Toxicokinetics of tetrabromobisphenol A in humans and rats after oral administration. Toxicological Sciences, 91, 49–58.
37. Stapleton H.M., Dodder N.G., Offenberg J.H., Schantz M.M., Wise S.A. 2005. Polybrominated diphenyl ethers in house dust and clothes dryer lint. Environmental Science & Technology, 39, 925-931.
38. Takigami H., Suzuki G., Hirai Y., Sakai S. 2009. Brominated flame retardants and other polyhalogenated compounds in indoor air and dust from two houses in Japan. Chemosphere, 76, 270–277.
39. Wang W., Abualnaja K.O., Asimakopoulos A.G., Covaci A., Gevao B., Johnson-Restrepo B. et. al. 2015. A comparative assessment of human exposure to tetrabromobisphenol A and eight bisphenols, including bisphenol A *via* indoor dust ingestion in twelve countries. Environment International, 83, 183–191.
40. Yang S., Wang S., Liu H., Yan Z. 2012. Tetrabromobisphenol A: tissue distribution in fish, and seasonal variation in water and sediment of Lake Chaohu, China. Environmental Science and Pollution Research, 19, 4090–4096.

# DOROBEK NAUKOWY

**Publikacje wchodzące w zakres rozprawy doktorskiej:**

1. **Włuka A.,**Woźniak A., Woźniak E., Michałowicz J. (2020).T*etrabromobisphenol A, terabromobisphenol S and other bromophenolic flame retardants cause cytotoxic effects and induce oxidative stress in human peripheral blood mononuclear cells (in vitro study).* Chemosphere, 261:127705.(MEiN = 140 pkt, IF = 7,086)
2. **Barańska A.,**Woźniak A., Mokra K., Michałowicz J. (2022).*Genotoxic mechanism of action of TBBPA, TBBPS and selected bromophenols in human peripheral blood mononuclear cells*. Frontiers in Immunology, 13:869741. (MEiN = 140 pkt, IF = 8,786)
3. **Barańska A.**, Sicińska P., Michałowicz J. (2022).Apoptosis-inducing potential of selected bromophenolic flame retardants: 2,4,6-tribromophenol and pentabromophenol in human peripheral blood mononuclear cells. Molecules, 27:5056. (MEiN = 140 pkt, IF = 4,927)
4. **Barańska A.,** Bukowska B., Michałowicz J. (2022).*Determination of apoptotic mechanism of action of tetrabromobisphenol A and tetrabromobisphenol S in human peripheral blood mononuclear cells*: A comparative study. Molecules, 27:6052.(MEiN = 140 pkt, IF = 4,927)
5. Michałowicz J., **Włuka A.,** Bukowska B. (2021). *A review on environmental occurrence, toxic effects and transformation of man-made bromophenols.* Science of the Total Environment, 811:152289. (MEiN = 200 pkt, IF = 10,754)

**Pozostałe publikacje:**

6. Jarosiewicz M., Duchnowicz P., **Włuka A.**, Bukowska B. (2017).*Evaluation of the effect of brominated flame retardants on hemoglobin oxidation and hemolysis in human erythrocytes.* Food and Chemical Toxicology, 109:264-271. (MEiN = 100 pkt, IF = 3,977)
7. Michałowicz J., **Włuka A.**, Cyrkler M., Maćczak A., Sicińska P., Mokra K. (2018). *Phenol and chlorinated phenols exhibit different apoptotic potential in human red blood cells (in vitro study).* Environmental Toxicology and Pharmacology, 61:95-101. (MEiN = 100pkt, IF = 3,061)
8. Szelenberger R., Dziedzic A., **Włuka A.**, Gralewska P., Janaszewska P. (2021). *Editorial. VI PhD Students National Conference of Life Sciences "BioOpen"*. Folia Biologica et Oecologica, 17:5-6. (MEiN = 20 pkt)

**Sumaryczny IF = 43,517 (980 pkt MEiN)**

**Rozdziały w monografiach pokonferencyjnych:**

1. **Włuka A.,** Jarosiewicz M. (2018).*Mitochondria – bioenergetic center in diabates*. Badania i Rozwój Młodych Naukowców w Polsce nr 3. ISBN: 978-83-66139-19-0.
2. **Włuka A.,** Jarosiewicz M. (2018). *Apoptosis in human erythrocytes*. Badania i Rozwój Młodych Naukowców w Polsce nr 3. ISBN: 978-83-66139-19-0.

**Doniesienia zjazdowe:**

1. Maćczak A., Jarosiewicz M., **Włuka A.,** Bukowska B., Michałowicz J. *Changes of acetylocholino esterase activity in human erythrocytes under the influence of bisphenol A and selected analogs*. III Ogólnopolska Konferencja Doktorantów Nauk o Życiu, 11-12 maja 2017, Łódź (Polska).
2. **Włuka A.,** Jarosiewicz M., Wolska A., Michałowicz J. *Evaluation of the effect of phenol and pentachlorophenol on the apoptosis process of human erythrocytes.*III Ogólnopolska Konferencja Doktorantów Nauk o Życiu, 11-12 maja 2017, Łódź (Polska).
3. Jarosiewicz M., Maćczak A., **Włuka A.,** Bukowska B. *The effects of tetrabromobisphenol S on hemolysis, hemoglobin oxidation and acetylcholinesterase activity in human erythrocytes.*III Ogólnopolska Konferencja Doktorantów Nauk o Życiu, 11-12 maja 2017, Łódź (Polska).
4. **Włuka A.,** Jarosiewicz M., Maćczak A., Michałowicz J. *Ocena wpływu 2,4-dichlorofenolu oraz 2,4,6-trichlorofenolu na apoptozę erytrocytów człowieka.* VI Międzyuczelniane Sympozjum Biotechnologiczne SYMBIOZA im. Prof. Krzysztofa W. Szewczyka, 26-28 maja 2017, Warszawa (Polska).
5. Maćczak A., Duchnowicz P., Koter-Michalak M., Sicińska P., Bukowska B.,**Włuka A.,** Michałowicz J. *The effect of bisphenol AF on human erythrocyte membrane.* 3rd International Conference of Cell Biology. 26-27 maja 2017,Kraków (Polska).
6. Jarosiewicz M., **Włuka A.,** Bukowska B. *Eryptosis-inducing activity of tetrabromobisphenol A and tetrabromobisphenol S in human red blood cells*. Chemistry for Agriculture. 26-29 listopada 2017,Karpacz (Polska).
7. Wolska A., **Włuka A.,** Jarosiewicz M., Michałowicz J. *The effect of phenol and pentachlorophenol on selected apoptotic parameters of human erythrocytes*. Chemistry for Agriculture. 26-29 listopada 2017, Karpacz (Polska).

8. **Włuka A.,** Michałowicz J. *Bromowane związki ograniczające palność – charakterystyka i toksyczność.* Badania I Rozwój Młodych Naukowców w Polsce 2018. 7 maja 2018, Poznań (Polska).
9. **Włuka A.,** Jarosiewicz M., Michałowicz J. *Tetrabromobisphenol A and tetrabromobisphenol S decrease viability and deplete ATP level in human peripheral blood mononuclear cells*. 11-12 maja 2018, 4th International Conference of Cell Biology, Kraków (Polska).
10. **Włuka A.**, Jarosiewicz M., Wolska A., Michałowicz J. *Ocena wpływu 2,4-dichlorofenolu oraz 2,4,6-trichlorofenolu na proces apoptozy erytrocytów człowieka..* IV Ogólnopolska Konferencja Doktorantów Nauk o Życiu, 24-25 maja 2018, Łódź (Polska).
11. Jarosiewicz M., **Włuka A.**, Bukowska B. *Tetrabromobisphenol A (TBBPA) and tetrabromobisphenol S (TBBPS) induce different oxidative stress in human red blood cells (in vitro study).* „Biomedical theories of aging" In memory of Prof. Tomasz Biliński, 21-22 czerwca 2018, Rzeszów (Polska).
12. **Włuka A.,** Jarosiewicz M., Wolska A., Michałowicz J. *The effect of phenol and pentachlorophenol on selected apoptotic parameters of human erythrocytes.* „Biomedical theories of aging" In memory of Prof. Tomasz Biliński, 21-22 czerwca 2018, Rzeszów (Polska).
13. **Włuka A.,** Michałowicz J. *Tetrabromobisphenol A and tetrabromobisphenol S enhance reactive oxygen species formation and induce lipid peroxidation in human peripheral blood mononuclear cells.*63rd Annual Meeting of Biophysical Society, 2-6 marca 2019, Baltimore (United States).
14. **Włuka A.**, Woźniak A., Michałowicz J. *Bromophenolic flame retardants*. 5th International Conference of Cell Biology, 10-12 maja 2019, Kraków (Polska).
15. **Włuka A.,** Michałowicz J. *2,4,6-Tribromophenol and pentabromophenol induce oxidative stress in human peripheral blood mononuclear cells.* Euro Summit on Toxicology and Pharmacology, 13-15 maja 2019, Rzym (Włochy).
16. **Włuka A.,** Woźniak A., Michałowicz J. *Ocena wpływu 2,4,6-tribromofenolu oraz pentabromofenolu na żywotność i poziom ATP w jednojądrzastych komórkach krwi obwodowej człowieka.* V Ogólnopolska Konferencja Doktorantów Nauk o Życiu, 30-31 maja 2019, Łódź (Polska).
17. Woźniak A., **Włuka A.,** Michałowicz J. *Ocena wpływu uniepalniaczy bromofenolowych na uszkodzenia DNA w jednojądrzastych komórkach krwi obwodowej człowieka*.

V Ogólnopolska Konferencja Doktorantów Nauk o Życiu, 30-31 maja 2019, Łódź (Polska).

18. Woźniak A., **Włuka A.,** Michałowicz J. *Ocena wpływu wybranych uniepalniaczy bromofenolowych na indukcję jedno- i dwuniciowych pęknięć DNA w jednojądrzastych komórkach krwi obwodowej człowieka.* X Sesja Magistrantów i Doktorantów Łódzkiego Środowiska Chemików 2019, 4 czerwca 2019, Łódź (Polska).
19. **Włuka A**., Woźniak A., Bukowska B., Sicińska P., Michałowicz J. *The effect of bromophenolic flame retardants on DNA damage in human peripheral blood mononuclear cells.*XVII Zjazd Polskiego Towarzystwa Biofizycznego, 24-27 czerwca 2019, Kortowo (Polska).
20. **Włuka A**., Michałowicz J. *Comparison of apoptotic inducing effect of selected bromophenolic flame retardants on human peripheral blood mononuclear cells.* 6th International Conference of Cell Biology, 6-8 listopada, Karków (Polska).
21. **Włuka A.,** Michałowicz J. *Ocena wpływu uniepalniaczy bromofenolowych na indukcję uszkodzeń dwuniciowych i naprawdę uszkodzonego DNA w jednojądrzastych komórkach krwi obwodowej człowieka.* VI Ogólnopolska Konferencja Doktorantów Nauk o Życiu - Bioopen 2021, 15-16 kwietnia 2021, Łódź (Polska).
22. **Włuka A**., Michałowicz J. *The effect of brominated flame retardants on selected apoptotic parameters of human peripheral blood mononuclear cells.*Symbioza – Intercollegate BiotechnologySymposium 2021, 21-23 maja 2021, Warszawa (Polska).

# KOPIE PUBLIKACJI WCHODZĄCYCH W ZAKRES ROZPRAWY DOKTORSKIEJ

Chemosphere 261 (2020) 127705

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

# Tetrabromobisphenol A, terabromobisphenol S and other bromophenolic flame retardants cause cytotoxic effects and induce oxidative stress in human peripheral blood mononuclear cells (*in vitro* study)

Anna Włuka [a], Agnieszka Woźniak [a], Ewelina Woźniak [b], Jaromir Michałowicz [a, *]

[a] *University of Lodz, Faculty of Biology and Environmental Protection, Department of Biophysics of Environmental Pollution, Pomorska Str. 141/143, 90-236, Lodz, Poland*

[b] *Medical University of Lodz, Department of Internal Diseases and Clinical Pharmacology, Laboratory of Tissue Immunopharmacology, Kniaziewicza Str. 1/5, 91-347, Lodz, Poland*

## HIGHLIGHTS

- Cytotoxic and oxidative effects of selected bromophenolic FRs on human PBMCs were studied.
- TBBPA, TBBPS, 2,4,6-TBP and PBP exhibited cytotoxic and oxidative potential in PBMCs.
- BFRs examined decreased cell viability and depleted intracellular ATP level.
- BFRs studied increased ROS level and caused damage to lipids and proteins in PBMCs.
- The greatest changes were noted for TBBPA, whereas the weakest for TBBPS.

## ARTICLE INFO

*Article history:*
Received 11 March 2020
Received in revised form
25 June 2020
Accepted 10 July 2020
Available online 19 July 2020

Handling Editor: Jian-Ying Hu

*Keywords:*
Cell viability
Oxidative damage
Peripheral blood mononuclear cells
Tetrabromobisphenol A
Tetrabromobisphenol S
Bromophenols

## ABSTRACT

Brominated flame retardants (BFRs) are the compounds used in the industry in order to decrease flammability of various everyday products. The use of BFRs leads to migration of these substances into the environment, which results in the exposure of humans to their action. Although BFRs are widespread in human surrounding, the effect of these compounds on human body has been very poorly assessed. The purpose of this study was to evaluate cytotoxic effects as well as oxidative potential of selected bromophenolic flame retardants such as tetrabromobisphenol A (TBBPA), tetrabromobisphenol S (TBBPS), 2,4,6-tribromophenol (2,4,6-TBP) and pentabromophenol (PBP) on human peripheral blood mononuclear cells (PBMCs) that are crucial for proper functioning of the immune system. The cells were treated with the substances studied in the concentrations ranging from 0.0001 to 100 μg/mL for 1 h or 24 h. The results have shown that the compounds examined reduced PBMCs viability and ATP level as well as increased reactive oxygen species (including hydroxyl radical) formation. Moreover, the substances tested induced lipid peroxidation and caused oxidative damage to proteins in the incubated cells. It has also been noticed that the greatest changes were provoked by tetrabromobisphenol A, while the weakest by TBBPS, which is used as a substitute of TBBPA in the manufacture.



## 1. Introduction

Brominated flame retardants (BFRs) are synthetic substances used in order to reduce the flammability or the burning rate of polymers (Bjermo et al., 2017). A demand for BFRs is continuing to grow, which is due to legislative provisions that are designed to improve the fire safety of consumer products, including textiles, furniture as well as electric and electronic devices (Alaee et al., 2003). BFRs account for approximately 25% of the total FRs market and more than 80 different chemicals in this group have been used. Generally, data concerning the occurrence of bromophenolic FRs in the environment and their toxic effects on living organisms,

* Corresponding author.
*E-mail address:* jaromir.michalowicz@biol.uni.lodz.pl (J. Michałowicz).

https://doi.org/10.1016/j.chemosphere.2020.127705
0045-6535/

including humans is very limited (Góralczyk et al., 2002; Staszowska, 2009; Jarosiewicz and Bukowska, 2017).

In 2012, European Food Safety Authority stated that it is impossible to determine the health risk posed by tetrabromobisphenol A (TBBPA) and other bromophenolic FRs because there is lack of data on the presence of these substances in the food chain and edibles as well as insufficient knowledge exists about their toxic action.

TBBPA is the most important bromophenolic FR. Nowadays, the annual production of TBBPA in the United States, Japan and Israel reached 170 thousand tons, which accounted for approximately 60% of the production of all BFRs (Staszowska, 2009; Covaci et al., 2009; Jarosiewicz and Bukowska, 2017). 2,4,6-Tribromophenol (2,4,6-TBP) is also produced in high quantities. Its synthesis takes place mainly in China, Japan (3,600 tons) and the United States (from 4,500 to 23,000 tons) (Covaci et al., 2011).

Recently, tetrabromobisphenol S (TBBPS) has been introduced into the market as the substitute of TBBPA. The studies showing its occurrence in the environment are scarce. Liu et al. (2017) determined TBBPS in a mean concentration of 3.1 μg/g of soil of Weifang City (China). In another study, TBBPS bis(allylether) was detected in industrial waste water in the range of concentrations from 0.5 to 8.2 μg/L, while it was not found in tap and surface water (Tian et al., 2014).

TBBPS has been shown to be toxic to some plants (weed) species, and thus it is used as a herbicide, whereas its toxic potential in animals and human has not been evaluated.

TBBPA, TBBPS and pentabromophenol (PBP) due to their hydrophobic nature may accumulate at various levels of the food chain, posing a threat to living organisms, including humans (Nakao et al., 2015). It has also been shown that workers employed in the production, packagings and transport of BFRs are strongly exposed to their action (Xie et al., 2007; Abdallah et al., 2008). Moreover, workers of sawmills where bromophenols are used in order to protect lumber against microorganisms, have been shown to be heavily exposed to these substances. For instance, 2,4,6-TBP was determined in the urine of Chilean sawmills workers in a mean concentration of 6.9 mg/g creatinine (approx. 6.9 mg per 1 L of urine) (Gutiérrez et al., 2005).

The studies carried out in the United States, Japan, Taiwan, Canada, and Northern Europe have shown the occurrence of TBBPA in dust and environmental samples (air, water, sewage) as well as in humans tissues and organs such as lungs, kidneys and liver (Alaee et al., 2003; DeWit, 2002; Hale et al., 2003; Remberger et al., 2004; Stapleton et al., 2005). TBBPA was determined in 44% of human milk samples in the concentrations range from 0.06 to 37.34 ng/g of fat and 30% of serum samples from both mothers and foetuses in comparable concentrations range (Cariou et al., 2008). TBBPA was also detected in plasma of Japanese men in a mean concentration of 950 pg/g fresh weight (Fujii et al., 2014). In addition, TBBPA was found (<0.0033–0.464 ng/g fat) in 69% of samples of adipose tissue obtained from patients in New York during liposuction (Johnson-Restrepo et al., 2008). Bromophenols have also been detected in significant concentrations in human body fluids. Dufour et al. (2017) found 2,4,6-TBP in the range of the concentrations from trace to 1.28 μg/L in blood of the general population of Belgium, while Feng et al. (2016) determined this substance at the concentration of 5.57 ± 4.05 μg/L in the urine of the general population of China..

Toxicological data has shown that bromophenolic FRs exhibit adverse effects in living organisms. For instance, TBBPA given to rats caused a decrease in serum thyroxine level, induced CYP2B1 activity and increased liver weight and constitutive expression of androstane receptors in the liver (Park et al., 2014). TBBPA has also been shown to induce development of uterine cancer in rats and adenocarcinoma, adenoma and Muller's mixed tumour in mice and rats (Dunnick et al., 2015; NTP, 2014). The studies have also documented toxic potential of 2,4,6-TBP. Lee et al. (2016) noticed that mice treated with 2,4,6-TBP had depleted deiodinase 1 level and reduced thyroid receptor lymphostasis (TRBβ 2) expression, while Koch and Sures (2018) observed that 2,4,6-TBP changed neuroblastoma cells differentiation by inhibiting their growth and increasing acetylocholinoesterase activity. In another study, PBP has been shown to suppress transforming growth factor-beta (TGF-β) signaling by increasing of degradation of type II TGF-β receptors *via* caveolae-mediated endocytosis in A549 and NMuMG cells, and thus being recognized as a potential risk factor for tumorigenesis and other TGF-β-related diseases (Chen et al., 2017). Bromophenolic FRs have also been shown to exhibit hemolytic, apoptotic and oxidative potential in human red blood cells (Jarosiewicz et al., 2017, 2019, 2020). Recent study has shown that TBBPA exhibited immunotoxic potential in murine macrophages by upregulation of expression of pro-inflammatory cytokines, like IL-1β, IL-6 and TNF-α (Wang et al., 2019).

White blood cells, and particularly lymphocytes (being the main population of peripheral blood mononuclear cells - PBMCs) are key cells of the immune system responsible for maintaining of the immune response. Damage to lymphocytes may contribute to disturbances in the immune system function, which possibly may result in cancer, asthma or allergy development (Ratomski et al., 2007). Moreover, oxidative stress in lymphocytes is believed to be implicated in aging and may play an important role in immunosenescence (Venza et al., 2012).

Taking the above into consideration, we decided to to compare cytotoxic and oxidative potential of TBBPA, TBBPS, 2,4,6-TBP and PBP in human PBMCs by evaluating changes in cell viability and ATP level and assessing alterations in reactive oxygen species (ROS) (including $^{\bullet}OH$) formation and lipid and protein oxidation.

## 2. Materials and methods

### 2.1. Chemicals

Tetrabromobisphenol A (2,2-bis[3,5-dibromo-4-hydroxyphenyl] propane) and pentabromophenol (2,3,4,5,6-pentabromophenol) were obtained from LGC Standards (Germany). Tribromophenol (2,4,6-tribromophenol) (pure ≤ 100%) was bought from Sigma-Aldrich (USA). Tetrabromobisphenol S (4,4′-sulfonylbis[2,6-dibromo-phenol]) (98.8%) was synthetized in the Institute of Industrial Organic Chemistry in Warsaw (Poland). Calcein-AM (95%), propidium iodide (PI) (95%), 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate ($H_2$DCFDA) and ethanol (ACS grade) were purchased from Sigma-Aldrich (USA). *Cis*-parinaric acid and bioluminescence assay kit for ATP determination, 3'-(p-hydroxyphenyl) fluorescein (HPF) (98%) were bought from Molecular Probes (USA). RPMI with L-glutamine and lymphocyte separation medium (LSM) (1.077 g/cm$^3$) were obtained from Cytogen (Germany). Potassium chloride (99.5%), sodium chloride (99.5%), sodium hydrogen carbonate (99%), ammonium chloride (99.5%), sodium wersenite (99.5%) and other chemicals were bought from POCH (Poland) and Roth (Germany).

### 2.2. Methods

#### 2.2.1. Cells isolation and treatment

Leucocyte buffy-coat was separated from blood purchased from the Regional Blood Center in Lodz (Poland). Blood was achieved from healthy volunteers (aged 18–55) showing no signs of

infection disease symptoms. The investigation was approved by the Bioethics Committee of the University of Lodz No. 1/KBBN-UŁ/II/2017.

PBMCs were separated using LSM (1.077 g/cm$^3$) gradient. Samples were centrifuged at 600*g* for 30 min at 20 °C. PBMCs were collected and suspended in erythrocyte lysis buffer (150 mM $NH_4Cl$, 1 mM EDTA, 10 mM $NaHCO_3$, pH 7.4) and incubated for 5 min at 20 °C. In the next step, PBS solution was added into the samples, which were centrifuged at 200*g* for 15 min at 20 °C. Then, the cells were washed twice with RPMI medium with L-glutamine (300*g* for 20 min at 20 °C), and suspended in RPMI medium with L-glutamine and FBS (10%). The final PBMCs density used in the experiments was $1 \times 10^6$ cells/mL. The cells were suspended in RPMI medium containing L-glutamine, FBS (10%) and penicillin-streptomycin (0.5%) during incubation. PBMCs were incubated in a humidified incubator at 37 °C in 5% $CO_2$ atmosphere in total darkness.

Cells were treated with the substances tested in the range of the concentrations from 0.0001 to 100 μg/mL for 1 h or 24 h. The concentration of 0.0001 μg/mL was selected as the lowest one that caused any statistically significant changes (for any compunds examined) in any parameter studied - ROS formation, while the concentration of 0.01 μg/mL was selected as the lowest one that caused any statistically significant changes in oxidative damage to biomacromolecules (protein/lipids) in the cells tested. Other parameters studied have been analyzed mainly within the concentrations range that had been used in our previous studies evaluating cytotoxic effects of chlorophenols and bisphenols on human PBMCs (Michałowicz, 2010a,b; Michałowicz et al., 2015).

The substances tested were dissolved in DMSO that concentration in negative control samples (without substances examined) and in the samples treated with TBBPA, TBBPS, 2,4,6-TBP or PBP was 0.2%. DMSO concentration used in the experiments (samples) was not toxic for PBMCs as assessed by all parameters examined.

#### 2.2.2. Cell viability

The calcein-AM/propidium iodide (PI) viability test is usually employed to assess the number of viable and necrotic cells. Calcein-AM is hydrolyzed to calcein, which has a negative charge and enters living cells, staining them green. PI is widely utilized fluorescent marker for staining necrotic cells. This substance enters the cells that lost membrane integrity, and binds with DNA (Papadopoulos et al., 1994).

PBMCs were incubated with TBBPA, TBBPS, 2,4,6-TBP or PBP in the range of the concentrations from 1 to 100 μg/mL for 1 h or 24 h. Then, the cells were centrifuged at 300*g* for 5 min at 4 °C and the supernatant was discarded. Finally, the cells were suspended in RPMI 1640 medium with L-glutamine. Calcein-AM (0.1 mM) and PI (1 mM) were added to the samples, which were incubated for 15 min at 37 °C in the dark. FCM gate on PBMCs has been established for data acquisition and the measurement was made using flow cytometer (LSR II, Becton-Dickinson) (excitation/emission maxima 494/517 for calcein and 535/617 for PI, respectively) for 10,000 cells.

#### 2.2.3. ATP level

ATP determination was based on oxidative decarboxylation of luciferin by luciferase at presence of ATP and magnesium ions with concomitant bioluminescence. The intensity of light emission was linearly associated with ATP level (Stanly and Williams, 1969). Intracellular level of ATP in PBMCs was determined using ATP assay kit (Molecular probes, USA).

After incubation of PBMCs for 1 h or 24 h with individual xenobiotic (1–100 μg/mL), the cells were pelleted by centrifugation at 12,000*g* for 10 min at 4 °C. Then, the cellular ATP was extracted by the addition of 1 mL of boiling deionized water to the cell pellet according to the method described by Yang et al. (2002). After vortexing and centrifugation (12,000*g* for 15 min at 4 °C), a volume of 45 μL of the lysed cell suspension was added to a volume of 100 μL of luciferin/luciferase mixture. The samples were incubated for 20 min at room temperature in total darkness. The light emission was measured for 0.1 min.

#### 2.2.4. $H_2$DCFDA and HPF oxidation

6-Carboxy-2′,7′-dichlorodihydrofluorescein diacetate ($H_2$DCFDA) was employed in order to determine the production of total ROS (Bartosz, 2009). $H_2$DCFDA is hydrolyzed by membrane esterases to $H_2$DCF when penetrates the cellular membrane. Inside the cell $H_2$DCF is oxidized to DCF, which fluorescence is analyzed by a flow cytometer (LSR II. Becton Dickinson) at excitation/emission wavelengths maxima at 488 nm and 530 nm, respectively. $H_2$DCFDA was used in the final concentration of 5 μM. In order to induce ROS formation (positive control), the cells were treated with hydrogen peroxide at 2 mM.

The fluorescent stain 3′-(p-hydroxyphenyl)-fluorescein (HPF) was employed to determine highly reactive oxygen species (mainly hydroxyl radical). The HPF concentration in PBMCs suspension was 4 μM. Inside the cell, HPF is oxidized to fluorescein, which emits strong fluorescence. Fluorescence was measured by a flow cytometer (LSR II. Becton Dickinson) at excitation/emission wavelengths maxima of 490 nm and 515 nm, respectively. In order to induce hydroxyl radical formation (positive control) a mixture of ferrous perchlorate(II) (0.1 mM) and hydrogen peroxide (1 mM) was added into the cell suspension.

During the above analyses, FCM gate has been established for data acquisition, and the data was recorded for a total of 10,000 events per sample.

PBMCs were incubated for 1 h with TBBPA, TBBPS, 2,4,6-TBP or PBP in the concentrations range from 0.0001 to 1 μg/mL and from 0.01 to 10 μg/mL to measure $H_2$DCFDA and HPF oxidation, respectively. Then, the cells were centrifuged at 300*g* for 5 min at 4 °C. The supernatant was removed, and PBMCs were resuspended in RPMI medium containing L-glutamine. Finally, individual fluorescent probe was added into the samples, which were incubated for 20 min at 37 °C in the dark.

#### 2.2.5. Lipid peroxidation and protein oxidation

The analyses of lipid peroxidation and protein oxidation were conducted according to previously described procedures (Michałowicz et al., 2015).

#### 2.2.6. Statistical analysis

Data was expressed as mean values with standard deviation. Statistical significance was examined on the basis of a comparison of averages using a one-way analysis of variance - ANOVA. To evaluate statistically significant differences between the tested samples, a multiple comparison test - the Tukey test (post-hoc) was used (Watała, 2002). Statistical significance was $p < 0.05$. Analysis was performed using the STATISTICA 13 software (StatSoft, Inc, Tulusa, USA). The tests were carried out on blood from 4 to 5 donors. For each individual experiment (one blood donor), an experimental point was a mean value from 2 to 3 replications.

## 3. Results

### 3.1. Cell viability

The compounds examined caused different decrease in PBMCs viability. After 1 h of incubation, TBBPA and particularly PBP at 50 μg/mL and 100 μg/mL slightly decreased cell viability (Fig. 1A).

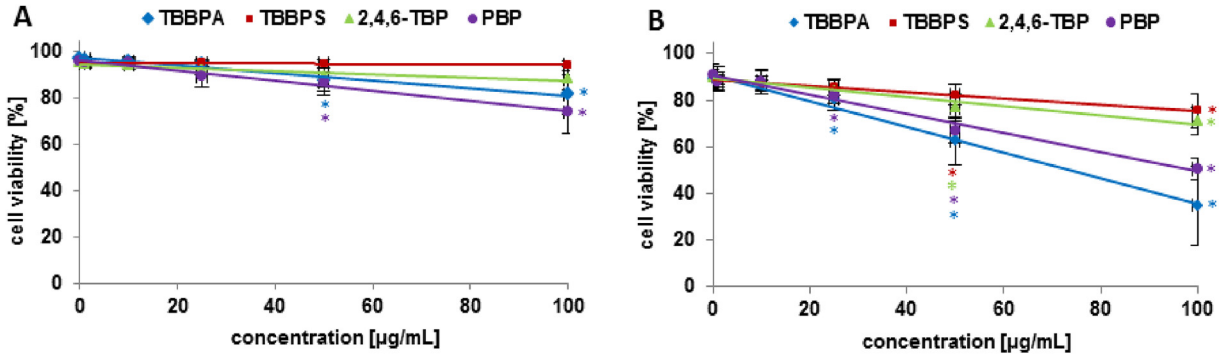



**Fig. 1.** Changes in viability of PBMCs incubated with TBBPA, TBBPS, 2,4,6-TBP and PBP in the concentrations ranging from 1 to 100 μg/mL for 1 h (A) and 24 h (B). Mean ± SD was calculated from 4 individual experiments (4 blood donors). Statistically different from negative control at *P < 0.05. Statistical analysis was conducted using one-way ANOVA and a posteriori Tukey test.

After 24 h of incubation, all compounds tested decreased viability of the cells studied. It has been found that 2,4,6-TBP and TBBPS in particular at 50 μg/mL and 100 μg/mL slightly reduced the parameter examined, while PBP and more strongly TBBPA from 25 to 100 μg/mL induced significant reduction in PBMCs viability (Fig. 1B).

### 3.2. ATP level

The substances tested reduced ATP level in PBMCs. After 1 h of incubation, PBP and TBBPA at 50 μg/mL and more strongly at 100 μg/mL caused substantial depletion of ATP level, whereas 2,4,6-TBP decreased this parameter only at 100 μg/mL (Fig. 2A). After longer incubation time (24 h), PBP and TBBPA at 25 μg/mL and 50 μg/mL depleted ATP level, whereas at their highest concentration of 100 μg/mL they potently reduced ATP pull in the cells studied (Fig. 2B). It has also been shown that 2,4,6-TBP at 50 μg/mL and 100 μg/mL significantly reduced ATP level in PBMCs, whereas TBBPS decreased ATP pull only at its highest concentration of 100 μg/mL (Fig. 2B).

### 3.3. Total ROS and hydroxyl radical formation

The compounds tested at low concentrations were capable of inducing significant increase in total ROS level in human PBMCs after 1 h of incubation. The greatest increase in ROS level has been noted in PBMCs treated with TBBPA in the concentrations range from 0.0001 to 1 μg/mL (Fig. 3A). Other compounds studied increased total ROS level mainly in the range of the concentrations from 0.001 to 0.1 μg/mL (Fig. 3A).

It has also been found that TBBPA at 1 μg/mL and 10 μg/mL most strongly increased highly reactive oxygen species (mainly hydroxyl radical) level in PBMCs after 1 h of incubation (Fig. 3B). Moreover, TBBPS and PBP negligibly increased this parameter at their highest concentration of 10 μg/mL (Fig. 3B).

### 3.4. Lipid peroxidation

It has been observed that the compounds examined induced lipid peroxidation (decreased cis-parinaric acid fluorescence) in PBMCs after 1 h, and particularly after 24 h of incubation. After 1 h of treatment, the greatest changes were provoked by TBBPA that in the range of the concentrations from 0.1 to 20 μg/mL provoked lipid peroxidation. Other substances tested only at 10 μg/mL and 20 μg/mL induced lipids damage (Fig. 4A). After 24 h of incubation, TBBPA, TBBPS and PBP at 0.1 μg/mL increased lipid oxidation. All substances tested in the concentrations range from 1 to 20 μg/mL substantially induced lipid peroxidation in PBMCs, with the strongest and weakest effects noted for TBBPA and TBBPS, respectively (Fig. 4B).

### 3.5. Protein oxidation

The compounds examined induced protein oxidation (caused a decrease in tryptophan fluorescence) in PBMCs after 1 h, and more strongly after 24 h of incubation. After 1 h of incubation, TBBPA and PBP in the concentrations range from 0.1 to 20 μg/mL induced protein oxidation, while TBBPS and 2,4,6-TBP only at 10 μg/mL and 20 μg/mL caused damage to protein in PBMCs (Fig. 5A). After longer incubation time (24 h), TBBPA, TBBPS and PBP in the range of the

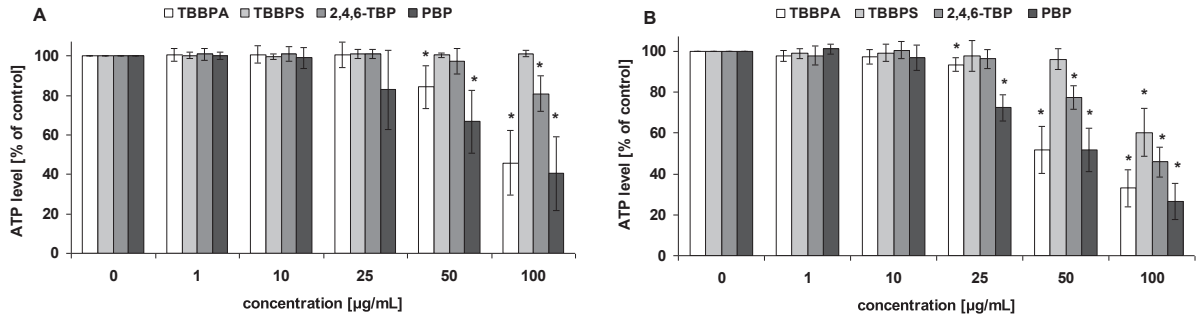



**Fig. 2.** Changes in ATP level in PBMCs incubated with TBBPA, TBBPS, 2,4,6-TBP and PBP in the concentrations ranging from 1 to 100 μg/mL for 1 h (A) and 24 h (B). Mean ± SD was calculated from 4 individual experiments (4 blood donors). Statistically different from negative control at *P < 0.05. Statistical analysis was conducted using one-way ANOVA and a posteriori Tukey test.

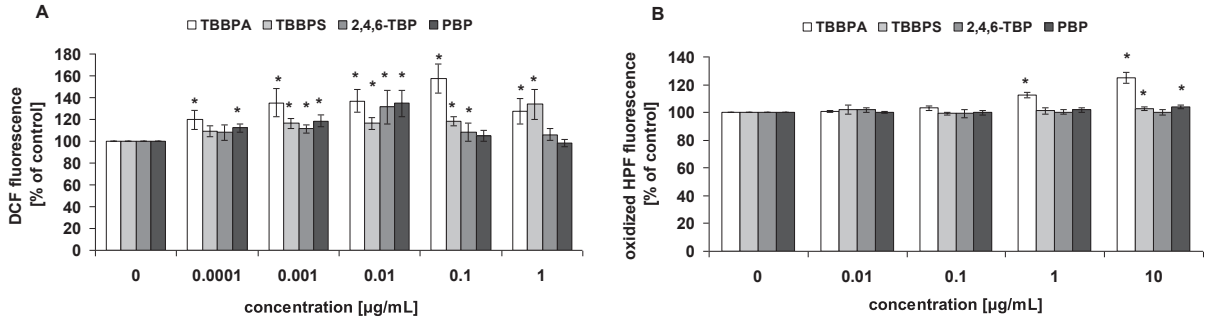



**Fig. 3.** Changes in total ROS (A) and highly reactive oxygen species (B) levels in PBMCs incubated with TBBPA, TBBPS, 2,4,6-TBP and PBP in the concentrations ranging from 0.0001 to 10 µg/mL for 1 h. Mean ± SD was calculated from 5 individual experiments (5 blood donors). Statistically different from negative control at *P < 0.05. Statistical analysis was conducted using one-way ANOVA and a posteriori Tukey test.

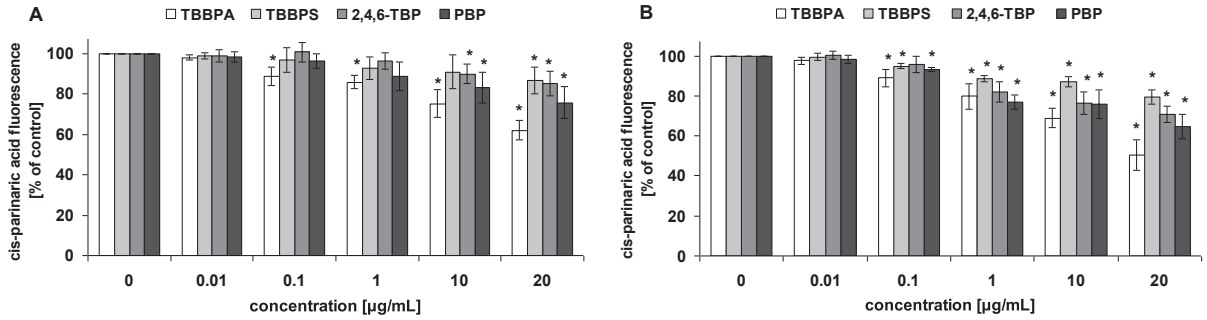



**Fig. 4.** Changes in lipid peroxidation (cis-parinaric acid oxidation) in PBMCs incubated with TBBPA, TBBPS, 2,4,6-TBP and PBP in the concentrations ranging from 0.01 to 20 µg/mL for 1 h (A) and 24 h (B). Mean ± SD was calculated from 4 individual experiments (4 blood donors). Statistically different from negative control at *P < 0.05. Statistical analysis was conducted using one-way ANOVA and a posteriori Tukey test.

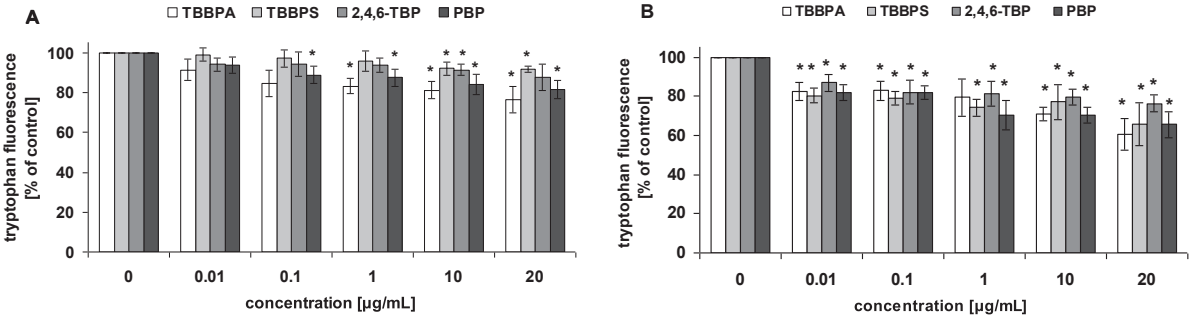



**Fig. 5.** Changes in protein damage (oxidation of tryptophan) in PBMCs incubated with TBBPA, TBBPS, 2,4,6-TBP and PBP in the concentrations ranging from 0.01 to 20 µg/mL for 1 h (A) and 24 h (B). Mean ± SD was calculated from 4 individual experiments (4 blood donors). Statistically different from negative control at *P < 0.05. Statistical analysis was conducted using one-way ANOVA and a posteriori Tukey test.

concentrations from 0.01 to 20 µg/mL induced substantial (comparable) oxidative damage to protein in PBMCs, while the smallest alterations were noted in cells treated with 2,4,6-TBP (Fig. 5B).

## 4. Discussion

Despite of increasing number of studies showing the occurrence of bromophenolic FRs in the environment and human body, little information exists concerning the effect of these compounds on living organisms, including humans.

In this study, we have evaluated cytotoxic and oxidative potential of selected brominated bisphenols (TBBPA, TBBPS) and brominated phenols (2,4,6-TBP, PBP) in human PBMCs, which are the key cells responsible for maintaining the body's homeostasis and responding to aggression of pathogens and cancer cells (Chaplin, 2010).

The results have shown that the compounds studied decreased to different extents PBMCs viability (caused different increase in the number of necrotic cells). The largest alterations were noted in cells incubated with TBBPA (particularly after 24 h of incubation), while the smallest in PBMCs treated with TBBPS, which is a substitute of TBBPA in the manufacture. These findings are in agreement with the study of Jarosiewicz et al. (2017) who showed that TBBPA exhibited the strongest, while TBBPS the weakest cytotoxic (hemolytic) potential in human erythrocytes. Similarly, Michałowicz et al. (2015) observed that bisphenol A (BPA) was

much more cytotoxic than bisphenol S (BPS) to human PBMCs.

A decrease in PBMCs viability was noted at relatively high concentrations (25–100 μg/mL) of TBBPS and TBBPA. Similarly, Wang et al. (2019) observed cytotoxic effect of TBBPA at similar (micromolar) concentrations on murine macrophages. In another study, Wu et al. (2018) showed that TBBPA in the range of the concentrations from 8 to 64 μg/mL decreased viability of A549 cell line, whereas Michałowicz et al. (2015) noticed that BPA in the concentrations from 25 to 100 μg/mL reduced viability of human PBMCs.

Similarly to brominated bisphenols, PBP and particularly 2,4,6-TBP decreased PBMCs viability only at substantial dosages. Previous studies conducted by Michałowicz (2010a,b) revealed that 2,4,5-trichlorophenol (2,4,5-TCP) and pentachlorophenol (PCP) only at significant concentrations caused a decrease in viability of human lymphocytes.

Moderate reduction of ATP level triggers oxidative stress, which may result in damage and dysfunction of the cell, and then progression or/and development of age-related disorders (Schutt et al., 2012). It has also been shown that significant decrease of ATP level in lymphocytes is associated with necrosis of this cell type (Nikotera et al., 1998). Therefore, it is highly probable that intracellular ATP depletion caused by bromophenolic FRs strongly contributed to a decrease in PBMCs viability. For instance, it was noticed that TBBPA, which caused the most substantial reduction in cell viability, simultaneously induced the strongest depletion in ATP level, whereas TBBPS, which exhibited the lowest necrotic potential caused the smallest decrease in ATP pull in PBMCs.

Similar results were obtained by Michałowicz et al. (2015) who showed that BPA much more strongly than BPS depleted ATP level in human PBMCs. They also observed positive association between intracellular ATP depletion and a decrease in PBMCs viability. In another studies, Maćczak et al. (2015, 2017b) noted that BPA caused a significant decrease in ATP level and viability of human erythrocytes, while BPS caused negligible reduction of both parameters studied. Taking the above findings into consideration, it may be suggested that sulphonyl group/methyl group(s) (but not bromine atoms) are mostly responsible for substantially different cytotoxic potential of TBBPA and TBBPS in human PBMCs.

This study also showed that PBP significantly depleted ATP level in human PBMCs. PBP is considered as a potent uncoupler of oxidative phosphorylation in mitochondria. This substance disturbs the coupling between the electron transport and phosphorylation reactions, and thus inhibits ATP synthesis in this organelle (Sweetman and Griffitths, 1971). Michałowicz (2010a) observed that PCP, which is a chlorinated derivative of PBP strongly reduced ATP level in human lymphocytes.

ROS are implicated in mitochondrial metabolism, response of the cell to cytokines or pathogens in the immune system and are involved in cell signaling, cell proliferation and cell death (Hirsch et al., 1997).

The highest increase in the level of ROS was observed in cells treated with TBBPA, while the smallest in cells incubated with TBBPS. It must be noted that TBBPA was able to promote ROS generation at very low concentration of 0.0001 μg/mL. In another study, Jarosiewicz et al. (2019) observed that bromophenolic FRs, including TBBPA at similar concentration of 0.001 μg/mL increased ROS formation in human erythrocytes. Similarly to our study, they also noticed that TBBPA caused the greatest, whereas TBBPS the smallest increase in ROS level. In other studies, Reistadt et al. (2005) noted that TBBPA caused an increase in ROS level in human neutrophil granulocytes, whereas Michałowicz et al. (2015) and Maćczak et al. (2017a) observed that BPA caused a significant rise in ROS level in human PBMCs and human erythrocytes, respectively.

This study has also shown that 2,4,6-TBP and PBP (similarly to bromobisphenols) at low concentrations induced ROS formation. These findings are in agreement with the results obtained by Jarosiewicz et al. (2019) who observed that bromophenols at comparably low concentrations increased ROS level in human red blood cells. Moreover, the studies conducted by Michałowicz (2010a,b) and Maheshwari et al. (2019) have revealed that chlorinated phenols at low concentrations were able to induce ROS formation in human lymphocytes and erythrocytes, respectively.

It was also found that PBP at its highest concentration decreased fluorescence intensity of the probe beneath the control value. This observation may suggest that PBP damaged (probably highly oxidized) $H_2$DCF, which partly quenched the emission of the probe. Similarly, Michałowicz (2010b) observed that PCP and particularly its derivative - tetrachlorocatechol at low concentrations (0.2–1 μg/mL) decreased fluorescence of the same probe in human lymphocytes.

Hydroxyl radical as a highly reactive oxygen species is implicated in the initiation of oxidative processes in cells and is responsible for damage to cellular biomolecules (Bartosz, 2009).

The recent study has shown that only TBBPA was able to induce hydroxyl radical substantially. Similarly, Xue et al. (2009) using electron paramagnetic resonance (EPR) observed that TBBPA even at low concentration of 0.01 μg/mL significantly promoted hydroxyl radical formation in earthworm *Eisenia fetida*, while Shi et al. (2005) revealed that TBBPA induced hydroxyl radical generation in liver of *Carassius auratus*. Moreover, the studies of Michałowicz et al. (2015) and Maćczak et al. (2017b) have documented that BPA was capable of inducing hydroxyl radical in human PBMCs and erythrocytes, respectively.

Accelerated ROS production leads to oxidative damage to lipids and proteins. Enhanced lipid peroxidation usually changes integrity and fluidity of biological membranes, which results in perturbations in transport of substrates and cell signaling. Moreover, it has been shown that products of lipids oxidation may exhibit genotoxicity, mutagenicity and carcinogenicity (Kim et al., 2000; Bartosz, 2009).

We have observed that bromophenolic FRs induced lipid peroxidation in PBMCs, while TBPPA caused much more substantial changes in this parameter in comparison to other compounds examined.

It has been documented that both phenols and bisphenols may potently induce lipid peroxidation in blood cells. Recent study of Maheshwari et al. (2019) revealed that PCP induced lipid peroxidation in human erythrocytes. Similarly, BPA and BPS have been shown to induce lipid peroxidation in human PBMCs (Michałowicz et al., 2015), whereas BPA also induced lipid damage in human erythrocytes (Maćczak et al., 2017a). In another study, Nakagawa et al. (2007) observed accumulation of malondialdehyde, a marker of lipid peroxidation in rat hepatocytes treated with TBBPA.

Oxidatively modified proteins are essentially liable to proteolytic enzymes activity, exhibit changed mechanical properties as well as reveal alterations in folding, conformation or in binding of co-factors and metal-ions that may lead to a loss of their functions. Furthermore, it has been shown that oxidation of protein may be involved in neurodegenerative diseases and cancer development (Castegna et al., 2002; Grosicka-Maciąg, 2011).

This study has revealed that the substances tested induced damage to proteins with stronger effect (particularly after 1 h of incubation) observed for TBBPA and PBP.

Recently Jarosiewicz et al. (2020) showed that bromophenolic FRs were able to damage tryptophan residues contained in proteins of the erythrocytes lipid bilayer (native system) and in the chemical system consisting of only the albumin (HAS). They also observed

that among the compounds studied, TBBPA and particularly PBP had stronger oxidative effect on the erythrocyte membrane and HAS, whereas 2,4,6-TBP and TBBPS in particular demonstrated weaker fluorescence-quenching effect of both membrane tryptophan and HSA. In another study, Shi et al. (2005) observed that TBBPA caused carbonylation of proteins in hepatocytes of fish C. *auratus*. Oxidative damage to proteins in human PBMCs and human erythrocytes treated with BPA and BPS has also been reported by Michałowicz et al. (2015) and Maćczak et al. (2017b). Moreover, the studies of Bukowska et al. (2009, 2016) and Michałowicz (2010a,b) have shown that phenol and chlorinated phenols, including 2,4-dichlorophenol (2,4-DCP), 2,4,5-TCP and PCP caused oxidative damage and carbonylation of proteins in human PBMCs and human red blood cells.

## 5. Conclusions

This study has shown that bromophenolic FRs, i.e. TBBPA, TBBPS, 2,4,6-TBP and PBP exhibited cytotoxic and oxidative potential in human PBMCs. The compounds tested depleted intracellular ATP level, which led to reduction of viability of the incubated cells. BFRs examined also increased ROS including hydroxyl radical level, causing oxidation of lipids and proteins in PBMCs. Among the compounds studied, TBBPA induced the strongest changes, while TBBPS, which is used as a substitute of TBBPA in the manufacture exhibited the lowest cytotoxic and oxidative potential. It may be concluded that oxidative changes in PBMCs were induced at environmentally or occupationally relevant concentrations of BFRs studied, while cytotoxic effects occurred under the influence of BFRs concentrations that were much higher than those detected in humans exposed to these toxicants. Further research, including *in vitro* studies are necessary for evaluating the risk caused by examined BFRs for human organism.

## Author statement

Anna Włuka: Investigation, Formal analysis, Writing. Agnieszka Woźniak: Investigation. Ewelina Woźniak: Visualisation. Jaromir Michałowicz: Conceptualization, Supervision, Writing - review & editing.

## 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.

## Acknowledgements

This work was granted from the Research 505/387/R admitted for the Department of Biophysics of Environmental Pollution University of Lodz.

## References

Abdallah, M.A.E., Harrad, S., Covaci, A., 2008. Hexabromocyclododecanes and tetrabromobisphenol-A in indoor air and dust in Birmingham, UK: implications for human exposure. Environ. Sci. Technol. 42, 6855–6861.
Alaee, M., Arias, P., Sjodin, A., Bergman, A., 2003. An overview of commercially used brominated flame retardants, their applications, their use patterns in different countries/regions and possible modes of release. Environ. Int. 2, 683–689.
Bartosz, G., 2009. Druga Twarz Tlenu. Wydawnictwo Naukowe PWN.
Bjermo, H., Aune, M., Cantillana, T., Glynn, A., Lind, P.M., Ridefelt, P., Darnerud, P., 2017. Serum levels of brominated flame retardants (BFRs: PBDE, HBCD) and influence of dietary factors in a population-based study on Swedish adults. Chemosphere 167, 485–491.
Bukowska, B., Marczak, A., Michałowicz, J., Wiśniewska, K., 2009. Effects of phenol, catechol, chloro- and methylphenol on human erythrocyte membrane (*in vitro*). Pol. J. Environ. Stud. 18, 569–577.
Bukowska, B., Wieteska, P., Kwiatkowska, M., Sicińska, P., Michalowicz, J., 2016. Evaluation of the effect of 2,4-dichlorophenol on oxidative parameters and viability of human blood mononuclear cells (*in vitro*). Hum. Exp. Toxicol. 35, 775–784.
Cariou, R., Antignac, J.P., Zalko, D., Berrebi, A., Cravedi, J.P., Maume, D., Marchand, P., Montenau, F., Riu, A., Andre, F., LeBizec, B., 2008. Exposure assessment of French women and their newborns to tetrabromobisphenol-A: occurrence measurements in maternal adipose tissue, serum, breast milk and cord serum. Chemosphere 73, 1036–1041.
Castegna, A., Aksenov, M., Aksenova, M., Thongboonkerd, V., Klein, J.B., Pierce, W.M., Booze, R., Marksesbery, W.R., Butterfield, D.A., 2002. Proteomic identification of oxidatively modificated proteins in Alzheimer disease brain. Part I. Creatine kinase BB, glutamine synthase, and ubiquitin carboxy-terminal hydrolase L-1. Free Radical Biol. Med. 33, 562–571.
Chaplin, D.D., 2010. Overview of the immune response. J. Allergy Clin. Immunol. 125. S3–23.
Chen, Ch, Yang, P., Kao, Y., Chen, P., Chung, Ch, Wang, S., 2017. Pentabromophenol suppresses TGF-β signaling by accelerating degradation of type II TGF-β receptors via caveolae-mediated endocytosis. Sci. Rep. 7, 43206.
Covaci, A., Voorspoels, S., Abdallah, M., Geens, T., Harrad, S., Law, R.J., 2009. Analytical and environmental aspects of the flame retardant tetrabromobisphenol-A and its derivatives. J. Chromatogr., A 1216, 346–363.
Covaci, A., Harrad, S., Abdallah, M.A.-E., Ali, N., Law, R.J., Herzke, D., de Wit, C.A., 2011. Novel brominated flame retardants: a review of their analysis, environmental fate and behaviour. Environ. Int. 37, 532–556.
DeWit, C.A., 2002. An overview of brominated flame retardants in the environment. Chemosphere 46, 583–624.
Dufour, P., Pirard, C., Charlier, C., 2017. Determination of phenolic organohalogens in human serum from a Belgian population and assessment of parameters affecting the human contamination. Sci. Total Environ. 599–600, 1856–1866.
Dunnick, J.K., Sanders, J.M., Kissling, G.E., Johnson, C.L., Boyle, M.H., Elmore, S.A., 2015. Environmental chemical exposure may contribute to uterine cancer development: studies with tetrabromobisphenol A. Toxicol. Pathol. 43, 464–473.
Feng, Ch, Xu, Q., Jin, Y., Lin, Y., Qiu, X., Lu, D., Wang, G., 2016. Determination of urinary bromophenols (BrPs) as potential biomarkers for human exposure to polybrominated diphenyl ethers (PBDEs) using gas chromatography-tandem mass spectrometry (GC–MS/MS). J. Chromatogr., B 1022, 70–74.
Fujii, Y., Harada, K.H., Hitomi, T., Kobayashi, H., Koizumi, A., Haraguchi, K., 2014. Temporal trend and age-dependent serum concentration of phenolic organohalogen contaminants in Japanese men during 1989–2010. Environ. Pollut. 185, 228–233.
Góralczyk, K., Struciński, P., Czaja, K., Hernik, A., Ludwicki, K.J., 2002. Uniepalniacze - zastosowanie i zagrożenie dla człowieka. Rocz Panstw Zakl Hig 53, 295–305.
Grosicka-Maciąg, E., 2011. Biologiczne skutki stresu oksydacyjnego wywołane działaniem pestycydów. Postep Hig Med Dosw 65, 357–366.
Gutiérrez, M., Becerra, J., Godoy, J., Barra, R., 2005. Occupational and environmental exposure to tribromophenol used for wood surface protection in sawmills. Int. J. Environ. Health Res. 15, 171–179.
Hale, R., Alaee, M., Manchester-Neesvig, J., Stapleton, H.M., Ikonomou, M.G., 2003. Polybrominated diphenyl ether flame retardants in the North American environment. Environ. Int. 29, 771–779.
Hirsch, T., Marzo, I., Kroemer, G., 1997. Role of the mitochondria permeability transition pore in apoptosis. Biosci. Rep. 17, 67–76.
Jarosiewicz, M., Michałowicz, J., Bukowska, B., 2019. In vitro assessment of eryptotic potential of tetrabromobisphenol A and other bromophenolic flame retardants. Chemosphere 215, 404–412.
Jarosiewicz, M., Miłowska, K., Krokosz, A., Bukowska, B., 2020. Evaluation of the effect of selected brominated flame retardants on human serum albumin and human erythrocyte membrane proteins. Int. J. Mol. Sci. 21, 3926.
Jarosiewicz, M., Bukowska, B., 2017. Tetrabromobisphenol A – toxicity, environmental and occupational exposure. Occup. Med. 68, 121–134.
Jarosiewicz, M., Duchnowicz, P., Włuka, A., Bukowska, B., 2017. Evaluation of the effect of brominated flame retardants on hemoglobin oxidation and hemolysis in human erythrocytes. Food Chem. Toxicol. 109, 264–271.
Jarosiewicz, M., Krokosz, A., Marczak, A., Bukowska, B., 2019. Changes in the activities of antioxidant enzymes and reduced glutathione level in human erythrocytes exposed to selected brominated flame retardants. Chemosphere 227, 93–99.
Johnson-Restrepo, B., Adams, D.H., Kannan, K., 2008. Tetrabromobisphenol A (TBBPA) and hexabromo-cyclododecanes (HBCDs) in tissues of humans, dolphins, and sharks from the United States. Chemosphere 70, 1935–1944.
Kim, H.S., Kwack, S.J., Lee, B.M., 2000. Lipid peroxidation, antioxidant enzymes, and benzo[a]pyrene-quinones in the blood of rats treated with benzo[a]pyrene. Chem-biol Interactions 127, 139–150.
Koch, Ch, Sures, B., 2018. Environmental concentrations and toxicology of 2,4,6-tribromophenol (TBP). Environ. Pollut. 233, 706–713.
Lee, D., Ahn, C., Hong, E., An, B.S., Hyun, S.H., Choi, K.C., Jeung, E.B., 2016. 2,4,6-Tribromophenol interferes with the thyroid hormone system by regulating thyroid hormones and the responsible genes in mice. Int. J. Environ. Res. Publ. Health 13, 697.
Liu, A., Zhaoshuang, S., Yong, T., Ronggnang, S., Lin, Y., Zhao, Z., 2017. Thin-layer chromatography coupled with high performance liquid chromatography for determining tetrabromobisphenol A/S and their derivatives in soils.

J. Chromatogr., A 1526, 151–156.
Maćczak, A., Bukowska, B., Michałowicz, J., 2015. Comparative study of the effect of BPA and its selected analogues on hemoglobin oxidation, morphological alterations and hemolytic changes in human erythrocytes. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 176, 62–70.
Maćczak, A., Cyrkler, M., Bukowska, B., Michałowicz, J., 2017a. Bisphenol A, bisphenol S, bisphenol F and bisphenol AF induce different oxidative stress and damage in human red blood cells (*in vitro* study). Toxicol. Vitro 41, 143–149.
Maćczak, A., Duchnowicz, P., Sicińska, P., Koter-Michalak, M., Bukowska, B., Michałowicz, J., 2017b. The in vitro comparative study of the effect of BPA, BPS, BPF and BPAF on human erythrocyte membrane; perturbations in membrane fluidity, alterations in conformational state and damage to proteins, changes in ATP level and Na+/K+ ATPase and AChE activities. Food Chem. Toxicol. 110, 351–359.
Maheshwari, N., Khan, F.H., Mahmood, R., 2019. Pentachlorophenol-induced cytotoxicity in human erythrocytes: enhanced generation of ROS and RNS, lowered antioxidant power, inhibition of glucose metabolism, and morphological changes. Environ. Sci. Pollut. Res. Int. 26, 12985–13001.
Michałowicz, J., 2010a. 2,4,5-Trichlororophenol and its derivatives induce biochemical and morphological changes in human peripheral blood lymphocytes *in vitro*. Arch. Environ. Contam. Toxicol. 59, 670–678.
Michałowicz, J., 2010b. Pentachlorophenol and its derivatives induce oxidative damage and morphological changes in human lymphocytes (*in vitro*). Arch. Toxicol. 84, 379–387.
Michałowicz, J., Mokra, K., Bąk, A., 2015. Bisphenol A and its analogs induce morphological and biochemical alterations in human peripheral blood mononuclear cells (in vitro study). Toxicol. Vitro 29, 1464–1472.
Nakagawa, Y., Suzuki, T., Ishii, H., Ogata, A., 2007. Biotransformation and cytotoxicity of a brominated flame retardant, tetrabromobisphenol A, and its analogues in rat hepatocytes. Xenobiotica 37, 693–708.
Nakao, T., Akiyama, E., Kakutani, H., Mizuno, A., Aozasa, O., Akai, Y., 2015. Levels of tetrabromobisphenol A, tribromobisphenol A, dibromobisphenol A, monobromobisphenol A, and bisphenol A in Japanese breast milk. Chem. Res. Toxicol. 28, 722–728.
Nikotera, P., Leist, M., Ferrnando-May, E., 1998. Intracellular ATP, a switch in the decision between apoptosis and necrosis. Toxicol. Lett. 102 (103), 139–142.
NTP, 2014. Technical Report on the Toxicology Studies of Tetrabromobisphenol A (CAS NO. 79–94-7) in F344/NTac Rats and B6C3F1/N Mice and Toxicology and Carcinogenesis Study of Tetrabromobisphenol A in Wistar Han [Crl:WI(Han)] Rats and B6C3F1/N Mice (Gavage Studies). TP TR, vol. 587. National Toxicology Program, Research Triangle Park, NC.
Papadopoulos, N., Dedoussis, G., Spanakos, G., Gritzapis, A., Baxevanis, C., Papamichail, M., 1994. An improved fluorescence assay for determination of lymphocyte-mediated cytotoxicity using flow cytometry. J. Immunol. Methods 177, 101–109.
Ratomski, K., Skotnicka, B., Kasprzycka, E., Żelazowska-Rutkowska, B., Wysocka, J., Anisimowicz, S., 2007. Evaluation of percentage of the CD19+CD5+ lymphocytes in hypertrophied adenoids at children with otitis media with effusion. Otolaryngol. Pol. 61, 962–966.
Reistad, T., Mariussen, E., Fonnum, F., 2015. The effect of a brominated flame retardant, tetrabromobisphenol-A, on free radical formation in human neutrophil granulocytes: the involvement of the MAP kinase pathway and protein kinase. C. Toxicol. Sci. 83, 89–100.
Remberger, M., Sternbeck, J., Palm, A., Kaj, L., Stromberg, K., Brorstrom-Lunden, E., 2004. The environmental occurrence of hexabromocyclododedecane in Sweden. Chemosphere 54, 9–21.
Schutt, F., Aretz, S., Auffarth, G., Kopitz, J., 2012. Moderately reduced ATP levels promote oxidative stress and debilitate autophagic and phagocytic capacities in human RPE cells. Retinal Cell Biol 53, 5354–5361.
Shi, H., Wang, X., Luo, Y., Su, Y., 2005. Electron paramagnetic resonance evidence of hydroxyl radical generation and oxidative damage induced by tetrabromobisphenol A in Carassius auratus. Aquat. Toxicol. 74, 365–371.
Stanly, P., Williams, S., 1969. Use of the liquid scintillation spectrometer for determining adenosine triphosphate by the luciferase enzyme. Anal. Biochem. 29, 381–392.
Stapleton, H.M., Dodder, N.G., Offenberg, J.H., Schantz, M.M., Wise, S.A., 2005. Polybrominated diphenyl ethers in house dust and clothes dryer lint. Environ. Sci. Technol. 39, 925–931.
Staszowska, A., 2009. Bromoorganiczne antypireny jako chemiczne zanieczyszczenia powietrza wewnętrznego. Polska Inżynieria Środowiska 59, 205–212.
Sweetman, A.J., Griffitths, D.E., 1971. Energy-linked reaction of oxidized nicotinamide-adenine dinucleotide by succinate in *Escherichia coli*. Biochem. J. 121, 117–124.
Tian, Y., Chen, J., Ouyang, Y.-Z., Qu, G.-B., Liu, A.-F., Wang, X.-M., Liu, Ch-X., Shi, J.-B., Chen, H.-W., Jiang, G.-L., 2014. Reactive extractive electrospray ionization tandem mass spectrometry for sensitive detection of tetrabromobisphenol A derivatives. Anal. Chim. Acta 814, 49–54.
Venza, I., Visalli, M., Cucinotta, M., Tedi, D., Venza, M., 2012. Association between oxidative stress and macromolecular damage in elderly patients with age-related macular degeneration. Aging Clin. Exp. Res. 24, 21–27.
Wang, X., Wei, L., Zhu, J., He, B., Kong, B., Xue, Z., Jin, X., Fu, Z., 2019. Environmentally relevant doses of tetrabromobisphenol A (TBBPA) cause immunotoxicity in murine macrophages. Chemosphere 236, 124413.
Watała, C., 2002. Biostatystyka – Wykorzystanie Metod Statystycznych W Pracy Badawczej W Naukach Biomedycznych. Alfa-Medica Press, Bielsko-Biała.
Wu, S., Wu, M., Qi, M., Zhong, L., Qiu, L., 2018. Effects of novel brominated flame retardant TBBPA on human airway epithelial cell (A549) *in vitro* and proteome profiling. Environ. Toxicol. 33, 1245–1253.
Xie, Z., Ebinghaus, R., Lohmann, R., Heemken, O., Caba, A., Püttmann, W., 2007. Trace determination of the flame retardant tetrabromobisphenol A in the atmosphere by gas chromatography–mass spectrometry. Anal. Chim. Acta 584, 333–342.
Xue, Y., Gu, X., Wang, X., Sun, C., Xu, X., Sun, J., Zhang, B., 2009. The hydroxyl radical generation and oxidative stress for the earthworm *Eisenia fetida* exposed to tetrabromobisphenol A. Ecotoxicology 18, 693–699.
Yang, N-Ch, Ho, W.-M., Chen, Y.-H., Hu, M.-L., 2002. A convenient onestep extraction of cellular ATP using boiling water for the luciferin-luciferase assay of ATP. Anal. Biochem. 306, 323–327.

frontiers | Frontiers in Immunology

ORIGINAL RESEARCH
published: 12 April 2022
doi: 10.3389/fimmu.2022.869741

# Genotoxic Mechanism of Action of TBBPA, TBBPS and Selected Bromophenols in Human Peripheral Blood Mononuclear Cells

*Anna Barańska, Agnieszka Woźniak, Katarzyna Mokra and Jaromir Michałowicz* *†

*Department of Biophysics of Environmental Pollution, Faculty of Biology and Environmental Protection, University of Lodz, Lodz, Poland*

Bromophenolic flame retardants (BFRs) are a large group of synthetic substances used in the industry in order to reduce the flammability of synthetic materials used in electrical and electronic devices, textiles, furniture and other everyday products. The presence of BFRs has been documented in the environment, food, drinking water, inhaled dust and the human body. Due to the widespread exposure of the general population to BFRs and insufficient knowledge on their toxic action, including genotoxic potential, we have compared the effect of tetrabromobisphenol A (TBBPA), tetrabromobisphenol S (TBBPS), 2,4,6,-tribromophenol (2,4,6-TBP) and pentabromophenol (PBP) on DNA damage in human peripheral blood mononuclear cells (PBMCs) (playing a crucial role in the immune system) as well as examined underlying mechanism of action of these substances. The cells were incubated for 24 h with studied compounds in the concentrations ranging from 0.01 to 10 µg/mL. The study has shown that examined BFRs induced single and, to a lesser extent, double strand-breaks formation and caused oxidative damage to pyrimidines, and particularly to purines in the incubated cells. PBMCs efficiently repaired the DNA strand-breaks induced by BFRs, but they were unable to remove completely damaged DNA (except cells treated with TBBPS). The greatest changes in the above-mentioned parameters were observed in cells incubated with TBBPA, while the smallest in PBMCs treated with TBBPS. The results have also revealed that tested compounds do not form adducts with DNA in PBMCs, while the observed changes were the most probably induced by indirect DNA-damaging agents, such as ROS and other reactive species.

**Keywords: tetrabromobisphenol A, tetrabromobisphenol S, pentabromophenol, Tribromophenol, DNA strand-breaks, DNA base oxidation, DNA adducts, peripheral blood mononuclear cells**

OPEN ACCESS

***Edited by:***
*Celine Ann Beamer, University of Montana, United States*

***Reviewed by:***
*Danielle Ferraz Mello, Duke University, United States*
*Sandra Helena Poliselli Farsky, University of São Paul, Brazil*

***Correspondence:**
*Jaromir Michałowicz*
*jaromir.michalowicz@biol.uni.lodz.pl*

†***ORCID:***
*Jaromir Michałowicz, orcid.org/0000-0002-7293-596X*

***Specialty section:***
*This article was submitted to Comparative Immunology, a section of the journal Frontiers in Immunology*

***Received:*** *04 February 2022*
***Accepted:*** *21 March 2022*
***Published:*** *12 April 2022*

***Citation:***
*Barańska A, Woźniak A, Mokra K and Michałowicz J (2022) Genotoxic Mechanism of Action of TBBPA, TBBPS and Selected Bromophenols in Human Peripheral Blood Mononuclear Cells. Front. Immunol. 13:869741. doi: 10.3389/fimmu.2022.869741*

## 1 INTRODUCTION

Bromophenolic flame retardants (BFRs) are ubiquitous chemicals widely used in the industry in the production of polymers, electrical and electronic equipment, textiles, furniture and other everyday products (1–3). Tetrabromobisphenol A (TBBPA) is the most widely used BFR. In 2004, it was estimated that the annual production of TBBPA in the United States, Japan and Israel reached

approx. 170 thousand tones, which accounted for approx. 60% of the production of all brominated FRs worldwide (2). Due to massive production of TBBPA, this substance has been detected in the air, inhaled dust as well as in terrestrial and aquatic ecosystems (2, 4, 5). Tetrabromobisphenol S (TBBPS) was introduced into the market as TBBPA substitute. There is very limited data on the presence of TBBPS in the environment and its effect on living organisms. In the study of Wang et al. (6), TBBPS was detected in significant concentrations (up to 12.1 μg/L) in wastewater, while Ding et al. (7) observed that TBBPS altered the circadian rhythm network in the early life stages of zebrafish and potentially caused developmental delays in zebrafish embryos. Some studies have suggested that TBBPS may not be less toxic than TBBPA in humans. For instance, Liang et al. (8) observed that TBBPA and TBBPS had similar toxicity towards human embryonic stem cells. Both compounds disturbed neural ectoderm development, influenced axon growth and neuron transmission as well as dysregulated the WNT and AHR signaling pathways.

Brominated phenols like pentabromophenol (PBP), and particularly 2,4,6-tribromophenol (2,4,6-TBP) are widely represented in the environment and human surrounding. These substances have been repeatedly determined in the air, surface water, soil as well as home dust, food and drinking water (9). 2,4,6-TBP and PBP have also been shown to provoke various adverse effects in animals and human (3, 9, 10).

BFRs have been found in human plasma, placental tissue, adipose tissue, and breast milk samples (11, 12). TBBPA was detected in samples of human milk in the concentrations of 0.06 to 37.34 ng/g of fat and serum samples from both mothers and fetuses in similar range of concentrations (13). TBBPA was also found in plasma of Japanese men in a mean concentration of 950 pg/g fresh weight (14), while TBBPS was determined in a mean concentration of 0.593 μg/L of serum samples from pregnant women in China (15). Dufour et al. (16) determined 2,4,6-TBP in the concentrations from trace to 1.28 μg/L of blood of the general population of Belgium. In other studies, Feng et al. (17) detected 2,4,6-TBP in the concentrations of 5.57 ± 4.05 μg/L in the urine of the general population of China, while Gutierrez et al. (18) found very high mean concentration of 2,4,6-TBP in the urine of Chilean sawmills workers, which was 6.9 mg/g creatinine (approx. 6.9 mg per 1 L of urine).

The mechanism of bromophenolic FRs genotoxicity has not been elucidated. Moreover, literature data often offers conflicting information about the effects of these compounds on DNA. For instance, earlier studies have shown no genotoxic effects of TBBPA that could have been associated with the use of very low doses of this compound (19–21). However, more recent studies have indicated genotoxic potential of TBBPA in spermatozoa of mice (22), blood cells of spotted snake (*Channa punctatus*) (23) and mouse testicular cell co-culture model (24). 2,4,6-TBP was not genotoxic in *in vitro* bacterial tests (25, 26); however, it caused chromosomal aberrations (with and without metabolic activation) in *in vitro* tests on Chinese hamster cells (27). In case of other studied BFRs, data on their genotoxic effects is negligible. It was shown that PBP was not mutagenic in *Salmonella typhimurium* with or without metabolic activation (28), while genotoxic potential of TBBPS has not been studied.

Each cell under normal conditions is subjected to thousands attacks on its DNA each day (29), which may lead to genetic instability contributing to an increase in the rate of spontaneous mutations (30). Reactive oxygen species (ROS), and mostly hydroxyl radical (•OH) have been recognized as critical factors to the DNA damage, and our previous study showed that BFRs increased ROS, including •OH levels in human PBMCs (31).

PBMCs play a key role in the body immune system. They are responsible for producing antibodies, killing virus-infected and cancerous cells, but also for regulating the immune system response (32). It has been proven that damage to PBMCs, and lymphocytes in particular (e.g. by xenobiotics) may contribute to the immune system dysfunction, which may result in autoimmune diseases (asthma, allergy) or cancer development (33, 34). Some studies have shown that BFRs may alter the immune system function. For instance, TBBPA has been shown to change tumor killing function of NK lymphocytes and alter secretion of various cytokines, including interferon gamma (IFNγ), interleukin-1β (IL-1β) and tumor necrosis factor (TNF) (35). In another study, microarray analysis of uterine tissue of female Wistar Han rats showed that TBBPA downregulated genes in pathways of the immune response, which could lead to estrogen-mediated immunosuppression in tested animals (36).

Taking the above into consideration, we have decided to compare genotoxic effect of TBBPA, TBBPS, 2,4,6-TBP and PBP in human PBMCs, and examine underlying mechanism of action of these substances by evaluating single and double strand-breaks formation, purines and pyrimidines oxidation and DNA adducts creation in the tested cells.

## 2 MATERIAL AND METHODS

### 2.1 Chemicals

Tetrabromobisphenol A (99%, 2,2-bis(3,5-dibromo-4-hydroxyphenyl)propane) and pentabromophenol (98%, 2,3,4,5,6-pentabromophenol) were obtained from LGC Standards (Germany). Tribromophenol (pure ≤100%, 2,4,6-tribromophenol) was bought from Sigma-Aldrich (USA). Tetrabromobisphenol S (98.8%) was synthetized in the Institute of Industrial Organic Chemistry in Warsaw (Poland). Low melting point (LMP), normal melting point (NMP) agarose, fetal bovine serum (FBS) and DAPI (98%) were bought in Sigma-Aldrich (USA). Lymphocyte separation medium (LSM) (1.077 g/cm$^3$) and RPMI 1640 with L-glutamine were purchased from Cytogen (Germany). Endonuclease III and human 8-oxoguanine DNA glycosylase were bought in New England BioLabs (USA). Potassium chloride (99.5%), sodium chloride (99.5%), sodium hydrogen carbonate (99%), ammonium chloride (99.5%), sodium wersenite (99.5%) and other chemicals were bought from POCH (Poland) and Roth (Germany).

## 2.2 Methods

### 2.2.1 PBMCs Isolation and Treatment

PBMCs were isolated from the buffy coat (concentrated suspension of leucocytes and platelets) separated from whole blood in the Blood Bank in Lodz, Poland. Blood was collected from healthy, non-smoking volunteers (aged 18-40) showing no signs of infection disease symptoms. The method of PBMCs isolation was described in detail by Włuka et al. (31). The use of human blood in the study of the effect of tested BFRs on leucocytes was approved by the Bioethical Commission of Scientific Research at the University of Lodz (contract no. KBBN-UŁ/I/7/2011).

The cells were treated with tested compounds in the concentrations range from 0.01 to 10 μg/mL for 24 h at 37°C in 5% $CO_2$ atmosphere in total darkness. The concentrations of examined BFRs corresponded to their levels determined in humans environmentally and occupationally exposed. Our previous study (31) proved that TBBPA, TBBPS, 2,4,6-TBP and PBP up to the concentration of 10 μg/mL did not decrease PBMCs viability below 80%. Cell viability (expressed in %) after treatment with TBBPA, TBBPS, 2,4,6-TBP and PBP at 10 μg/mL was 86.3 ± 3.55, 85.4 ± 1.98, 83.4 ± 2.47 and 81.2 ± 2.27, respectively (31). The analysis of cell viability was conducted using calcein-AM and propidium iodide stains. The samples were analyzed by means of flow cytometry.

The examined compounds were dissolved in DMSO. Final concentration of DMSO in untreated samples (negative control) and samples treated with TBBPA, TBBPS, 2,4,6-TBP or PBP was 0.2%. The above DMSO concentration was not toxic for PBMCs as assessed by all studied parameters.

All analyses of DNA damage included positive controls. The positive controls for alkaline and neutral comet assay were done based on previous experiments performed in our laboratory (37, 38).

Hydrogen peroxide at 20 μM was used in a positive control during analysis of single strand breaks (SSBs) formation and DNA bases oxidation (the cells were incubated with $H_2O_2$ for 15 min on ice). In order to induce double strand breaks (DSBs) formation, the samples were irradiated with 1.8 Gy/min for 5 min at room temperature.

### 2.2.2 Comet Assay – Alkaline Version

A comet assay has been accepted as a simple, rapid and sensitive visual technique for assessing DNA damage. Alkaline version of comet assay can determine chemically or physically induced SSBs/DSBs and alkali labile sites, while neutral version of comet assay enables to determine selectively DSBs in the DNA of individual cells. In the comet assay, the cells are embedded in agarose on a microscope slide, and then are lysed with detergent and high salt to form nucleoids containing supercoiled loops of DNA linked to the nuclear matrix. After DNA staining, the release of DNA from a highly supercoiled DNA–protein complex is visually determined, which correlates with DNA damage detection (39, 40).

Alkaline version of the comet assay was carried out according to Singh et al. (40) with modifications (41), as described by Błasiak and Kowalik (42). A freshly prepared cells suspension in 0.75% LMP agarose dissolved in PBS was layered onto microscope slides, which was pre-coated with 0.5% NMP agarose. Then, the cells were lysed for 1 h at 4°C in a buffer containing 2.5 M NaCl, 0.1 M $Na_2EDTA$, 10 mM Tris, 1% Triton X-100, pH 10. After cells lysis, the slides were placed in an electrophoresis unit. DNA was allowed to unwind for 20 min in the solution containing 300 mM NaOH and 1 mM $Na_2EDTA$, pH > 13.

Electrophoretic separation was performed in the solution containing 30 mM NaOH and 1 mM EDTA, pH > 13 at ambient temperature of 4°C (the temperature of the running buffer did not exceed 12°C) for 20 min at an electric field strength of 0.73 V/cm (28 mA).

### 2.2.3 Comet Assay - Neutral Version

A neutral version of the comet assay was used to assess DSBs formation (43). The electrophoresis was run in a buffer containing 100 mM Tris and 300 mM sodium acetate at pH 9.0 adjusted by glacial acetic acid. Electrophoresis was conducted for 60 min, after a 20 min equilibrium period, at electric field strength of 0.41 V/cm (50 mA) at 4°C.

### 2.2.4 Oxidized Purines and Pyrimidines Detection (DNA Repair Enzyme Treatment)

Detection of oxidative DNA damage was conducted with the comet assay using endonuclease III (Endo III) and human 8-oxoguanine DNA glycosylase (hOGG1). The slides after cell lysis were washed three times (5 min, 4°C) in an enzyme buffer containing 40 mM HEPES–KOH, 0.1 M KCl, 0.5 mM EDTA, and 0.2 mg/mL bovine serum albumin, pH 8.0. Then, agarose on slides was covered with a volume of 50 μL of buffer containing 1 U of Endo III or hOGG1 or without the enzyme. Then, the slides were covered with cover glasses and incubated for 30 min at 37°C in a moist chamber. The cover glasses were removed and the slides were placed in an electrophoresis unit (44). DNA was allowed to unwind for 20 min in a solution containing 300 mM NaOH and 1 mM EDTA (pH > 13). The procedure was then conducted according to alkaline version of the comet assay.

We did not decide to calibrate the enzymes. According to New England BioLabs protocol, on which our experiment based on, dilution of hOGG1 and endoIII enzyme should be from 1: 102 to 1: 103 and from 1: 104 to 1: 105, respectively. It means that 50 μL of enzyme buffer with proper enzyme is equivalent of 0.08–0.8 U for hOOG1 and 0.05–0.5 U for endo III. Based on literature data (45) we decided to use 1 U of each enzyme per gel, which guaranteed their use in excess.

### 2.2.5 DNA Repair

After 24 h of incubation, untreated cells (negative controls) and cells treated with TBBPA, TBBPS, 2,4,6-TBP or PBP at 10 μg/mL were washed and resuspended in RPMI 1640 medium with L-glutamine pre-heated to 37°C. Aliquots of the suspension were taken immediately ("time zero") and 120 min later. In order to stop DNA repair, the samples were placed in an ice bath. DNA repair was assessed by the extent of residual DNA damage

detection at time-point '0 min' and '120 min' using alkaline version of the comet assay.

### 2.2.6 Comets Analysis

After electrophoresis, the slides were washed with deionized water, dried, stained with DAPI at 2 μg/mL and covered with cover slides. In order to prevent additional DNA damage, this procedure was carried out in limited light or darkness.

From each sample, 50 comets were randomly selected and the mean DNA value in the comet tail was taken as an indicator of DNA damage (expressed as a percentage). For one blood donor, two parallel tests with aliquots of the sample of the cells were performed for a total number of 100 comets. A total number of 300 comets (3 blood donors, n=3) was recorded to calculate mean ± SD.

The comets were observed at 200× magnification in an Eclipse fluorescence microscope AXIO SCOPE.A1 (Carl Zeiss, Germany) attached to Axiocam 305 color camera (Carl Zeiss, Germany) equipped with UV-1 filter block (an excitation filter of 359 nm and a barrier filter of 461 nm) and connected to a personal computer-based image analysis system Lucia-Comet v. 7.3 (Laboratory Imaging, Praha, Czech Republic).

### 2.2.7 Plasmid Relaxation Assay

The plasmid relaxation assay was conducted to evaluate the effect of studied compounds on changes in DNA structure and their ability to form adducts with DNA. For this purpose, DNA plasmid from *E. coli* (pUC19) was used. Plasmid may be represented *via* various structural forms: super coiled (SC, completely intact DNA strands), linear (L, both DNA strands damaged) and open coiled (OC, damaged one of the DNA strands). During electrophoretic separation, the highest rate of migration is represented by super coiled form. Slower migration is shown by open coiled form, whereas the slowest rate is represented by linear form.

Plasmid pUC19 was incubated with tested compounds at 0.1 μg/mL, 1 μg/mL and 10 μg/mL. Negative control referred to a plasmid treated with DMSO (0.2%). A positive control was obtained by the exposure of the plasmid to a hydroxyl radicals ($^{\bullet}OH$) formed as a result of the Fenton reaction; $^{\bullet}OH$ induce DNA strand breaks formation, which lead to relaxation of supercoiled plasmid (observed as a DNA linear form – L). To initiate the Fenton reaction, a mixture of $H_2O_2$ at 200 μM and $Fe^{2+}$ at 20 μM were added to the plasmid, which was incubated for 20 min at 37°C. After incubation, DNA gel loading buffer and Tris-EDTA buffer were added to the samples. Then, the samples were loaded onto 1% agarose gel and stained with ethidium bromide (0.5 μg/mL). Electrophoresis was performed in TrisAcetate-EDTA buffer for 60 min, at electric field strength of 5 V/cm (115 mA).

Gel was imaged in a Syngene Imagine Gels Documentation System under UV light and *via* Gel Documentation System Software Phoretix 1D. Image was saved as a TIFF file with a size of 16-bit. Then, image was evaluated using the Gel Analyzer tool of ImageJ, a public domain program from the National Institute of Health (NIH). Images were cropped from 1,280 x 1,020 pixels to 865 × 365 pixels to zoom into the gel. Before density analysis was done, background subtraction had been arranged. The profiles plot represents the average density value across a set of horizontal slices of each lane.

### 2.2.8 Statistical Analysis

The tests by comet assay were carried out on blood from 3 donors. For each individual experiment (one blood donor), an experimental point was a mean value from 2 replications. Moreover, 3 experiments were conducted to assess DNA adducts formation. Data was expressed as mean value with standard deviation. The first step was to check data normality using the Shapiro-Wilk test. Statistical significance was examined on the basis of a comparison of averages using a one-way analysis of variance - ANOVA. In order to evaluate statistically significant differences between the tested samples, a multiple comparison test - the Tukey test (*post-hoc*) was used (46). The differences were considered to be statistically significant when $p < 0.05$. Analysis was performed using the STATISTICA 13 software (StatSoft, Inc, Tulusa, USA).

## 3 RESULTS

### 3.1 DNA SSBs and DSBs Formation

The tested compounds induced SSBs/DSBs in DNA (**Figures 1A, B**). After 24-h of incubation the greatest changes were noted in cells treated with PBP, which even at 0.01 μg/mL caused DNA lesions. Much stronger DNA damage were noted in PBMCs incubated with PBP in the concentrations from 0.1 to 10 μg/mL. TBBPA at 0.1 μg/mL and 1 μg/mL also caused substantial damage to DNA, while at 10 μg/mL it caused greater DNA lesions than other tested BFRs. 2,4,6-TBP exhibited moderate genotoxic potential at 1 μg/mL and 10 μg/mL, while TBBPS only at 10 μg/mL induced relatively small DNA lesions (**Figure 1A**).

Selected photographs of damaged DNA (comets) of human PBMCs incubated with DMSO at 0.2% (negative control) and BFRs at 1 μg/mL were presented in **Figure 1B**.

After 24 h of incubation, all tested BFRs at their highest concentration of 10 μg/mL slightly increased DSBs levels in PBMCs. Among studied compounds, only TBBPA at lower concentration of 1 μg/mL was capable of inducing DNA DSBs formation in the incubated cells (**Figure 2**).

### 3.2 Oxidative Damage to DNA Bases

Tested compounds after 24 h of incubation induced oxidative damage to pyrimidines and purines in PBMCs (**Figures 3**, **4**). Among studied BFRs, only TBBPA at 0.1 μg/mL caused slight increase in oxidized pyrimidines level, while all tested substances, and particularly TBBPA and PBP at highest concentration of 1 μg/mL induced oxidative damage to pyrimidines in the incubated cells (**Figure 3**).

It was observed that examined compounds caused greater damage to purines than pyrimidines in the PBMCs (**Figures 3**, **4**). TBBPA induced the greatest changes in the parameter examined increasing oxidized purines level even at 0.01 μg/mL, and more strongly at 0.1 μg/mL and 1 μg/mL. Similarly, 2,4,6-

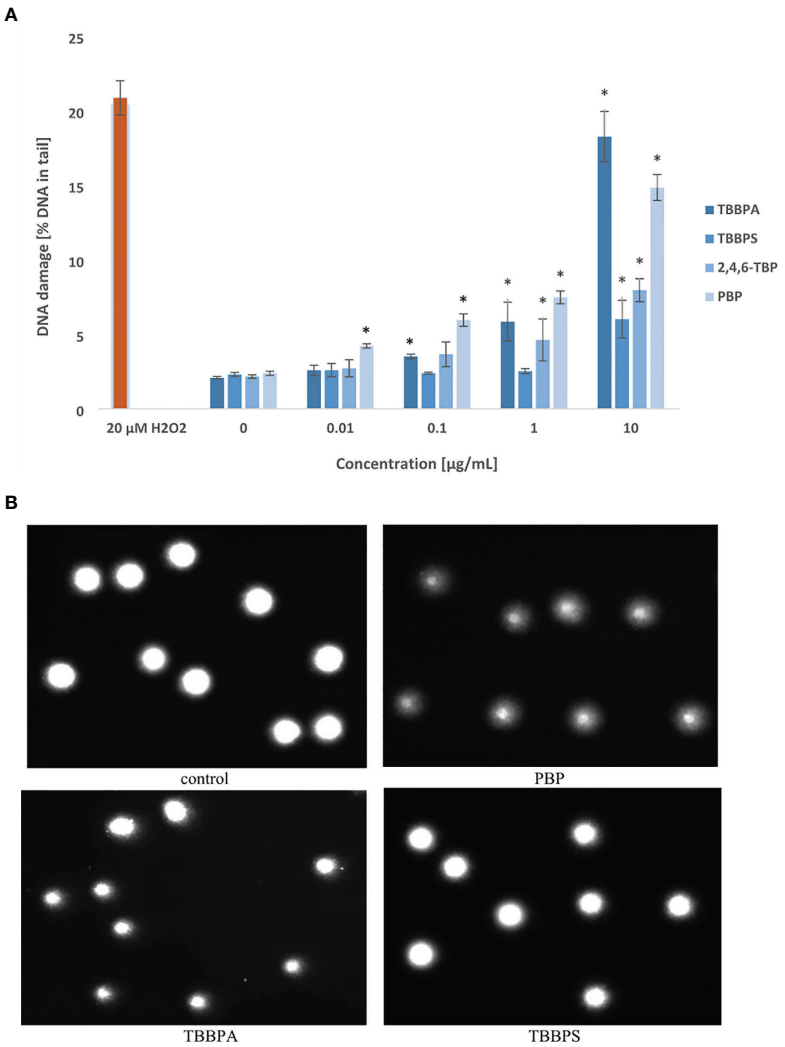



**FIGURE 1** | Total DNA strand breaks formation. **(A)** SSBs and DSBs formation in human PBMCs treated with TBBPA, TBBPS, 2,4,6-TBP and PBP at the concentrations of 0.01 µg/mL, 0.1 µg/mL, 1 µg/mL and 10 µg/mL for 24 h DNA damage was measured as the *percentage of DNA* in the comet *tail* using the alkaline version of the comet assay. Mean ± SD was calculated from 3 individual experiments (3 blood donors). Statistically different from negative control at *P<0.05. Statistical analysis was conducted using one-way ANOVA and a posteriori Tukey test. **(B)** Selected photographs of damaged DNA (comets) of human BPMCs incubated with DMSO at 0.2% (negative control) and tested BFRs at 1 µg/mL (comet assay, alkaline version). The photos were obtained using fluorescent microscope with 200x magnification.

TBP in the concentrations range from 0.01 to 1 µg/mL was capable of provoking purines lesions, while PBP at 0.1 µg/mL and 1 µg/mL caused purines oxidation (**Figure 4**). The smallest changes were noted in cells treated with TBBPS, which only at 1 µg/mL caused small oxidative purine and pyrimidine oxidation in the studied cells (**Figures 3**, **4**).

## 3.3 DNA Repair

Tested compounds at the concentration of 10 µg/mL caused substantial SSBs/DSBs formation in PBMCs after 24 h of incubation (**Figures 1**, **5**). It was observed that PBMCs efficiently repaired DNA lesions, but they were unable to remove completely damaged DNA (except cells treated with TBBPS) after 120 min. post-incubation period (**Figure 5**).

## 3.4 Plasmid Relaxation Assay

The results achieved during electrophoretic separation of pUC19 plasmid DNA revealed that neither brominated bisphenols nor bromophenols bound directly to DNA (**Figure 6A**). Similarly, densitometric analysis showed no changes in the amount of various plasmid forms after BFRs exposure, when compared to the negative control. That is why, it was concluded that tested

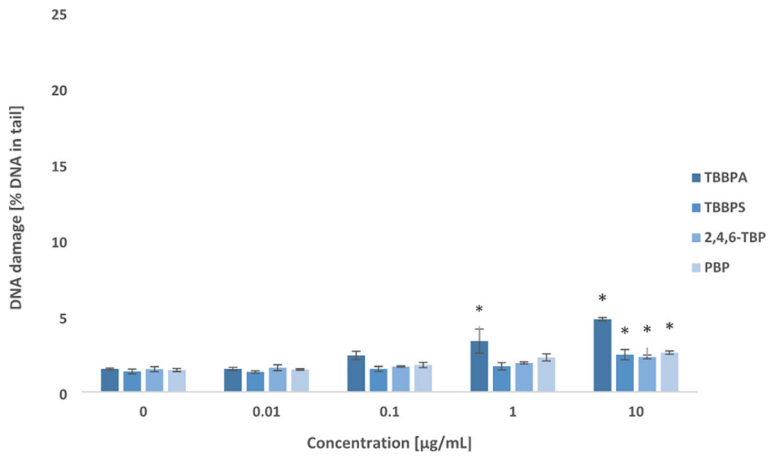



**FIGURE 2** | Double DNA strand breaks formation. DSBs formation in human PBMCs treated with TBBPA, TBBPS, 2,4,6-TBP and PBP at the concentrations of 0.01 µg/mL, 0.1 µg/mL, 1 µg/mL and 10 µg/mL for 24 h. DNA damage was measured as the *percentage of DNA* in the comet *tail* using the neutral version of the comet assay. Mean ± SD was calculated from 3 individual experiments (3 blood donors). Statistically different from negative control at *P<0.05. Statistical analysis was conducted using one-way ANOVA and a posteriori Tukey test.

compounds were incapable of creating adducts with DNA (**Figure 6B**).

## 4 DISCUSSION

There are limited and inconsistent results concerning genotoxic potential of bromophenolic FRs (20, 23, 25, 27). Moreover, according to our best knowledge, no study has been conducted to describe genotoxic mechanism of action of these substances in any cell type or organism.

In this study, we have decided to assess genotoxic potential of TBBPA, TBBPS, 2,4,6-TBP and PBP in human PBMCs, which play a key role in protecting the body from pathogens and cancer cells as well as are involved in maintaining of body homeostasis (47).

The results have shown that some of examined compounds at relatively low concentrations (from 0.01 µg/mL) caused SSBs formation in PBMCs, while DSBs were detected in cells incubated with much higher concentrations (from 1 µg/mL) of

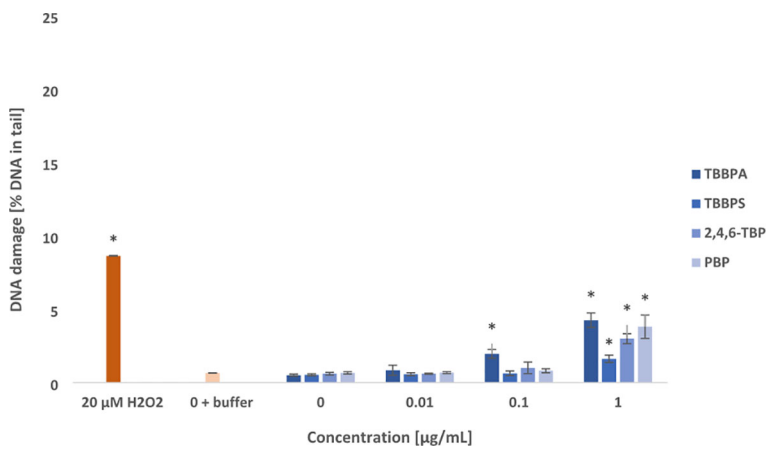



**FIGURE 3** | Oxidative damage to pyrimidines in DNA. Oxidative damage to DNA pyrimidines in human PBMCs treated with TBBPA, TBBPS, 2,4,6-TBP and PBP at the concentrations of 0.01 µg/mL, 0.1 µg/mL and 1 µg/mL for 24 h. DNA damage was measured as the *percentage of DNA* in the comet *tail* using the enzyme endo III and the alkaline version of the comet assay. The mean ± SD was calculated for 3 experiments (3 blood donors). Statistically different from negative control at *P<0.05. Statistical analysis was conducted using one-way ANOVA and a posteriori Tukey test.

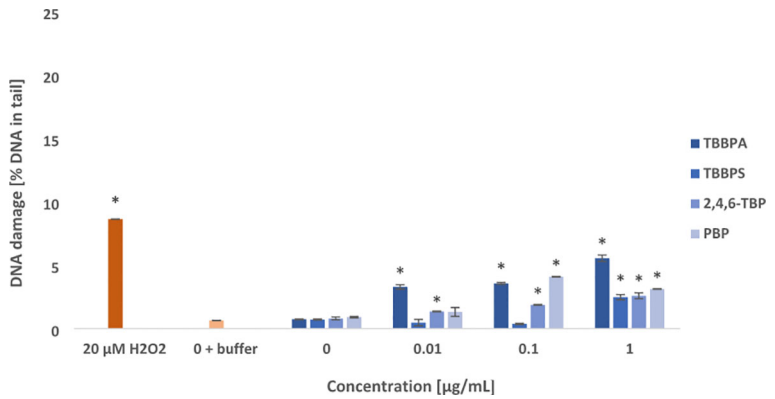



**FIGURE 4** | Oxidative damage to purines in DNA. Oxidative damage to DNA purines in human PBMCs treated with TBBPA, TBBPS, 2,4,6-TBP and PBP at the concentration of 0.01 µg/mL, 0.1 µg/mL and 1 µg/mL for 24 h. DNA damage was measured as the *percentage of DNA* in the comet *tail* using the enzyme hOGG1 and the alkaline version of the comet assay. The mean ± SD was calculated for 3 experiments (3 blood donors). Statistically different from negative control at *P<0.05. Statistical analysis was conducted using one-way ANOVA and a posteriori Tukey test.

tested substances (**Figures 1**, **2**). Among studied BFRs, TBBPA and PBP caused the greatest DNA damage.

Inconsistent literature results on TBBPA genotoxicity may be associated with usage of different research models, concentrations/doses or methods in evaluation of genotoxic potential of this substance. Earlier studies have shown no genotoxic potential of TBBPA (19–21), while more recent research works revealed DNA damaging potential of this substance. Yin et al. (24) determined an early DNA damage response marker γ-H2AX to assess the genotoxicity of TBBPA in mouse testicular cell co-culture model. They observed that after 24 h of incubation, TBBPA at 15 µM (8.1 µg/mL) caused significant increase of the number of γ-H2AX positive cells. Similarly, Liang et al. (48) observed that TBBPA at 25 µM (13.5 µg/mL) after 72 h of incubation increased the number of γ-H2AX positive mouse C18-4 spermatogonial cells. In another study, TBBPA at 20 µM (16.2 µg/mL) after 24 h of incubation caused DNA SSBs/DSBs formation in the IAR20 cell line (epithelial cells isolated from liver) (49). In *in vivo* study, Zatecka et al. (22) assessed DNA damage in spermatozoa of C57Bl/6J inbred mouse administrated with TBBPA at 200 µg/L dissolved in drinking water. Using terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL), they detected significantly higher number of TUNEL-positive cells from TBBPA-treated animals. Similarly, Linhartova et al. (50) using comet assay showed that TBBPA in the concentrations range from 1.75 to 10 µg/L induced DNA fragmentation in

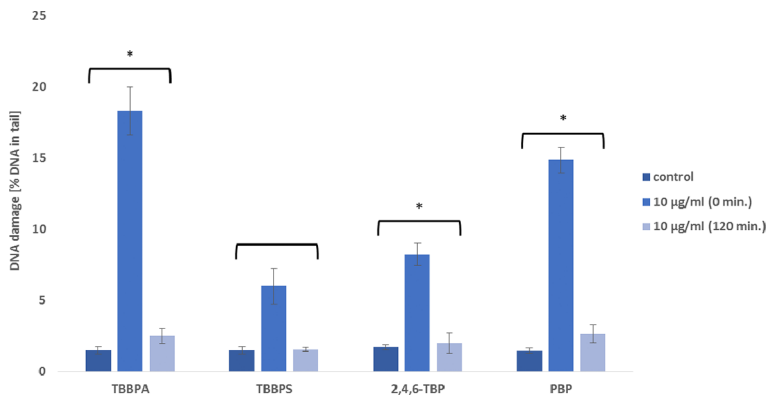



**FIGURE 5** | DNA repair capacity. Repair of damaged DNA in human PBMCs after 24 h of incubation with TBBPA, TBBPS, 2,4,6-TBP and PBP at 10 µg/mL. The repair was assessed after 120 min. of post incubation of the cells in medium deprived of these substances as a decrease in the extent of DNA damage (measured as the percentage of the DNA in comet tail) using the alkaline version of the comet assay. Mean ± SD was calculated from 3 individual experiments (3 blood donors). Statistically different from negative control at *P<0.05. Statistical analysis was conducted using one-way ANOVA and a posteriori Tukey test.

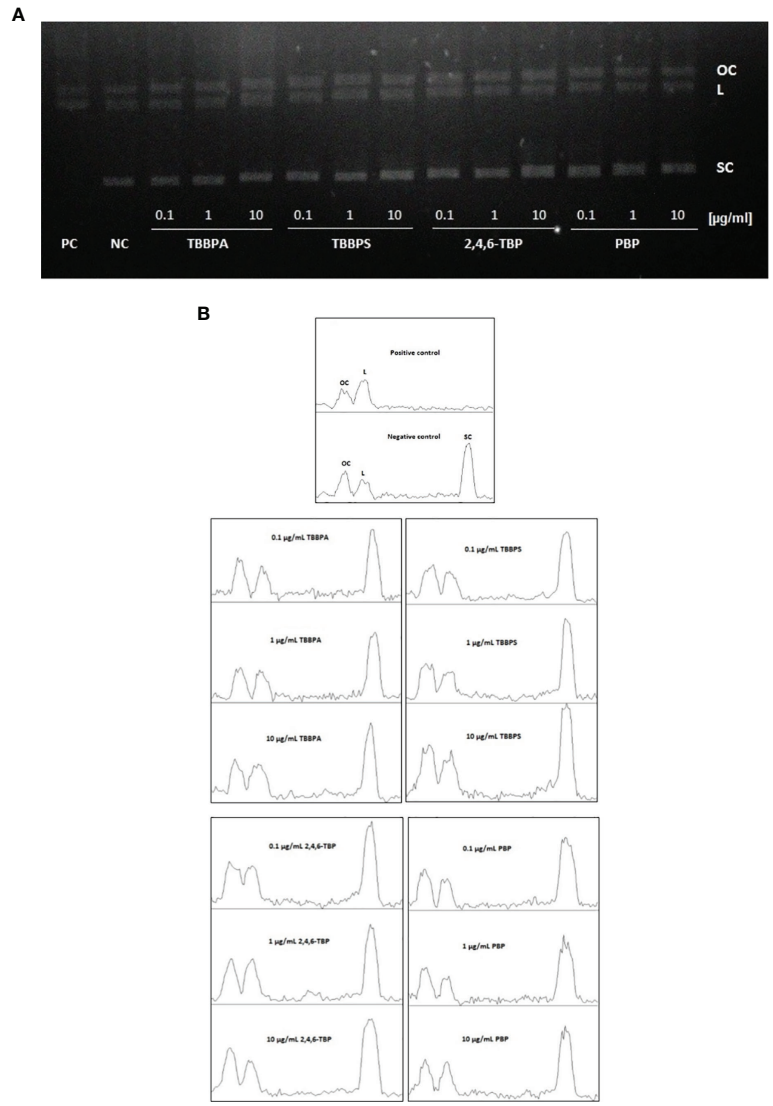



**FIGURE 6** | DNA adducts formation. Plasmid relaxation assay. **(A)** Plasmid DNA pUC19 was resolved on a 1% agarose gel, stained with ethidium bromide and visualized in UV light; line 1 - positive control (PC) (the plasmid was exposed to hydroxyl radicals generated in Fenton reaction), line 2 - negative control (NC) (pUC19 plasmid), lines 3-14 - pUC19 plasmid incubated with TBBPA, TBBPS, 2,4,6-TBP and PBP at 0.1 μg/mL, 1 μg/mL and 10 μg/mL. Structural differences between supercoiled (SC), open circular (OC) and linear (L) forms of the plasmid accounted for their different electrophoretic mobility. **(B)** Densitometric analysis of agarose gel was presented below the gel image. Open circular (OC) (as a consequence of DNA single strand breaks), linear (L) (as a consequence of DNA double strand-breaks) and supercoiled (SC) (undamaged DNA) forms of DNA plasmid are presented as peaks. Densitometric analysis was performed with the Gel Analyzer tool of ImageJ.

spermatozoa nuclei of sterlet (*Acipenser ruthenus*), while Sharma et al. (23) observed that TBBPA at 5.09 μg/mL caused DNA damage in blood cells of snake head (*Channa punctatus*). Finally, TBBPA (0.2-0.8 μg/mL) has been shown to induce DNA SBs formation in gill and digestive gland cells of bivalve Farrer's scallop (*Chlamys farreri*) (51).

There is almost no research on genotoxic potential of PBP. One study showed that PBP was not mutagenic in *Salmonella typhimurium* with or without metabolic activation (28). It is also worth noting that pentachlorophenol (PCP), which is a chlorinated analogue of PBP was capable of inducing significant increase in SBs formation in human peripheral blood lymphocytes (52).

This study showed that 2,4,6-TBP and particularly TBBPS induced lower level of SSBs/DSBs formation in comparison to other tested bromophenolic FRs (**Figures 1**, **2**).

Literature data does not provide any information on TBBPS genotoxicity; however Mokra et al. (53) revealed that its debrominated analogue bisphenol S (BPS) induced DNA SBs formation in human PBMCs.

Several studies have been conducted in order to assess 2,4,6-TBP genotoxicity, but no research work aimed to describe genotoxic mechanism of action of this substance. Analysis of 2,4,6-TBP mutagenicity in *Salmonella typhimurium* and *Escherichia coli* provided negative results (25, 54). Similarly, 2,4,6-TBP given intraperitoneally (75-300 mg/kg b.w.) to mice did not increase micronuclei formation in their bone marrow (54). Nevertheless, in most of eukaryotic models, 2,4,6-TBP has been shown to exhibit genotoxic potential. *In vitro*, in Chinese hamster lung cells (CHL/IU) 2,4,6-TPB in very high concentrations up to 1.6 mg/mL, induced chromosomal aberrations with and without metabolic activation (27, 54). Similarly, 2,4,6-TBP in high concentrations from 400 to 500 μg/mL was able to induce chromosomal aberrations in human peripheral blood lymphocytes both in the absence and the presence of metabolic activation (S9-mix) (20). More recently, *in vivo*, Lebaron et al. (55) using comet assay observed that 2,4,6-TBP mixed with bromoform and tribromoacetic acid induced DNA SBs in larvae of sea urchin (*Paracentrotus lividus*), while Heberle et al. (56) showed that 2,4,6-TBP significantly increased frequency of chromosomal aberrations in root cells of onion (*Allium cepa*). It was also reported that trichlorophenol, a chlorinated analog of TBP, caused DNA SSBs/DSBs formation in human lymphocytes (57).

The tested compounds caused oxidative damage to pyrimidines, and more strongly to purines (**Figures 3**, **4**). Generally, most of examined BFRs at lower concentrations (from 0.01 μg/mL) caused oxidative damage to DNA bases when compared with SSBs, and particularly DSBs induction. It was also observed that TBBPA caused the greatest oxidative damage to purines and pyrimidines, while TBBPS induced the lowest DNA bases lesions.

There is scarce data concerning oxidative DNA damage caused by BFRs. Choi et al. (58) observed that TBBPA given orally to Sprague-Dawley male rats strongly induced the production of oxidative DNA biomarker 8-hydroxy-2'-deoxyguanosine (8-OHdG) in the testis and kidney of the tested animals. In another study, 2,6-dibromohydroquinone, which is metabolite of TBBPA and 2,4,6-TBP at presence of Cu(II) caused 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) formation (59). Moreover, Michałowicz and Majsterek (60) observed that PCP and TCP were capable of inducing of oxidative DNA bases lesions in human peripheral blood lymphocytes, while Mokra et al. (37) showed that BPA and BPS induced pyrimidines and purines oxidation in human PBMCs.

DNA repair, including mismatch repair, the nucleotide excision repair or the base excision repair is responsible for the removal of DNA lesions (61, 62). Unrepaired DNA damage leads to a loss of genome integrity, and in the consequence increased risk of errors in the synthesis of both RNA and protein products. It has been shown that such increase of unrepaired lesions in DNA might be responsible for ageing process, cancer, atherosclerosis and degenerative diseases (63–66). For instance, inefficient oxidative DNA bases modifications by base excision repair (BER) may contribute to expansion of DNA trinucleotide repeat (TNR), which results in various neurodegenerative diseases development (67).

Our study has revealed that PBMCs efficiently repaired DNA lesions induced by tested BFRs, but they were not able to remove completely damaged DNA (except cells treated with TBBPS) (**Figure 5**). Similarly, Mokra et al. (50) assessed genotoxic potential of bisphenols in human PBMCs, and observed that tested cells completely removed DNA damage induced by BPS, but not by bisphenol A (BPA).

In order to elucidate the mechanism of the observed DNA damage, we explored the ability of tested BFRs to form DNA adducts. DNA adducts are created during interaction of physical factors and electrophilic chemical compounds with DNA (68). Using the conformation test, we evaluated the impact of examined compounds on the structure of DNA plasmid to find out whether DNA damage resulted from direct interaction between DNA and studied compounds.

The results have shown that none of tested compounds bound directly to DNA (created adducts) as no formation of linear structure of DNA plasmid was observed in any case (**Figures 6A, B**); therefore we suggested that DNA was damaged indirectly by ROS or/and other reactive species generated by tested BFRs.

ROS have been shown to be implicated in DNA damage, while hydroxyl radical ($^{\bullet}OH$) has the strongest ability to provoke oxidative DNA lesions (69). For instance, the creation of 8-hydroxylated purine in DNA is connected with addition of $^{\bullet}OH$ to the C8 of the purine base (70).

DNA-damaging effect of ROS in PBMCs treated with tested compounds is all the more likely because our previous study (31) showed that TBBPA, TBBPS, 2,4,6-TBP and PBP at very low (non-cytotoxic) concentrations (from 0.001 μg/L) were capable of generating total ROS and hydroxyl radical (at higher concentrations) in human PBMCs. Moreover the above study showed that PBP, and particularly TBBPA at 0.001 μg/L and 0.01 μg/L most strongly increased ROS level, which correlates with the results of this study showing that these substances exhibited the strongest genotoxic potential in the incubated cell. Similarly, Gao et al. (71) observed a correlation between SSBs/DSBs and 8-OHdG formation and an increase in ROS level in the SH-SY5Y cell line treated with brominated flame retardant PBDE-47 at non-cytotoxic concentration of 2.5 μg/L and 5 μg/L.

It must also be noted that TBBPA exhibited much stronger genotoxic potential than TBBPS in tested cells. Similar differences were observed by Mokra et al. (51) who showed that BPA caused stronger oxidative damage to DNA than BPS in human PBMCs. Taking the above findings into consideration, it may be suggested that sulphonyl group/methyl group(s) (but not bromine atoms) are mostly responsible for substantially different genotoxic effects provoked by TBBPA and TBBPS in tested cells.

It is worth noting that in physiological state, lymphocytes generate numerous DNA DSBs, which activate cellular DNA damage response (DDR). Interestingly, DDR capacity is different in various lymphocyte subsets being the strongest in NK cells, and the weakest in B lymphocytes, which correlates inversely

with DNA damage-related survival (72). Recent studies have revealed that physiologic DNA SBs formation and DDR can initiate a genetic program that is unique and important for developing and maturation of lymphocytes. Nevertheless, elevated DNA DSBs formation that occur as a result of the exposure of lymphocytes to genotoxic agents may lead to improper activation of cell type-specific genetic programs, and thus disturb normal functions of lymphocytes (73). For instance, Innes and co-workers (74) observed that an increased DSBs formation in lymphocytes accelerated normal B cell maturation as well as induced a unique cancer-prone phenotype and the process that activated B cell response to antigen agent.

## CONCLUSIONS

(1) The results of this study have shown that bromophenolic FRs, such as TBBPA, TBBPS, 2,4,6-TBP and PBP caused SSBs, and to a much lesser extent DSBs formation in DNA of human PBMCs. (2) Tested compounds at low concentrations caused oxidative damage to purines, and to a lesser extent to pyrimidines. (3) The greatest changes in the above-mentioned parameters were observed in cells incubated with TBBPA, while the smallest in PBMCs treated with its commercial substitute TBBPS (4) PBMCs efficiently repaired DNA SBs induced by BFRs, but they were unable to remove completely damaged DNA (except cells treated with TBBPS). (5) It was revealed that tested compounds did not form adducts with DNA in PBMCs, while detected DNA lesions were the most probably induced by indirect DNA-damaging agents, such as ROS and other reactive species (6) Purines oxidation was induced by TBBPA and 2,4,6-TBP in the concentrations that were found in humans environmentally exposed to these substances, while all DNA damage types (excluding DSBs) occurred in PBMCs exposed to 2,4,6-TBP in the concentrations found in humans occupationally exposed to this compound.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The use of human blood in the study of the effect of tested BFRs on leucocytes was approved by the Bioethical Commission of Scientific Research at the University of Lodz (contract no. KBBN-UŁ/I/7/2011). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

## AUTHOR CONTRIBUTIONS

AB analyzed the samples, interpreted the data and drafted the manuscript. AW analyzed the samples. KM prepared the samples and JM designed the study and revised the manuscript. All authors contributed to the article and approved the submitted version.

## FUNDING

This work was supported by statutory research (B2011000000191.01) admitted for Department of Biophysics of Environmental Pollution, Faculty of Biology and Environmental Protection, University of Lodz.

## REFERENCES

1. Lai YD, Kacew S, Dekant W. Tetrabromobisphenol A (TBBPA): Possible Modes of Action of Toxicity and Carcinogenicity in Rodents. *Food Chem Toxicol* (2015) 80:206–14. doi: 10.1016/j.fct.2015.03.023
2. Jarosiewicz M, Bukowska B. Tetrabromobisphenol A - Toxicity, Environmental and Occupational Exposure. *Occup Med* (2017) 68:121–34. doi: 10.13075/mp.5893.00491
3. Michałowicz J, Włuka A, Bukowska B. A Review on Environmental Occurrence, Toxic Effects and Transformation of Man-Made Bromophenols. *Sci Total Environ* (2022) 811:152289. doi: 10.1016/j.scitotenv.2021.152289
4. Wang X, Li C, Yuan X, Yang S. Contamination Level, Distribution Characteristics, and Ecotoxicity of Tetrabromobisphenol A in Water and Sediment From Weihe River Basin, China. *Int J Environ Res Public Health* (2020) 17:3750. doi: 10.3390/ijerph17113750
5. Sunday OK, Bin H, Guanghua M, Yao C, Zhengja Z, Xian Q, et al. Review of the Environmental Occurrence, Analytical Techniques, Degradation and Toxicity of TBBPA and Its Derivatives. *Environ Res* (2022) 206:112594. doi: 10.1016/j.envres.2021.112594
6. Wang X, Liu J, Liu A, Liu Q, Du X, Jiang G. Preparation and Evaluation of Mesoporous Cellular Foams Coating of Solid-Phase Microextraction Fibers by Determination of Tetrabromobisphenol A, Tetrabromobisphenol S and Related Compounds. *Anal Chim Acta* (2012) 753:1–7. doi: 10.1016/j.aca.2012.09.030
7. Ding Y, Dong X, Feng W, Mao G, Chen Y, Oiu X, et al. Tetrabromobisphenol S Alters the Circadian Rhythm Network in the Early Life Stages of Zebrafish. *Sci Total Environ* (2022) 806:150543. doi: 10.1016/j.scitotenv.2021.150543
8. Liang S, Liang S, Yin N, Hu B, Faiola F. Toxicogenomic Analyses of the Effects of BDE-47/209, TBBPA/S and TCBPA on Early Neural Development With a Human Embryonic Stem Cell *In Vitro* Differentiation System. *Toxicol Appl Pharmacol* (2019) 379:114685. doi: 10.1016/j.taap.2019.114685
9. Jarosiewicz M, Michałowicz J, Bukowska B. *In Vitro* Assessment of Eryptotic Potential of Tetrabromobisphenol A and Other Bromophenolic Flame Retardants. *Chemosphere* (2019) 215:404–12. doi: 10.1016/j.chemosphere.2018.09.161
10. Koch C, Sures B. Environmental Concentrations and Toxicology of 2,4,6-Tribromophenol (TBP). *Environ Pollut* (2018) 233:706–13. doi: 10.1016/j.envpol.2017.10.127
11. Smeds A, Saukko P. Brominated Flame Retardants and Phenolic Endocrine Disrupters in Finnish Human Adipose Tissue. *Chemosphere* (2003) 53:1123–30. doi: 10.1016/S0045-6535(03)00589-7
12. Leonetti C, Butt CM, Hoffmann K, Miranda ML, Stapleton HM. Concentrations of Polybrominated Diphenyl Ethers (PBDEs) and 2,4,6-Tribromophenol in Human Placental Tissues. *Environ Int* (2016) 88:23–9. doi: 10.1016/j.envint.2015.12.002
13. Cariou R, Antignac JP, Zalko D, Berrebi A, Cravedi JP, Maume D, et al. Exposure Assessment of French Women and Their Newborns to Tetrabromobisphenol-A: Occurrence Measurements in Maternal Adipose

Tissue, Serum, Breast Milk and Cord Serum. *Chemosphere* (2008) 73:1036–41. doi: 10.1016/j.chemosphere.2008.07.084
14. Fujii Y, Harada KH, Hitomi T, Kobayashi H, Koizumi A, Haraguchi K. Temporal Trend and Age-Dependent Serum Concentration of Phenolic Organohalogen Contaminants in Japanese Men During 1989-2010. *Environ Pollut* (2014) 185:228–33. doi: 10.1016/j.envpol.2013.11.002
15. Li A, Zhuang T, Shi W, Lian Y, Ch L, Song M, et al. Serum Concentration of Bisphenol Analogues in Pregnant Women in China. *Sci Total Environ* (2020) 707:136100. doi: 10.1016/j.scitotenv.2019.136100
16. Dufour P, Pirard C, Charlier C. Determination of Phenolic Organohalogens in Human Serum From a Belgian Population and Assessment of Parameters Affecting the Human Contamination. *Sci Total Environ* (2017) 599-600:1856–66. doi: 10.1016/j.scitotenv.2017.05.157
17. Feng C, Xu Q, Jin Y, Lin Y, Qui X, Lu D, et al. Determination of Urinary Bromophenols (BrPs) as Potential Biomarkers for Human Exposure to Polybrominated Diphenyl Ethers (PBDEs) Using Gas Chromatography-Tandem Mass Spectrometry (GC–MS/ Ms). *J Chromatogr B* (2016) 1022:70–4. doi: 10.1016/j.jchromb.2016.03.041
18. Gutierrez M, Becerra J, Godoy J, Barra R. Occupational and Environmental Exposure to Tribromophenol Used for Wood Surface Protection in Sawmills. *Int J Environ Health Res* (2005) 15:171–79. doi: 10.1080/09603120500105828
19. COT. *COT Statement on Tetrabromobisphenol A-Review of Toxicological Data*. London: UK Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (2004).
20. Alexander J, Benford D, Boobis A, Ceccotelli S, Cottrill B, Cravedi J-P. European Food Safety Authority, Scientific Opinion on Brominated Flame Retardants (BFRs) in Food: Brominated Phenols and Their Derivatives. *EFSA J* (2012) 10:2634–76. doi: 10.2903/j.efsa.2012.2634
21. Colnot T, Kacew S, Dekant W. Mammalian Toxicology and Human Exposures to the Flame Retardant 2,2′,6,6′-Tetrabromo-4,4′-Isopropylidenediphenol (TBBPA): Implications for Risk Assessment. *Arch Toxicol* (2013) 88:553–73. doi: 10.1007/s00204-013-1180-8
22. Zatecka E, Castillo J, Elzeimova F, Kubatova A, Ded L, Peknicova J, et al. The Effect of Tetrabromobisphenol A on Protamine Content and DNA Integrity in Mouse Spermatozoa. *Andrology* (2014) 2:910–17. doi: 10.1111/j.2047-2927.2014.00257.x
23. Sharma P, Chadha P, Saini SH. Tetrabromobisphenol A Induced Oxidative Stress and Genotoxicity in Fish Channa Punctatus. *Drug Chem Toxicol* (2019) 42:559–64. doi: 10.1080/01480545.2018.1441864
24. Yin L, Siracusa JS, Measel E, Guan X, Edenfield C, Liang L, et al. High-Content Image-Based Single-Cell Phenotypic Analysis for the Testicular Toxicity Prediction Induced by Bisphenol A and Its Analogs Bisphenol S, Bisphenol AF, and Tetrabromobisphenol A in a Three-Dimensional Testicular Cell Co-Culture Model. *Toxicol Sci* (2020) 173:313–35. doi: 10.1093/toxsci/kfz233
25. Shibuya T, Hara T, Kawakami K. *Reverse Mutation Test of 2,4,6-Tribromophenol on Bacteria*. Kanagawa: Japan Ministry of Health & Welfare, Hatano Research Institute (Toxicity Testing Reports of Environmental Chemicals (1999). No. 7.
26. Litton Bionetics. *Mutagenicity Evaluation of 2,4,6-Tribromophenol Lot 3287 in the Ames Salmonella/microsome Plate Test (Final)*. Kensington, MD: Litton Bionetics Inc (1978).
27. Sasaki K, Kusakabe H, Takahashi T, Hashimoto K. *In Vitro Chromosomal Aberration Test of 2,4,6-Tribromophenol on Cultured Chinese Hamster Cells*. Kanagawa: Ministry of Health & Welfare, Hatano Research Institute. Toxicity Testing Reports of Environmental Chemicals (1999). No. 7.
28. Zeiger E, Anderson B, Haworth S, Lawlor T, Mortelmans K, Speck W. *Salmonella* Mutagenicity Tests: III. Results From the Testing of 255 Chemicals. *Environ Mutagen* (1987) 9:61–109. doi: 10.1002/em.2860090603
29. Olive PL. Impact of Comet Assay in Radiobiology. *Mutat Res* (2009) 681:13–23. doi: 10.1016/j.mrrev.2007.11.001
30. Moon JJ, Lu A, Moon C. Role of Genomic Instability in Human Carcinogenesis. *Exp Biol Med* (2019) 244:227–40. doi: 10.1177/1535370219826031
31. Włuka A, Woźniak A, Woźniak E, Michałowicz J. Tetrabromobisphenol A, Terabromobisphenol S and Other Bromophenolic Flame Retardants Cause Cytotoxic Effects and Induce Oxidative Stress in Human Peripheral Blood Mononuclear Cells (*In Vitro* Study). *Chemosphere* (2020) 261:127705. doi: 10.1016/j.chemosphere.2020.127705
32. LaRosa FD, Orange SJ. Lymphocytes. *J Allergy Clin Immunol* (2008) 121:364–69. doi: 10.1016/j.jaci.2007.06.016
33. Ratomski K, Skotnicka B, Kasprzycka E, Żelazowska-Rutkowska B, Wysocka J, Anisimowicz S. Evaluation of Percentage of the $CD19^+$ $CD5^+$ Lymphocytes in Hypertrophied Adenoids at Children With Otitis Media With Effusion. *Otolaryngol Pol* (2007) 61:962–66. doi: 10.1016/S0030-6657(07)70561-9
34. Halit S, Salameh P. Exposure to Toxins During Pregnancy and Childhood and Asthma in Children: A Pilot Study. *J Epidemiol Global Health* (2017) 7:147–54. doi: 10.1016/j.jegh.2017.04.004
35. Feiterio J, Mariana M, Cairrao E. Health Toxicity Effects of Brominated Flame Retardants: From Environmental to Human Exposure. *Environ Pollut* (2021) 285:117475. doi: 10.1016/j.envpol.2021.117475
36. Hall SM, Coulter SJ, Knudsen GA, Sanders JM, Birnbaum LS. Gene Expression Changes in Immune Response Pathways Following Oral Administration of Tetrabromobisphenol A (TBBPA) in Female Wistar Han Rats. *Toxicol Lett* (2017) 272:68–74. doi: 10.1016/j.toxlet.2017.03.008
37. Mokra K, Kuźmińska-Surowaniec A, Woźniak K, Michałowicz J. Evaluation of DNA Damaging Potential of Bisphenol A and Its Selected Analogs in Human Peripheral Blood Mononuclear Cells (*In Vitro* Study). *Food Chem Toxicol* (2017) 100:62–9. doi: 10.1016/j.fct.2016.12.003
38. Woźniak E, Sicińska P, Michałowicz J, Woźniak K, Reszka E, Huras B, et al. The Mechanism of DNA Damage Induced by Roundup 360 PLUS, Glyphosate and AMPA in Human Peripheral Blood Mononuclear Cells - Genotoxic Risk Assessement. *Food Chem Toxicol* (2018) 120:510–22. doi: 10.1016/j.fct.2018.07.035
39. Singh NP, McCoy T, Tice RR, Schneider EL. A Simple Technique for Quantitation of Low Levels of DNA Damage in Individual Cells. *Exp Cell Res* (1988) 175:184–92. doi: 10.1016/0014-4827(88)90265-0
40. Fairbairn DW, Olive PL, O'Neill KL. The Comet Assay: A Comprehensive Review. *Mutat Res Rev Genet Toxicol* (1995) 229:37–59. doi: 10.1016/0165-1110(94)00013-3
41. Klaude M, Eriksson S, Nygren J, Ahnstrom G. Polyribose Polymerase-Deficient V79 Chinese Hamster. The Comet Assay: Mechanisms and Technical Cell Line. *Int J Oncol* (1996) 17:955–62.
42. Błasiak J, Kowalik J. A Comparison of the DNA Damage Recognition After UV-Irradiation by *In Vitro* Genotoxicity of Tri- and Hexavalent Chromium. *Mutat Res* (2000) 469:135–45. doi: 10.1016/S1383-5718(00)00065-6
43. Singh NP, Stephens RP. Microgel Electrophoresis: Sensitivity, Mechanism and DNA Electrostretching. *Mutat Res* (1997) 383:167–75. doi: 10.1016/S0921-8777(96)00056-0
44. Drozdz K, Wysokinski D, Krupa R, Wozniak K. Bisphenol A-Glycidyl Methacrylate Induces a Broad Spectrum of DNA Damage in Human Lymphocytes. *Arch Toxicol* (2011) 85:1453–61. doi: 10.1007/s00204-010-0593-x
45. Czarny P, Kwiatkowski D, Kacperska D, Kawczyński D, Talarowska M, Orzechowska A, et al. Elevated Level of DNA Damage and Impaired Repair of Oxidative DNA Damage in Patients With Recurrent Depressive Disorder. *Med Sci Monit* (2015) 6:412–18. doi: 10.12659/MSM.892317
46. Watała C. *Biostatystyka – Wykorzystanie Metod Statystycznych W Pracy Badawczej W Naukach Biomedycznych*. Bielsko-Biała: Alfa-Medica Press (2002). 234 p.
47. Ch Kleiveland, K Verhoeckx, P Cotter, I Lopez-Expositi, T Lea, A Mackie, T Requena, D Swiatecka, H Wichers eds. *The Impact of Food Bioactives on Health: In Vitro and Ex Vivo Models*. Cham, CH: Springer (2015). Chapter 15.
48. Liang S, Yin L, Yu KS, Hofmann M-C, Yu X. High-Content Analysis Provides Mechanistic Insights Into the Testicular Toxicity of Bisphenol A and Selected Analogues in Mouse Spermatogonial Cells. *Toxicol Sci* (2017) 155:43–60. doi: 10.1093/toxsci/kfw178
49. Samuelsen H. Master Thesis. Norwegian University of Life Sciences (NMBU) (2014).
50. Linhartova P, Gazo I, Shaliutina-Kolesova A, Hulak M, Kaspar V. Effects of Tetrabromobisphenol A on DNA Integrity, Oxidative Stress, and Sterlet (*Acipenser Ruthenus*) Spermatozoa Quality Variables. *Environ Toxicol* (2015) 30:735–45. doi: 10.1002/tox.21953
51. Hu F, Pan L, Xiu M, Jin Q. Exposure of *Chlamys Farreri* to Tetrabromobisphenol A: Accumulation and Multibiomarker Responses. *Environ Sci Pollut Res* (2015) 22:12224–34. doi: 10.1007/s11356-015-4487-6
52. Michałowicz J. Pentachlorophenol and Its Derivatives Induce Oxidative Damage and Morphological Changes in Human Lymphocytes (*In Vitro*). *Arch Toxicol* (2010) 84:379–87. doi: 10.1007/s00204-010-0515-y

53. World Health Organization. Concise International Chemical Assessment Document (CICAD) 66. In: *2,4,6-Tribromophenol and Other Simple Brominated Phenols*. Geneva: WHO (2005).
54. Lebaron K, Mechiri L, Richard S, Austruy A, Boudenne J-L, Coupé S. Assessment of Individual and Mixed Toxicity of Bromoform, Tribromoacetic-Acid and 2,4,6 Tribromophenol, on the Embryo-Larval Development of *Paracentrotus Lividus* Sea Urchin. *Environ Sci Pollut Res* (2019) 26:20573–80. doi: 10.1007/s11356-019-05279-8
55. Heberle ANA, Alves MEP, da Silva SW, Klauck CR, Rodrigues MAS, Bernardes AM. Phytotoxicity and Genotoxicity Evaluation of 2,4,6-Tribromophenol Solution Treated by UV-Based Oxidation Processes. *Environ Pollut* (2019) 249:354–61. doi: 10.1016/j.envpol.2019.03.057
56. Michałowicz J. 2,4,5-Trichlororophenol and Its Derivatives Induce Biochemical and Morphological Changes in Human Peripheral Blood Lymphocytes In Vitro. *Arch Environ Contam Toxicol* (2010) 59:670–78. doi: 10.1007/s00244-010-9508-3
57. Choi JS, Lee YJ, Kim TH, Lim HJ, Ahn MY, Kwack SJ, et al. Molecular Mechanism of Tetrabromobisphenol A (TBBPA)-Induced Target Organ Toxicity in Sprague-Dawley Male Rats. *Toxicol Res* (2011) 27:61–70. doi: 10.5487/TR.2011.27.2.061
58. Shao B, Mao L, Qu N, Wang Y-F, Gao H-Y, Li F, et al. Mechanism of Synergistic DNA Damage Induced by the Hydroquinone Metabolite of Brominated Phenolic Environmental Pollutants and Cu(II): Formation of DNA-Cu Complex and Site-Specific Production of Hydroxyl Radicals. *Free Radic Biol Med* (2017) 80:206–14. doi: 10.1016/j.freeradbiomed.2016.12.050
59. Michałowicz J, Majsterek I. Chlorophenols, Chlorocatechols and Chloroguaiacols Induce DNA Base Oxidation in Human Lymphocytes (*In Vitro*). *Toxicol* (2010) 268:171–75. doi: 10.1016/j.tox.2009.12.009
60. Mokra K, Woźniak K, Bukowska B, Sicińska P, Michałowicz J. Low-Concentration Exposure to BPA, BPF and BPAF Induces Oxidative DNA Bases Lesions in Human Peripheral Blood Mononuclear Cells. *Chemosphere* (2018) 201:119–26. doi: 10.1016/j.chemosphere.2018.02.166
61. Gillet LC, Schärer OD. Molecular Mechanisms of Mammalian Global Genome Nucleotide Excision Repair. *Chem Rev* (2006) 106:253–76. doi: 10.1021/cr040483f
62. Bauer NC, Corbett AH, Doetsch PW. The Current State of Eukaryotic DNA Base Damage and Repair. *Nucleic Acids Res* (2015) 43:10083–101. doi: 10.1093/nar/gkv1136
63. Wang Z, Wang F, Tang T, Guo C. The Role of PARP1 in the DNA Damage Response and Its Application in Tumor Therapy. *Front Cardiovas Med* (2012) 6:156–64. doi: 10.1007/s11684-012-0197-3
64. Borghini A, Cervelli T, Galli A, Andreassi MG. DNA Modifications in Atherosclerosis: From the Past to the Future. *Atherosclerosis* (2013) 230:202–9. doi: 10.1016/j.atherosclerosis.2013.07.038
65. Chow HM, Herrup K. Genomic Integrity and the Ageing Brain. *Nat Rev Neurosci* (2015) 16:672–84. doi: 10.1038/nrn4020
66. Dizdaroglu M. Oxidatively Induced DNA Damage and Its Repair in Cancer. *Mutat Res* (2015) 763:212–45. doi: 10.1016/j.mrrev.2014.11.002
67. Cilli P, Ventura I, Minoprio A, Meccia E, Martire A, Wilson SH, et al. Oxidized dNTPs and the Hogg1 and MUTYH DNA Glycosylases Combine to Induce CAG/CTG Repeat Instability. *Nucleic Acids Res* (2016) 44:5190–203. doi: 10.1093/nar/gkw170
68. Keith G, Dirheimer G. Postlabeling: A Sensitive Method for Studying DNA Adducts and Their Role in Carcinogenesis. *Curr Opin Biotechnol* (1995) 6:3–11. doi: 10.1016/0958-1669(95)80002-6
69. Jena NR. DNA Damage by Reactive Species: Mechanisms, Mutation and Repair. *J Biosci* (2012) 37:503–17. doi: 10.1007/s12038-012-9218-2
70. Bergeron F, Auvre F, Radicella JP, Ravanat JL. HO Radicals Induce an Unexpected High Proportion of Tandem Base Lesions Refractory to Repair by DNA Glycosylases. *Proc Natl Acad Sci USA* (2010) 107:5528–33. doi: 10.1073/pnas.1000193107
71. Gao P, He P, Wang A, Xia T, Xu B, Xu Z, et al. Influence of PCB153 on Oxidative DNA Damage and DNA Repair–Related Gene Expression Induced by PBDE-47 in Human Neuroblastoma Cells *In Vitro*. *Toxicol Sci* (2009) 107:165–70. doi: 10.1093/toxsci/kfn224
72. Felgentreff K, Schuetz C, Baumann U, Klemann Ch, Viemann D, Ursu S, et al. Differential DNA Damage Response of Peripheral Blood Lymphocyte Populations. *Front Immunol* (2021) 12:739675. doi: 10.3389/fimmu.2021.739675
73. Bednarski JJ, Sleckman BP. Lymphocyte Development: Integration of DNA Damage Response Signaling. *Adv Immunol* (2012) 116:175–204. doi: 10.1016/B978-0-12-394300-2.00006-5
74. Innes CL, Hesse JE, Palii SS, Helmink BA, Holub AJ, Sleckman BP, et al. DNA Damage Activates a Complex Transcriptional Response in Murine Lymphocytes That Includes Both Physiological and Cancer-Predisposition Programs. *BMC Genomics* (2013) 14:163. doi: 10.1186/1471-2164-14-163


**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Publisher's Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

molecules

MDPI

*Article*

# Apoptosis-Inducing Potential of Selected Bromophenolic Flame Retardants 2,4,6-Tribromophenol and Pentabromophenol in Human Peripheral Blood Mononuclear Cells

**Anna Barańska, Paulina Sicińska and Jaromir Michałowicz ***

Department of Biophysics of Environmental Pollution, Faculty of Biology and Environmental Protection, University of Lodz, Pomorska Str. 141/143, 90-236 Lodz, Poland
* Correspondence: jaromir.michalowicz@biol.uni.lodz.pl; Tel.: +48-42-6354453

**Abstract:** (1) Background: 2,4,6-Tribromophenol (2,4,6-TBP) and pentabromophenol (PBP) are utilized as brominated flame retardants (BFRs) in order to reduce the combustion of materials used in various utility products. The presence of 2,4,6-TBP and PBP has been reported in environmental samples as well as in inhaled air, dust, food, drinking water, and the human body. To date, there are limited data concerning the toxic action of 2,4,6-TBP and particularly PBP, and no study has been conducted to assess the apoptotic mechanism of action of these substances in human leukocytes. (2) Methods: PBMCs were isolated from leukocyte–platelet buffy coat and treated with tested substances in concentrations ranging from 0.01 to 50 µg/mL for 24 h. The apoptotic mechanism of action of the tested BFRs was assessed by the determination of phosphatidylserine exposure on the PBMCs surface, the evaluation of mitochondrial potential and cytosolic calcium ion levels, and the determination of caspase-8, -9, and -3 activation. Moreover, poly (ADP-ribose) polymerase-1 (PARP-1) cleavage, DNA fragmentation, and chromatin condensation were analyzed. (3) Results: 2,4,6-TBP and, more strongly, PBP induced apoptosis in PBMCs, changing all tested parameters. It was also found that the mitochondrial pathway was mainly involved in the apoptosis of PBMCs exposed to the studied compounds. (4) Conclusions: 2,4,6-TBP and PBP triggered apoptosis in human PBMCs, and some observed changes occurred at 2,4,6-TBP concentrations that were detected in humans occupationally exposed to this substance.

**Keywords:** 2,4,6-tribromophenol; pentabromophenol; peripheral blood mononuclear cells; apoptosis; cytosolic calcium ion level; transmembrane mitochondrial potential; caspase activation; PARP-1 cleavage; chromatin condensation



**Citation:** Barańska, A.; Sicińska, P.; Michałowicz, J. Apoptosis-Inducing Potential of Selected Bromophenolic Flame Retardants 2,4,6-Tribromophenol and Pentabromophenol in Human Peripheral Blood Mononuclear Cells. *Molecules* **2022**, *27*, 5056. https://doi.org/10.3390/molecules27165056

Academic Editor: Baljinder Kandola

Received: 21 July 2022
Accepted: 7 August 2022
Published: 9 August 2022



## 1. Introduction

Bromophenolic flame retardants (BFRs), including 2,4,6-tribromophenol (2,4,6-TBP) and pentabromophenol (PBP), are the chemicals widely used in the industry in order to reduce flammability or the burning rate of various materials, including plastics, textiles, and furniture, as well as electric and electronic equipment [1,2].

2,4,6-TBP is the most commonly synthesized bromophenol in the world. Moreover, its production is still increasing because it is widely utilized in the manufacturing of BFRs. In the United States, the production of 2,4,6-TBP was evaluated to be 4500 to <23,000 tons, while in the EU this substance has been recognized as a high-production-volume chemical (HPV) [3,4]. The production volume of PBP is not known, although 55 sources of PBP manufacturing, mainly situated in the USA and China, have been identified [5].

2,4,6-TBP and PBP are mostly utilized as FRs, but they are also used as the intermediates for the manufacturing of allyl ethers [6,7], vinyl-aromatic polymers, and epoxy-phenolic polymers [5]. Moreover, 2,4,6-TBP is used as a wood preservative and fungicide [8], while PBP is utilized as a molluscicide, bactericide, and chemical intermediate for the synthesis of pentabromophenoxy compounds [9,10].

The intensive use of 2,4,6-TBP and PBP has caused the prevalence of these substances in the environment [11–13], including biota [14], as well as their presence in indoor air, dust, food, and drinking water [15–18]. 2,4,6-TBP has been repeatedly identified in humans. For instance, Feng et al. [19] detected 2,4,6-TBP at a mean concentration of 5.57 ± 4.05 μg/L in the urine of the general population of China, whereas Dufour et. al. [20] detected this substance in the range of concentrations from a trace to 1.28 μg/L in the blood of the general population of Belgium. In another study, Gutierrez et al. [21] found high concentrations of 2,4,6-TBP in the urine of Chilean sawmills workers, which were from 1.9 to 12.3 mg/g creatinine (approx. mean 6 mg of 2,4,6-TBP per 1 L of urine). Other studies have shown the presence of 2,4,6-TBP in solid tissues of humans environmentally exposed to this substance. 2,4,6-TBP was found by Smeds and Saukko [22] in adipose tissue (2.16–53.8 μg/kg lipids) collected from people in Finland, whereas Gao et al. [23] detected this compound in concentrations up to 54.3 μg/kg lipids of adipose tissue in inhabitants of New York City, USA. In another study, Leonetti et al. [24] identified 2,4,6-TBP in placental tissue collected from women who delivered term infants (Durham, UK) in significant concentrations ranging from 1.31 to 316 μg/kg lipids.

It has been proven that BPs are toxic for living organisms, including humans. 2,4,6-TBP and PBP are structurally similar to thyroxine [25], and therefore they are able to bind to nuclear receptors and alter metabolism and the transport of thyroid hormones [26]. Moreover, 2,4,6-TBP has been shown to bind to estrogen and androgen receptors, changing testosterone and estradiol levels in animals [27]. The scientists suggested that the endocrine-disrupting activity and other adverse effects provoked by 2,4,6-TBP and PBP, such as changes in cellular calcium ions or transforming growth factor (TGF-β) signaling pathways, may contribute to cancer development [28–30]. In red blood cells, 2,4,6-TBP and PBP increased the level of reactive oxygen species (ROS), oxidized hemoglobin, and induced eryptosis [31–33]. Similarly, our previous research revealed that 2,4,6-TBP and PBP induced ROS formation, depleted ATP levels [34], and induced DNA strand breaks and DNA base oxidation in human peripheral blood mononuclear cells (PBMCs) [35]. It is known that ROS induction, the perturbation of energy production, and DNA damage are involved in apoptotic cell death [36].

Apoptosis is a highly regulated process in which the body removes unwanted (old and damaged) cells without an inflammatory state. Nevertheless, it has been shown that various factors, such as toxicants, can accelerate apoptotic cell death, which may lead to the development of different disorders [37].

The assessment of the mechanism of proapoptotic action of toxicants, such as BFRs is crucial for the recognition of biochemical targets that are affected by tested compounds. It must also be taken into consideration that changes in some apoptotic parameters, such as the calcium ion level or transmembrane mitochondrial potential (which usually occur at relatively low toxicant concentrations), are linked to other cellular processes (e.g., cell signaling, the energy state of the cell, etc.), and their disturbance may change cellular function before apoptosis occurs.

It should also be underlined that halogenated phenols and some BFRs have been shown to exhibit proapoptotic potential in blood cells and other cell types, while their mechanism of action appeared to be complex. Studies have shown that halogenated phenols, such as 2,4,5-trichlorophenol (2,4,5-TCP) and pentachlorophenol (PCP), are capable of inducing apoptosis in human lymphocytes by changing cell membrane permeability and reducing mitochondrial potential and caspase-3 activation [36]. Similarly, Jarosiewicz et al. [37] noticed that 2,4,6-TBP and PBP triggered apoptosis in human erythrocytes through increasing the calcium ion level, caspase-3 activation, and phosphatidylserine translocation on the cell surface. In another study, halogenated brominated diphenyl ethers (PBDEs), such as PBDE-47, PBDE-99, PBDE-209, and particularly 2,2′,4,4′-tetrabromodiphenyl ether (PBDE-47), triggered apoptosis in bronchial epithelial cells by decreasing the transmembrane mitochondrial potential and caspase-3 activation [38].

Recently, Dong et al. [39] reported that methylated and acetylated derivatives of natural bromophenols were able to trigger apoptosis in leukemia K562 cells and immortalized human keratocytes (HaCaT cell line).

PBMCs play a key role in the immune system and are permanently exposed to toxicants entering the human body. They are involved in the production of antibodies and responsible for killing virus-infected cells and cancer cells. Moreover, PBMCs participate in the regulation of the immune system response [40].

It has been shown that the accelerated apoptosis of PBMCs adversely affects the immune system, causing depressed human immunity [41], which may finally lead to autoimmune diseases, such as type 1 diabetes, allergy, or asthma, as well as cancer development [42–45].

Some studies have shown that BPs are capable of disturbing the immune system. Xie et al. [46] observed that 2,4,6-TBP caused changes in the production of cytokines, such as tumor necrosis factor (TNF) and interleukins 6 and 10 (IL-6 and IL-10) in RAW264.7 mouse macrophages, as well as the altered polarization of these cells. The authors of this study suggested that the observed effects could have led to a distinct immunomodulatory outcome. In another study, Bowen et al. [47] noticed that PBP changed the phagocytic capability of murine microglial BV-2 cells.

According to our best knowledge, no study has been conducted to assess the effect of BRs, including 2,4,6-TBP and PBP, on apoptosis induction in human leukocytes. In nucleated cells, only Rios et al. [48] and Bowen et al. [47] observed that 2,4,6-TBP and PBP induced apoptosis in neuroblastoma cell cultures (SH-SY5Y) and BV-2 cells, respectively.

Taking the above into account, we compared the apoptotic potential of 2,4,6-TBP and PBP in human PBMCs and examined the underlying mechanism of action of these substances. In this study, changes in phosphatidylserine exposure (PS) on PBMCs membranes (quantitative determination of apoptosis) and alterations in intracellular calcium ion and transmembrane mitochondrial potential levels, as well as the activation of caspase 8, -9, and -3 were evaluated. Furthermore, the impacts of the examined compounds on poly (ADP-ribose) polymerase-1 (PARP-1) cleavage, DNA fragmentation, and chromatin condensation were studied.

## 2. Results

### *2.1. Quantitative Analysis of Apoptosis*

The percent of apoptotic cells in the negative control after 24 h of incubation was approx. 10% (Figure 1), which was due to the significant spontaneous apoptosis of human PBMCs that occurs even when these cells are not exposed to any toxicant.

2,4,6-TBP and PBP increased the PS exposure of the PBMCs surface, as determined by staining the cells with annexin V-FITC and PI. After 24 h of incubation, PBP at 1 μg/mL caused an increase in the number of apoptotic cells; however, this change was not statistically significant. PBP at higher concentrations of 5 μg/mL and particularly at 25 μg/mL and 50 μg/mL induced substantial (concentration-dependent) statistically significant increases in the number of apoptotic PBMCs. It was also noticed that 2,4,6-TBP induced smaller changes (than PBP) of the tested parameter. 2,4,6-TBP only at 25 μg/mL and 50 μg/mL significantly increased the percentage of apoptotic cells (Figure 1).

### *2.2. Cytosolic Calcium Ion Level*

Alterations in intracellular calcium ion level were detected using Fluo-3/AM. This stain is hydrolyzed by membrane esterases to Fluo-3, which after the complexation of calcium ions, shows intense fluorescence. Statistically significant (concentration-dependent) increases in the intracellular calcium ion level were noted in PBMCs treated with PBP at 0.1 μg/mL, 1 μg/mL, and 5 μg/mL after 24 h of incubation. Smaller increases in the cytosolic $Ca^{2+}$ level were noted in PBMCs exposed for 24 h to 2,4,6-TBP, which slightly increased the tested parameter at 1 μg/mL and 5 μg/mL (Figure 2).

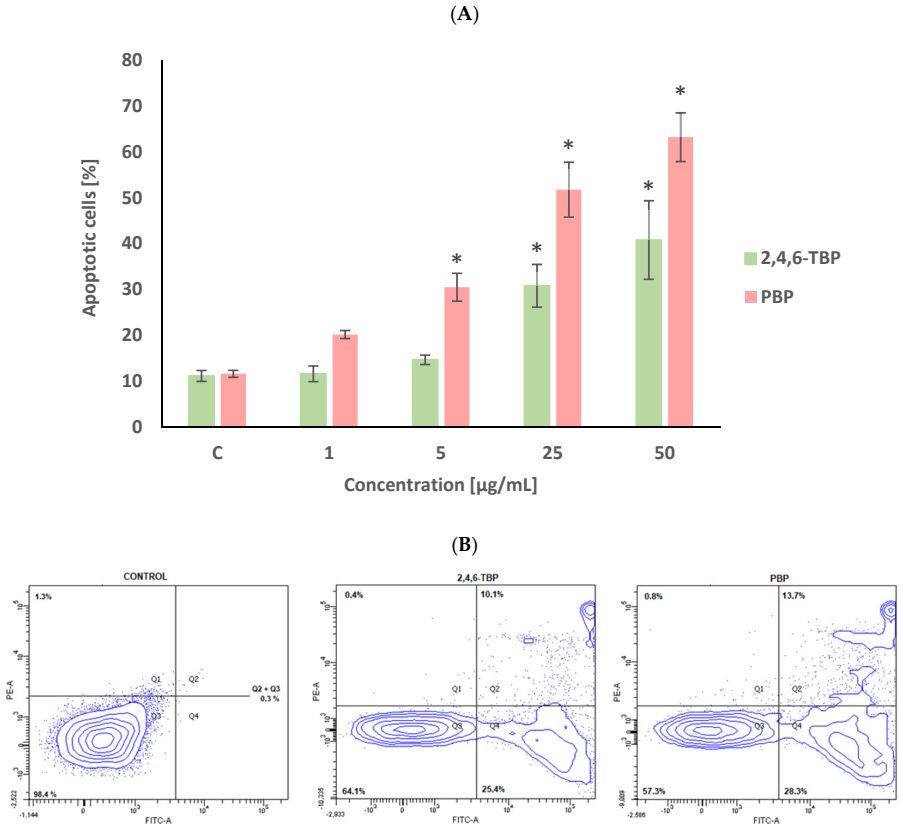



**Figure 1.** Apoptotic changes in human PBMCs incubated with 2,4,6-TBP and PBP in concentrations ranging from 1 to 50 µg/mL for 24 h (**A**). The cells were stained with Annexin V-FITC and propidium iodide. Exemplary dot plot showing apoptotic changes in human PBMCs unexposed (negative control), as well as exposed to 2,4,6-TBP at 50 µg/mL and PBP at 25 µg/mL for 24 h, Q1—necrotic cells, Q2 + Q3—apoptotic cells, Q4—live cells (**B**). (*) Means ± SDs were calculated from four individual experiments (four blood donors). Results were statistically different from negative control at * $p < 0.05$. Statistical analysis was conducted using one-way ANOVA and a posteriori Tukey test.

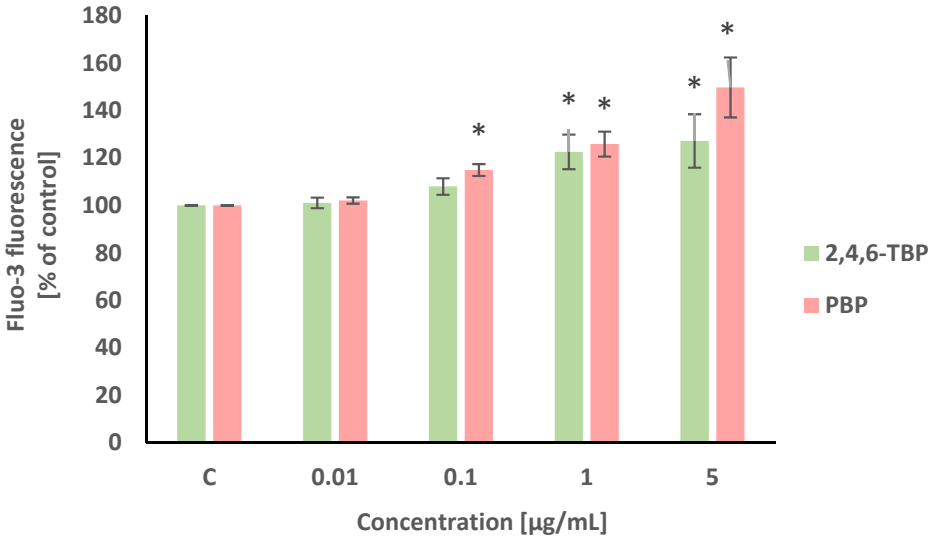



**Figure 2.** Changes in intracellular calcium ion levels in human PBMCs incubated with 2,4,6-TBP and PBP in concentrations ranging from 0.01 to 5 µg/mL for 24 h. Means ± SDs were calculated from four individual experiments (four blood donors). Results were statistically different from negative control at * $p < 0.05$. Statistical analysis was conducted using one-way ANOVA and a posteriori Tukey test.

### 2.3. Changes in Transmembrane Mitochondrial Potential

The transmembrane mitochondrial potential ($\Delta\Psi m$) was evaluated using the MitoLite Red CMXRos stain. Alterations in the fluorescence intensity of this probe were associated with changes in the $\Delta\Psi m$ level. It was revealed that 2,4,6-TBP and PBP, in a concentration-dependent manner, reduced $\Delta\Psi m$ in the tested cells after 24 h of incubation. It was shown that PBP at 1 µg/mL, 5 µg/mL, and particularly at 25 µg/mL substantially decreased $\Delta\Psi m$, whereas 2,4,6-TBP at the same concentrations caused smaller reductions in the tested parameter (Figure 3).

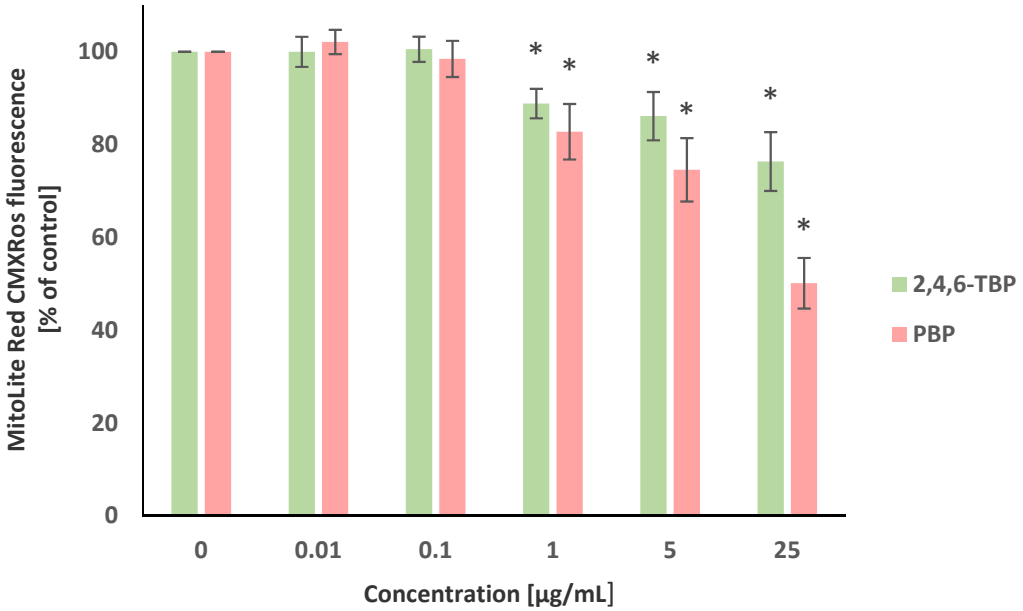



**Figure 3.** Changes in transmembrane mitochondrial potential ($\Delta\Psi m$) of PBMCs incubated with 2,4,6-TBP and PBP in concentrations ranging from 0.01 to 25 µg/mL for 24 h. Means ± SDs were calculated from four individual experiments (four blood donors). Results were statistically different from negative control at * $p < 0.05$. Statistical analysis was conducted using one-way ANOVA and a posteriori Tukey test.

### 2.4. Caspase-8, -9, and -3 Activation

Caspase-8 and caspase-9 are initiator caspases of programmed cell death that are involved in the receptor and mitochondrial pathways of apoptosis, respectively. This study showed that both 2,4,6-TBP and PBP more strongly activated caspase-9 than caspase-8 in human PBMCs.

2,4,6-TBP and PBP at 5 µg/mL and 25 µg/mL caused small increases in caspase-8 activation in tested cells after 24 h of incubation (Figure 4A).

After 24 h of incubation, 2,4,6-TBP and PBP at 5 µg/mL and particularly at 25 µg/mL caused significant increases in caspase-9 activity in PBMCs. It was also noted that 2,4,6-TBP at its highest concentration increased caspase-9 activation more strongly than PBP in the tested cells (Figure 4B).

Caspase-3 is an executioner caspase of apoptosis. It was found that after 24 h of incubation 2,4,6-TBP and PBP at 5 µg/mL and, more strongly, at 25 µg/mL increased caspase-3 activation in the tested cells, and the effects induced by these substances were similar (Figure 4C).

In all three experiments, preincubation with caspase-8, caspase-9, and caspase-3 inhibitors was performed. The additions of the appropriate caspase inhibitors caused decreases in the enzyme activities to the control values (data not shown).

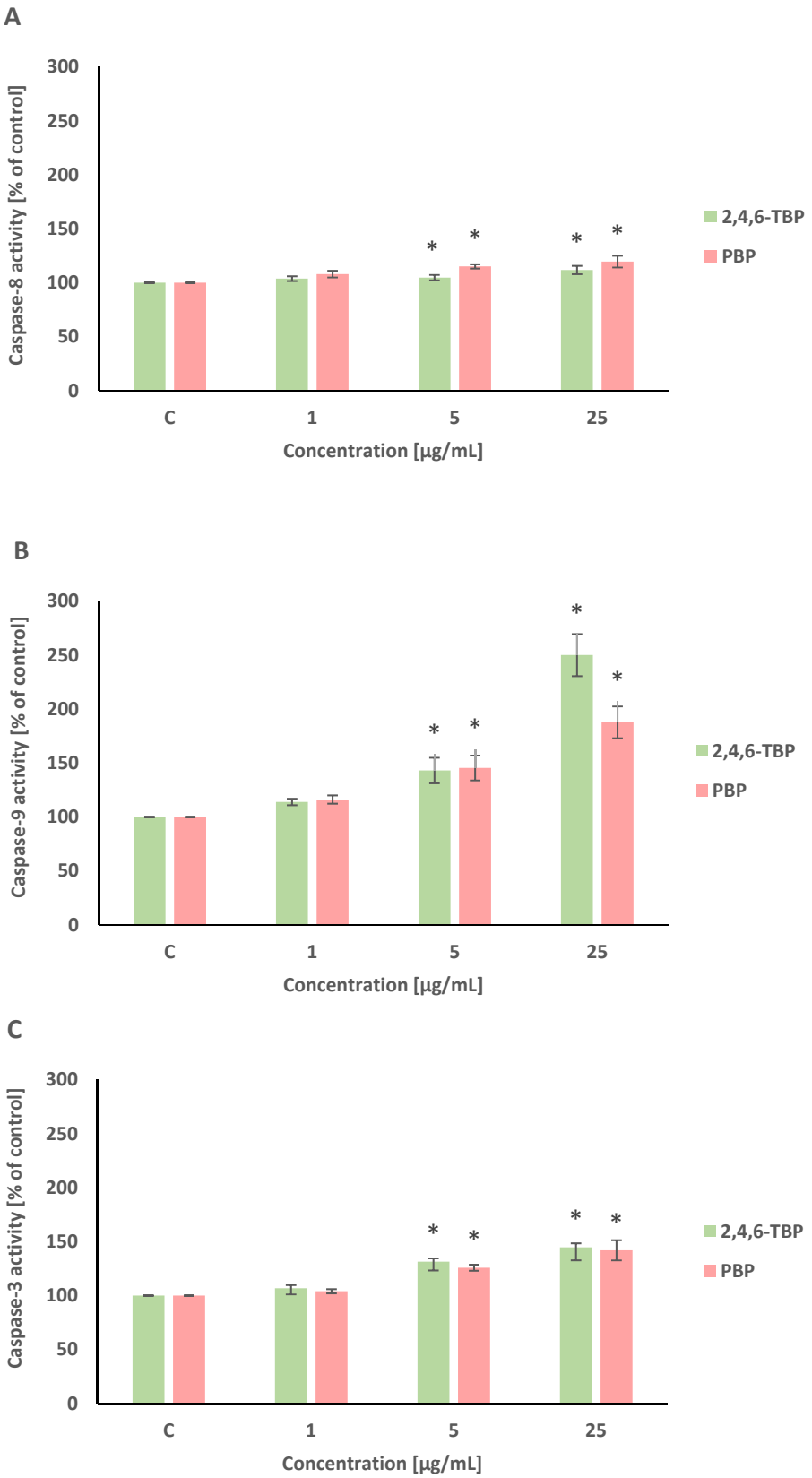



**Figure 4.** Changes in the activity of caspase-8 (**A**), -9 (**B**), and -3 (**C**) in human PBMCs incubated with 2,4,6-TBP and PBP in concentrations ranging from 1 to 25 µg/mL for 24 h. Means ± SDs were calculated from four individual experiments (four blood donors). Results were statistically different from negative control at * $p < 0.05$. Statistical analysis was conducted using one-way ANOVA and a posteriori Tukey test.

### 2.5. PARP-1 Cleavage and DNA Fragmentation

PARP-1 is a substrate for caspase-3. It is known that PARP-1 cleavage leads to its inactivation and thus helps the cells undergoing apoptosis. In order to detect PARP-1 degradation, 2,4,6-TBP and PBP were used in the concentration of 25 µg/mL that most efficiently induced caspase-3 activation in the tested cells.

It was found that after 24 h of incubation 2,4,6-TBP and, more strongly, PBP at 25 µg/mL caused PARP-1 cleavage in human PBMCs (Figure 5A).

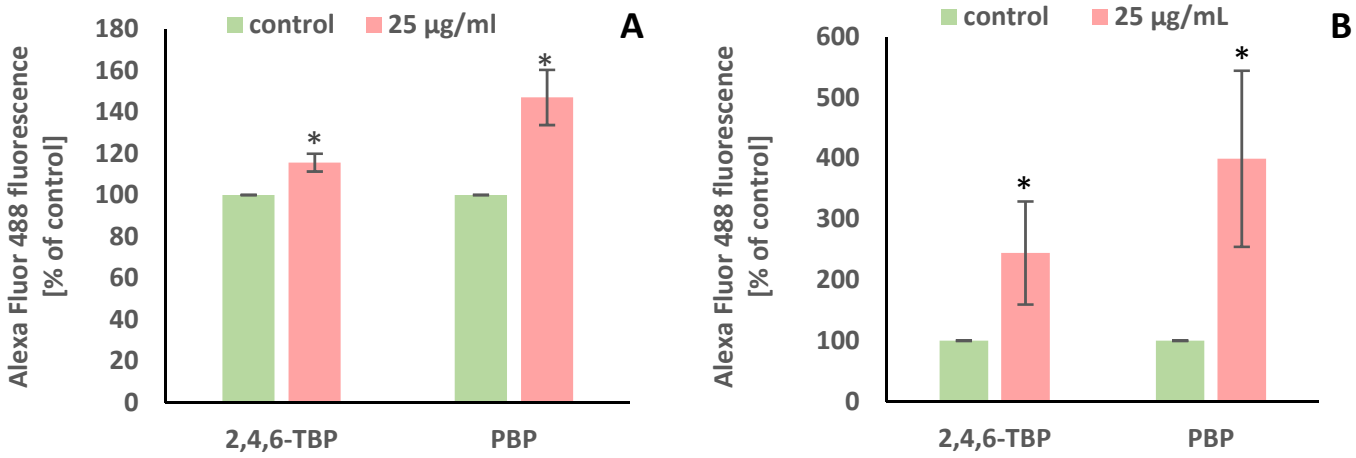



**Figure 5.** Changes in PARP-1 cleavage (**A**) and DNA fragmentation (**B**) in human PBMCs incubated with 2,4,6-TBP and PBP in the concentration of 25 µg/mL for 24 h. Means ± SDs were calculated from four individual experiments (four blood donors). Results were statistically different from negative control at * $p < 0.05$. Statistical analysis was conducted using Welch's test.

The APO-BrdU TUNEL assay was chosen for labeling DNA strand breaks along with total cellular DNA to determine apoptotic PBMCs. Similarly to the detection of PARP-1 cleavage, the concentration of 25 µg/mL of the tested BRs was selected.

It was found that after 24 h of incubation 2,4,6-TBP and, more strongly, PBP at 25 µg/mL caused statistically significant increases in the number of TUNEL-positive cells, which proved that the tested BPs caused DNA fragmentation in human PBMCs (Figure 5B).

### 2.6. Apoptosis Detection *by Fluorescence* Microscopy

Figure 6 shows representative photomicrographs of PBMCs stained with Hoechst 33324/PI. Before the cells were stained, they had been treated for 24 h with DMSO at 0.2% (negative control), as well as 2,4,6-TBP or PBP at 5 µg/mL or 50 µg/mL. In control probes, only viable PBMCs were detected. In probes treated with 2,4,6-TBP at 5 µg/mL, mostly viable cells were observed, while in the probes treated with PBP at 5 µg/mL, mainly early apoptotic PBMCs (cells with condensed chromatin) were noticed. The samples treated with 2,4,6-TBP at 50 µg/mL consisted of both viable and late apoptotic cells, while the probes incubated with PBP at 50 µg/mL contained mainly late apoptotic cells.

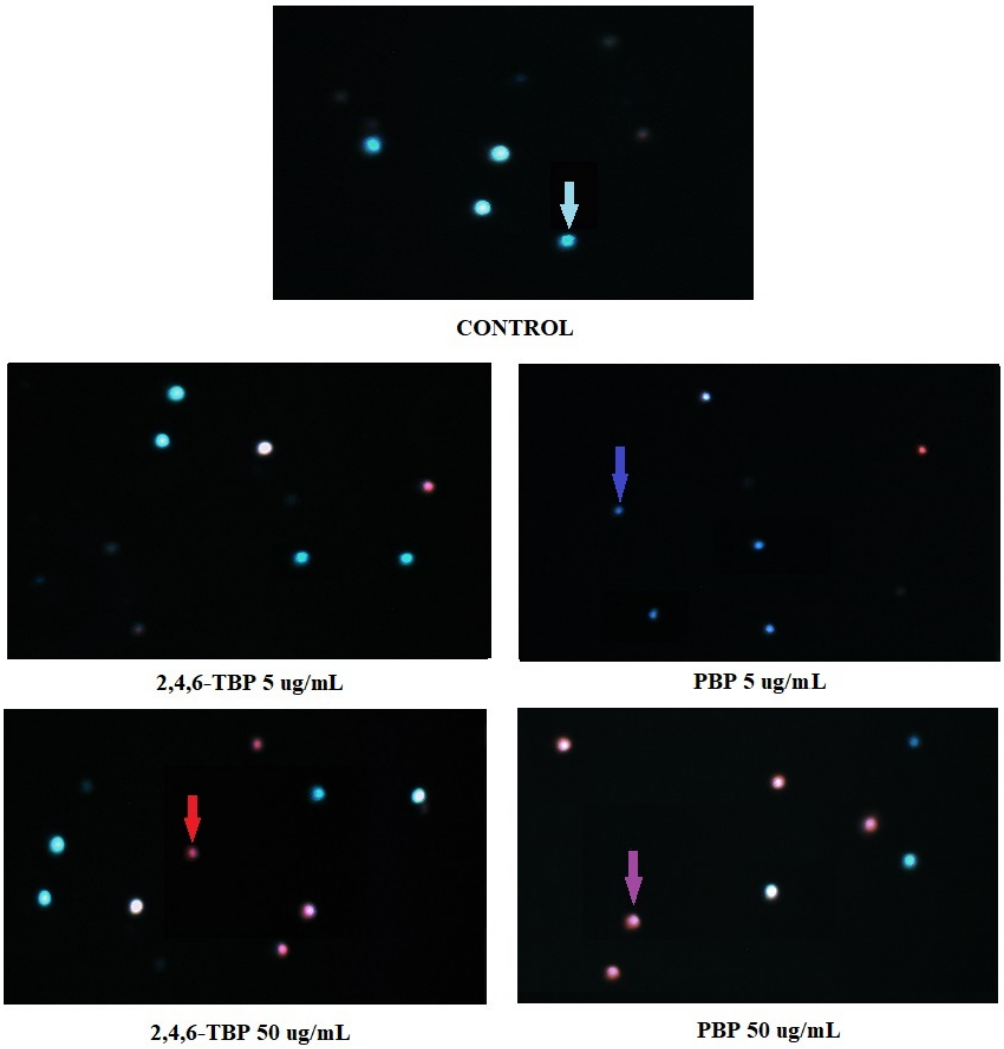



**Figure 6.** Apoptotic changes in PBMCs incubated with DMSO (control) as well as 2,4,6-TBP and PBP at 5 µg/mL and 50 µg/mL. The cells were stained with Hoechst 33342 and PI. Viable cells (blue fluorescence), early apoptotic cells (intense bright-blue fluorescence), late apoptotic cells (blue/violet fluorescence), and necrotic cells (red fluorescence).

## 3. Discussion

Apoptosis is a tightly regulated process in which the body eliminates aging or damaged cells without an inflammatory response. Various xenobiotics, such as BFRs can enhance apoptosis, which may lead to the development of infection, heart failure, neurodegenerative disorders, or myocardial ischemia [36]. PBMCs are the primary cells of the immune system, and their excessive removal may contribute to the development of cancer and autoimmune disorders, including diabetes, asthma, or allergy [49].

In this study, the apoptotic mechanism of action of selected BFRs, such as 2,4,6-TBP and PBP, in human PBMCs was studied by analyzing changes in PS translocation and alterations in cytosolic calcium ion and $\Delta\Psi m$ levels, as well as caspase-8, -9, and -3 activation. Moreover, changes in PARP-1 degradation, DNA fragmentation, and chromatin condensation were evaluated.

It was observed that the tested BPs (5–50 µg/mL after 24 h of incubation) increased the number of apoptotic PBMCs, as assessed by changes in PS translocation, but PBP showed a stronger apoptotic potential than 2,4,6-TBP (Figure 1). Similarly, Jarosiewicz et al. [37] observed that 2,4,6-TBP and, more strongly, PBP (50–100 µg/mL, 48 h of incubation) increased the number of apoptotic red blood cells, as determined by PS exposure on cell surface. In another study, brominated diphenyl ethers (PBDEs), which are used as

BFRs, triggered the apoptosis of nucleated cells. Montalbano et al. [38] assessed the toxic effects of PBDE-47, PBDE-99, and PBDE-209 in bronchial epithelial cells, showing that the tested compounds, particularly 2,2′,4,4′-tetrabromodiphenyl ether (PBDE-47) at a low concentration of 1 μM, triggered apoptosis in incubated cells.

The studies have shown that phenol and chlorophenols induce apoptotic alterations in various cell types. Phenol at relatively high concentrations (1.5–2 mM, 24 h of incubation) triggered apoptosis in erythroid progenitor-like K562 cells by increasing the PS exposure on cell surfaces [50], whereas 2,4,5-trichlorophenol (2,4,5-TCP) and pentachlorophenol (PCP) (5–100 μg/mL, 4 h of incubation) induced the apoptosis of human lymphocytes, which was associated with changes in cell membrane permeability that are characteristic for this process [51]. In other studies, Wispriyono et al. [52] observed that PCP at 20 μM (10 h of incubation) induced apoptosis in the Jurkat T-cell line by the stimulation of extracellular signal-regulated kinases and p38 mitogen-activated protein kinases, whereas Chen et al. [53] noticed that 4-chlorophenol (4-CP) 2,4-dichlorophenol (2,4-DCP) and 2,3,6-trichlorophenol (2,3,6-TCP) (0.23–1.09 mM, 6–48 h of incubation) induced concentration- and time-dependent increases in the number of apoptotic mouse L929 fibroblasts, which were associated with the presence of condensed nuclei, segregated nuclei, and apoptotic body formation.

One of the critical moments in apoptosis is the increase in the cytosolic $Ca^{2+}$ level. The release of $Ca^{2+}$ ions from the cellular compartments of the endoplasmic reticulum into the cytosol can lead to the uptake and accumulation of these ions in the mitochondria, thereby resulting in the reduction in $\Delta\Psi m$ and subsequent apoptotic cell death [54–56].

Our study showed that 2,4,6-TBP and, more strongly, PBP caused an increase in the intracellular calcium ion level. PBP and 2,4,6-TBP increased this parameter from concentrations of 0.1 μg/mL and 1 μg/mL, respectively (Figure 2). An increase in the $Ca^{2+}$ ion level was observed in the neuroendocrine cell line (PC12) treated with 2,4,6-TBP at 300 μM [26]. In another study, Jarosiewicz et al. [37] noticed that 2,4,6-TBP and PBP at 50 μg/mL (24 h of incubation) increased the cytosolic $Ca^{2+}$ level in human erythrocytes, while Mokra et al. [57] showed that bisphenol A (BPA) and its analogs increased the calcium ion level in the cytosol of PBMCs, which finally led to the apoptosis of treated cells.

The tested BPs (1–25 μg/mL) caused a decrease in $\Delta\Psi m$ in treated cells, while PBP showed greater changes than 2,4,6-TBP (Figure 3). Bowen et al. [47] observed mitochondria-related effects in the BV-2 microglial cell line exposed to PBP (10–40 μM) for 18 h, whereas Michałowicz and Sicińska [51] reported that 2,4,5-TCP and PCP (1 to 25 μg/mL, 4 h of incubation) depleted $\Delta\Psi m$ in human lymphocytes. In another study, Yan et al. [58] observed that brominated diphenyl ether PBDE-47 in concentrations ranging from 25 to 100 μM (48 h of incubation) induced apoptosis in Jurkat cells (immortalized line of T lymphocytes), which was associated with the overproduction of ROS and a reduction in the $\Delta\Psi m$.

An increase in reactive oxygen species (ROS) formation (as observed in our previous study on the prooxidative effects of 2,4,6-TBP and PBP on human PBMCs [34]) and raised cytosolic calcium ion level impair electrolyte transport across the mitochondrial membrane, causing the opening of the mitochondrial permeability transition pores (PTPs), which results in the release of various proapoptotic molecules, such as apoptosis-inducing factor (AIF), cytochrome c, and procaspase-9, into the cytosol [36]. As a consequence, an apoptosome is formed, which leads to caspase-9 activation, and subsequently to the initiation of the intrinsic (mitochondrial) pathway of apoptosis. On the other hand, the external (receptor) pathway of apoptosis may be activated due to the interaction of ligands, including ROS with transmembrane receptors, which results in the activation of caspase-8 [36,56]. Both caspase-8 and caspase-9 may activate executioner caspase-3 [56].

In this study, we observed changes in the activation of initiator caspase-8 and -9, as well as executory caspase-3. We found that 2,4,6-TBP and PBP (5–25 μg/mL) slightly increased caspase-8 activity in human PBMCs, while they more strongly (particularly 2,4,6-TBP) caused caspase-9 activation. The obtained results indicate that the mitochondrial pathway was mainly involved in the apoptosis induction of human PBMCs exposed to the tested

compounds. The obtained results also showed that the examined BFRs (5–25 μg/mL), to the same extent, increased caspase-3 activation in incubated cells (Figure 4).

Jarosiewicz et al. [37] revealed that 2,4-dibromophenol (2,4-DBP), 2,4,6-TBP, and PBP, from the concentration of 10 μg/mL (48 h of incubation), were capable of increasing caspase-3 activity in red blood cells. Other studies have shown that phenol at high concentrations (1.5–2 mM, 24 h of incubation) increased caspase-3, -8, and -9 activities in erythroid progenitor-like K562 cells [47], whereas 2,4,5-TCP and PCP (5–50 μg/mL, 4 h of incubation) induced caspase-3 activation in human lymphocytes [51].

Caspase-3 can cleave PARP-1 into 89 kDa and 24 kDa fragments. The 89 kDa fragments (with PAR polymers) are translocated from the nucleus to the cytoplasm and interact with AIF, which results in shrinkage of the nucleus and apoptosis induction. In contrast, 24 kDa fragments bind to the DNA breaks that are formed during apoptotic cell death [59].

This study showed that both 2,4,6-TBP and, more strongly, PBP at 25 μg/mL induced PARP-1 cleavage (Figure 5A). Mokra et al. [57] reported that BPA and its analogues at 5 μg/mL caused PARP-1 cleavage in human PBMCs. In another study, triphenyl phosphate (TPP), which is a phenol derivative used as a FR, caused hepatocyte apoptosis by altering PARP-1 activity [60].

The TUNEL assay is considered to be one of the most-studied and well-known methods for the determination of apoptotic cells, and it is based on the detection of DNA strand breaks in tested cells [61].

We noted that PBP and, less strongly, 2,4,6-TBP caused significant increases in the number of TUNEL-positive cells, which confirmed that these substances were able to induce apoptosis in human PBMCs (Figure 5B). The study of Chen et al. [55] showed that 4-CP, 2,4-DCP, and 2,3,4-TCP (0.23–1.09 mM, 6–48 h of incubation) induced apoptosis in mouse L929 fibroblasts, which was associated with DNA fragmentation.

In order to visualize apoptotic changes, we stained the cells with Hoechst 33342/PI and analyzed them using fluorescence microscopy (Figure 6). The conducted experiment allowed the observation of live, early apoptotic, late apoptotic, and necrotic cells.

We noticed that PBP at 5 μg/mL caused an increase in the number of early apoptotic cells (cells with clear condensed chromatin), while 2,4,6-TBP at the same concentration almost did not induce apoptosis. Both tested compounds at 50 μg/mL significantly increased the number of apoptotic cells, although PBP induced stronger changes. It was also observed that 2,4,6-TBP and PBP at 50 μg/mL mostly induced the formation of late apoptotic PBMCs (Figure 6). These findings are in agreement with the results of the quantitative determination of apoptosis by means of flow cytometry (Figure 1). Mokra et al. [59] observed that BPA, bisphenol S (BPS), and bisphenol F (BPF) at 100 μg/mL (24 h of incubation) caused chromatin condensation and changes in cell nuclei. Moreover, Chen et al. [53] noticed that mouse L929 fibroblasts incubated with chlorophenols (0.23–1.09 mM, 6–48 h of incubation) showed distinct condensed nuclei, segregated nuclei, and apoptotic bodies formation.

It is worth noting that derivatives of natural BPs also exhibit proapoptotic potential, and this property may be useful in cancer treatment. Dong et al. [39] observed that methylated and acetylated derivatives of natural BPs (5–20 μg/mL, 24 h of incubation) were capable of triggering apoptosis in leukemia K562 cells and immortalized human keratocytes (HaCaT cell line), showing potential anticancer activity. In another study, a natural BP derivative named HPN at a very low concentration (below 1 μM, 12 h of incubation) caused apoptosis in a human liver cancer cell line (HepG2), which was associated with caspase-3 activation, changes in the BAX/Bcl2 ratio, and chromatin condensation [62].

Summing up, the tested BPs were capable of inducing apoptosis in human PBMCs. The mechanism of apoptotic action was assessed, which showed that 2,4,6-TBP and PBP, by changing the cytosolic calcium ion and $\Delta\Psi m$ levels, caused caspase-9 and subsequent caspase-3 activation. The cleavage of PARP-1 by caspase-3 resulted in DNA fragmentation and chromatin condensation in the tested cells. The observed activation of caspase-8 seems to have minor importance for the apoptotic changes observed in PBMCs treated with the tested BFRs.

It must be underlined that changes in the intracellular calcium ion level, $\Delta\Psi m$, and caspase-3, -8, and -9 activation were observed in human PBMCs treated with 2,4,6-TBP in the concentrations that were determined in humans occupationally exposed to this substance [21]. As no data, according to our best knowledge, on the presence of PBP in the human body exist, it is difficult to conclude whether this substance, in the concentrations used in this study, may cause apoptotic changes in humans. Nevertheless, it must be noted that PBP caused stronger apoptotic alterations than 2,4,6-TBP, increasing the intracellular $Ca^{2+}$ level and the number of apoptotic cells, even at concentrations of 0.1 μg/mL and 5 μg/mL, respectively.

## 4. Conclusions

In conclusion, (1) 2,4,6-TBP and, more strongly, PBP exhibited apoptotic potential in human PBMCs. (2) The studied BPs triggered apoptosis by inducing PS exposure on cell surface; they also elevated the cytosolic calcium ion level, depleted the $\Delta\Psi m$, activated caspase-8, -9, and -3 and caused PARP-1 cleavage, DNA fragmentation, and chromatin condensation. (3) 2,4,6-TBP and PBP induced apoptosis, mainly by the involvement of the mitochondrial pathway, while the receptor pathway was of minor importance for apoptotic cell death activation. (4) Apoptotic changes in PBMCs were not observed at BFRs concentrations determined in humans environmentally exposed to these substances; however, changes in the intracellular calcium ion level and $\Delta\Psi m$, as well as alterations in caspases activities occurred in studied cells treated with 2,4,6-TBP in the concentrations that were determined in humans occupationally exposed to this compound.

## 5. Materials and Methods

### *5.1. Chemicals*

2,4,6-Tribromophenol (2,4,6-TBP) and pentabromophenol (PBP) of 99% purity were purchased from LGC Standards (Teddington, UK). HBSS solution, valinomycin, pluronic F-127, propidium iodide, Hoechst 33342, and a Caspase-3 Fluorimetric Assay Kit were bought from Sigma-Aldrich (St. Louis, MO, USA). FITC Annexin V Apoptosis Detection Kit and Perm/Wash Buffer were bought from Becton Dickinson (Franklin Lakes, NJ, USA). MitoLite Red CMXRos was obtained from AAT Bioquest (Sunnyvale, CA, USA). Fluo-3/AM was obtained from PromoCell (Heidelberg, Germany). A Caspase-8 Fluorimetric Assay Kit, as well as a caspase-9 chromogenic substrate and caspase-9 inhibitor were purchased from BioVision (San Francisco, CA, USA). An APO-BrdU TUNEL Assay Kit and PARP-1 (cleaved Asp214) monoclonal antibody (HLNC4) were obtained from Thermo-Fisher (Waltham, MA, USA). Lymphocyte separation medium (LSM) (1.077 g/$cm^3$) and RPMI medium with L-glutamine were purchased from Cytogen (Seoul, South Korea). Camptothecin was obtained from Pol-Aura, Poland, while ionomycin was purchased from Biokom (Janki, Poland). Other chemicals were of analytical grade and were obtained from POCH, Poland, and Roth, Germany.

### *5.2. Methods*

#### 5.2.1. Cell Isolation and Treatment

PBMCs were isolated from the leukocyte–platelet buffy coat that was achieved from the whole blood in the Blood Bank in Lodz, Poland. Blood was collected from healthy non-smoking volunteers (aged 18–45) who did not show any signs of symptoms of infectious disease. The method of isolation of PBMCs was described in detail in the study conducted by Włuka et. al. [31]. This research was approved by the Bioethical Commission of Scientific Research at the University of Lodz, no. 1/KBBN-UŁ/II/2017.

The examined compounds were dissolved in DMSO that had a final concentration of 0.2% in the untreated samples (negative control), as well as in the samples treated with 2,4,6-TBP or PBP. The DMSO concentration used in this study was not toxic for PBMCs, as assessed by all analyzed parameters.

The cells were treated with the examined substances in concentrations ranging from 0.01 to 50 μg/mL (depending on the method used) for 24 h at 37 °C in 5% $CO_2$ atmosphere in total darkness. The final density of PBMCs used in the experiments (after BFR solution addition) was 4 × $10^6$ cells/mL for the caspase analysis and 1 × $10^6$ cells/mL for the determination of the other examined parameters. The viability of PBMCs in the negative control samples was over 95%.

The lowest concentration of examined BFRs (0.01 μg/mL) used in this research (the analysis of the cytosolic calcium ion level and the transmembrane mitochondrial potential) corresponded to the mean 2,4,6-TBP level detected in the general human population of China, as reported by Feng et al. [19].

#### 5.2.2. Quantitative Determination of Apoptosis (Annexin V-FITC/PI Staining)

This method is based on the ability of annexin V labeled with fluorescein isothiocyanate (FITC) to bind to PS, which is transferred on the outer monolayer of the cellular membrane of apoptotic cells. PI is used to determine necrotic cells, as it penetrates damaged membranes and binds with DNA. This experiment was performed according to the procedure given by the manufacturer for the Annexin V-FITC apoptosis detection kit.

PBMCs were treated with 2,4,6-TBP or PBP in a range of concentrations from 1 to 50 μg/mL and incubated for 24 h at 37 °C in total darkness. After incubation, the cells were centrifuged (300× *g*) for 5 min at 4 °C and suspended in RPMI medium. Then, PBMCs were stained with the mixture of Annexin V-FITC and PI (1 μM each) dissolved in Annexin V-binding buffer and incubated for 20 min at room temperature in total darkness. In the cells, apoptosis was triggered with camptothecin at 10 μM (positive control). The probes were determined using flow cytometry (LSR II, Becton Dickinson) (excitation/emission maxima: 488/525 nm for annexin V and 530/620 nm for PI, respectively). The FMC gate on the PBMCs was established, and the data were recorded for a total of 10,000 cells per sample.

#### 5.2.3. Cytosolic Calcium Ion Level

An increase in the intracellular $Ca^{2+}$ level has been recognized as one of the primary features of apoptosis. This parameter was determined by means of Fluo-3/AM staining. Fluo-3/AM shows negligible fluorescence; however, after hydrolysis by membrane esterases (fluo-3 formation) and complexation with calcium ions, it shows about a 100-fold increase in green fluorescence intensity.

The cells were treated with 2,4,6-TBP or PBP in a range of concentrations from 0.01 to 5 μg/mL and incubated for 24 h at 37 °C in total darkness. Then, PBMCs were centrifuged (300× *g*) for 5 min at 4 °C, resuspended in Fluo-3 AM solution (1 μM), and incubated for 20 min at 37 °C in total darkness. In the next step, HBSS containing 1% BSA was added to the cells that were incubated for 40 min at 37 °C in total darkness. PBMCs were washed twice using HEPES buffer and centrifuged (300× *g*) for 5 min at 4 °C. In the final step, the cells were resuspended in HEPES buffer and incubated for 10 min at 37 °C in total darkness. The positive control consisted of PBMCs exposed to ionomycin at 1 μM (calcium ionophore). The probes were analyzed by flow cytometry (LSR II, Becton Dickinson) (excitation/emission maxima: 488/525 nm for fluo-3). The FMC gate on PBMCs was established, and the data were recorded for a total of 10,000 cells per sample.

#### 5.2.4. Mitochondrial Transmembrane Potential ($\Delta\Psi m$)

A depletion of $\Delta\Psi m$ is considered to be a feature of early apoptosis. $\Delta\Psi m$ was detected based on alterations in the intensity of the fluorescence of MitoLite Red CMXRos (excitation/emission maxima: 579/599 nm). This stain is a cationic dye that is capable of entering living cells and bioaccumulating in mitochondria, depending on the $\Delta\Psi m$ level. The stain is able to remain in mitochondria because it consists of thiol-reactive chloromethyl moieties.

PBMCs were treated with 2,4,6-TBP or PBP in a range of concentrations from 0.01 to 25 μg/mL for 24 h at 37 °C in total darkness. Nigericin and valinomycin (1 μM), which

are capable of increasing and decreasing $\Delta\Psi m$, respectively, were used as positive controls. After the probes had been incubated, they were centrifuged (300× *g*) for 5 min at 4 °C. The supernatant was discarded, and the cells were resuspended in PBS solution. PBMCs were stained with MitoLite CMXRos at 1 μM and then incubated for 20 min at 37 °C in total darkness. The probes were determined in 96-well plates using a microplate reader (Cary Eclipse, Varian).

#### 5.2.5. Caspase-3, -8, and -9 Activation

Apoptosis is regulated directly and indirectly by caspases. A fluorimetric analysis of caspase-3 and caspase-8 activity was conducted according to the manufacturers′ protocols. The methods of caspase-3 and caspase-8 determination are based on the hydrolysis (by these enzymes) of peptide substrates, such as acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) and acetyl-Ile-GluThr-Asp-7-amino-4-methylcoumarin (Ac-IETD-AMC), respectively. The hydrolysis of the substrates leads to the release of the fluorescent 7-amino-4-methylcoumarin (AMC) (excitation/emission maxima: 360/460 nm). The colorimetric evaluation of caspase-9 activity was due to the hydrolysis (by this enzyme) of the substrate acetyl-Leu-Glu-His-Asp-p-nitroaniline (Ac-LEHD-pNA), which caused a release of p-nitroaniline (pNA) (absorption at 405 nm). In all experiments, positive controls were used that consisted of PBMCs suspensions incubated with camptothecin (10 μM). Preincubation with caspase-3, caspase-8, and caspase-9 inhibitors was also conducted for all experiments. The detection of caspase-3 and caspase-8 activities was performed using a fluorescent microplate reader (Fluoroskan Ascent FL, Labsystem), while the determination of caspase-9 activity was performed using an absorbance microplate reader (BioTek ELx808, Bio-Tek) (Winooski, VT, USA).

#### 5.2.6. PARP-1 Cleavage

PARP-1 is the enzyme present in the nucleus that is involved in DNA repair and is implicated in other essential cellular processes. When caspase-3 is activated during apoptosis, it cleaves PARP-1 (between Asp214 and Gly215), leading to the formation of two fragments of 85 kDa and 25 kDa. The addition of an HLNC4 antibody (conjugated with Alexa Fluor 488) allows the specific detection of the 85 kDa PARP-1 fragment.

The cells were treated with 2,4,6-TBP or PBP at 25 μg/mL and incubated for 24 h at 37 °C in total darkness. Then, PBMCs were washed and suspended in 1% paraformaldehyde (dissolved in PBS solution). Finally, an HLNC4 antibody conjugated with Alexa Fluor 488 was added to the samples that were incubated for 30 min at 37 °C in total darkness. In the cells, apoptosis was induced by camptothecin at 10 μM (positive control). A cytometric analysis of the samples was performed (LSR II, Becton Dickinson) at excitation/emission maxima of 494/519 nm for Alexa Fluor 488. The FMC gate on PBMCs was established, and the data were recorded for a total of 10,000 cells per sample.

#### 5.2.7. APO-BrdU TUNEL Assay

The TUNEL method allows the determination of apoptosis by labeling the 3′-OH ends of single- and double-stranded DNA fragments with labeled brominated deoxyuridine triphosphate nucleotides (Br-dUTP). The reaction is catalyzed by terminal deoxynucleotidyl transferase (TdT).

The cells were treated with 2,4,6-TBP or PBP at 25 μg/mL and incubated for 24 h at 37 °C in total darkness. Then, PBMCs were fixed in paraformaldehyde (1%). In the next step, the samples were incubated in a DNA labeling solution containing BrdUTP and TdT for 1 h at 37 °C in total darkness. Finally, an anti-BrdUTP antibody was added to the probes that were incubated for 30 min at room temperature in total darkness. In the cells, apoptosis was induced by camptothecin at 10 μM (positive control). The samples were determined by flow cytometry (LSR II, Becton Dickinson) at excitation/emission maxima of 494/519 nm for Alexa Fluor 488. The FMC gate on PBMCs was established, and the data were recorded for a total of 10,000 cells per sample.

#### 5.2.8. Hoechst 33342/PI Staining

Apoptotic changes (particularly chromatin condensation) were observed using fluorescence microscopy. PBMCs were incubated with the tested BFRs and then stained with Hoechst 33342 and PI. Based on morphological features, PBMCs were described as viable (blue fluorescence), early apoptotic (intense bright-blue fluorescence), late apoptotic (blue-violet fluorescence), and necrotic (red fluorescence) [63]. PBMCs were treated with 2,4,6-TBP or PBP at 5 μg/mL or 50 μg/mL and incubated for 24 h at 37 °C in total darkness. After incubation, the cells were centrifuged (200× *g*) for 3 min at 4 °C, then the supernatant was discarded. PBMCs were resuspended in a PBS solution (0.5 mL), and the mixture of 1 μL of Hoechst 33342 and 1 μL of PI (1 mg/mL each) was added to the probes. Finally, the cells were incubated for 1 min at 37 °C in total darkness and analyzed using a fluorescence microscope (Olympus IX70, Japan) at 400× magnification.

#### 5.2.9. Statistical Analysis

Data are shown as average values with standard deviations. The ANOVA (one-way analysis of variance) test and Tukey's post hoc test or Welch's test were employed to evaluate the statistical significance between the examined probes [64]. Statistical significance was considered to be $p < 0.05$. All statistical evaluations were conducted using STATISTICA 13 software (StatSoft, Inc, Tulusa, OK, USA). The tests were conducted on blood from four donors, while for each individual experiment (one blood donor), an experimental point was the mean value of 2–3 replications.

In the statistical analyses, the results (Figures 2–5) were recounted as the % of control. The raw control data did not differ in any significant way between each another; however, the standardization of control values allowed for a better assessment of the impact of the tested compounds on the examined parameters. According to the literature data (Watała, 2002), the results recalculated as the % of control are considered to be relative values, which are always recognized in statistical analysis as unpaired. The statistical analysis of this type of data included sequentially testing for the normality of the distribution (Shapiro–Wilk test) and variance (Brown–Forsythe test). Finally, due to the normal distribution and homogeneity of variance, our data were analyzed using an ANOVA and post hoc test (Tukey's test).

**Author Contributions:** Conceptualization, J.M.; methodology, A.B.; validation, A.B.; investigation, A.B. and P.S.; writing—original draft preparation, A.B. and J.M.; writing—review and editing, J.M. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was carried out under the research project PRELUDIUM 17 no. 2019/33/N/NZ7/01202, awarded by the National Science Centre, Poland, and the Grant for Young Scientists no. B1911000002126.02, awarded by the Dean of the Faculty of Biology and Environmental Protection, University of Lodz.

**Institutional Review Board Statement:** The study was approved by Bioethical Commission of Scientific Research at the University of Lodz, no. 1/KBBN-UŁ/II/2017.

**Informed Consent Statement:** PBMCs were isolated from the leukocyte buffy coat separated from blood bought in the Regional Centre of Blood Donation and Blood Treatment in Lodz, Poland. All the procedures related to blood donation were executed at the Regional Centre of Blood Donation and Blood Treatment in Lodz, Poland. Blood donor recruitment was performed at the Centre according to the national legal procedures and European Union regulations (incl. regulation (EU) 2016/679 of the European parliament and of the council of 27 April 2016 on the protection of natural persons with regard to the processing of personal data and on the free movement of such data).

**Data Availability Statement:** The raw data supporting the conclusions of this paper are deposited in the Department of Biophysics of Environmental Pollution, University of Lodz, and will be made available by the authors without undue reservation.

**Conflicts of Interest:** The authors declare no conflict of interest.

**Sample Availability:** Samples of the compounds are not available from the authors.

## References


1. Yu, G.; Bu, Q.; Cao, Z.; Du, X.; Xia, J.; Wu, M.; Huang, J. Brominated flame retardants (BFRs): A review on environmental contamination in China. *Chemosphere* **2016**, *150*, 479–490. [CrossRef]
2. Covaci, A.; Voorspoelsb, S.; Abdallahc, M.A.E.; Geensa, T.; Harradc, S.; Lawd, E.J. Analytical and environmental aspects of the flame retardant tetrabromobisphenol-A and its derivatives. *J. Chromat. A* **2009**, *1216*, 346–363. [CrossRef] [PubMed]
3. Howe, P.D.; Dobson, S.; Malcolm, H.M. 2,4,6-Tribromophenol and other simple brominated phenols. *IPCS Concise Int. Chem. Assess. Doc.* **2005**, *66*, 1–35.
4. Okon, D.E.; Choo, G.; Barcelo, D.; Oh, J.-E. Chapter One-Introduction of emerging halogenated flame retardants in the environment. *Comprehensive Anal. Chem.* **2020**, *88*, 1–39.
5. Michałowicz, J.; Włuka, A.; Bukowska, B. A Review on environmental occurrence, toxic effects and transformation of man-made bromophenols. *Sci. Total Environ.* **2022**, *811*, 152289. [CrossRef]
6. European Food Safety Authority. Scientific opinion on brominated flame retardants (BFRs) in food: Brominated phenols and their derivatives. *EFSA J.* **2012**, *10*, 2634–2676. [CrossRef]
7. Zhu, Q.; Maeno, S.; Nishimoto, R.; Miyamoto, T.; Fukushima, M. Oxidative degradation of pentabromophenol in the presence of humic substances catalyzed by a SBA-15 supported iron-porphyrin catalyst. *J. Mol. Catalysis A Chem.* **2014**, *385*, 31–37. [CrossRef]
8. Nichkova, M.; Germani, M.; Marco, M.P. Immunochemical analysis of 2,4,6-tribromophenol for assessment of wood contamination. *J. Agric. Food Chem.* **2008**, *56*, 29–34. [CrossRef] [PubMed]
9. World Health Organisation. 2,4,6-Tribromophenol and Other Simple Brominated Phenols. In *Concise International Chemical Assessment Document 66*; World Health Organisation: Geneva, Switzerland, 2005.
10. Mizukawa, H.; Nomiyama, K.; Nakatsu, S.; Yamamoto, M.; Ischizuka, M.; Ikenaka, Y.; Nakayama, S.; Tanabe, S. Anthropogenic and naturally produced brominated phenols in pet blood and pet food in Japan. *Environ. Sci. Technol.* **2017**, *51*, 11354–11362. [CrossRef] [PubMed]
11. Schlabach, M.; Remberger, M.; Brorström-Lundén, E.; Norström, K.; Kaj, L.; Andersson, H.; Herzke, D.; Borgen, A.; Harju, M. *Brominated Flame Retardants (BFR) in the Nordic Environment*; TemaNord, 528; Nordic Council of Ministers: Copenhagen, Denmark, 2011; p. 86.
12. Xiong, J.K.; An, T.C.; Zhang, C.S.; Li, G.Y. Pollution profiles and risk assessment of PBDEs and phenolic brominated flame retardants in water environments within a typical electronic waste dismantling region. *Environ. Geochem. Health* **2015**, *37*, 457–473. [CrossRef] [PubMed]
13. Xiong, J.; Li, G.; An, T.; Zhang, C.; Wei, C. Emission patterns and risk assessment of polybrominated diphenyl ethers and bromophenols in water and sediments from the Beijiang River, South China. *Environ. Pollut.* **2016**, *219*, 596–603. [CrossRef] [PubMed]
14. Huber, S.; Warner, N.A.; Nygard, T.; Remberger, M.; Harju, M.; Uggerud, H.T.; Kaj, L.; Hanssen, L. A broad cocktail of environmental pollutants found in eggs of three seabird species from remote colonies in Norway. *Environ. Toxicol. Chem.* **2015**, *34*, 1296–1308. [CrossRef] [PubMed]
15. Saito, I.; Onuki, A.; Seto, H. Indoor organophosphate and polybrominated flame retardants in Tokyo. *Indoor Air* **2007**, *17*, 28–36. [CrossRef] [PubMed]
16. Takigami, H.; Suzuki, G.; Hirai, Y.; Sakai, S. Brominated flame retardants and other polyhalogenated compounds in indoor air and dust from two houses in Japan. *Chemosphere* **2009**, *76*, 270–277. [CrossRef] [PubMed]
17. Malysheva, S.V.; Goscinny, S.; Malarvannan, G.; Poma, G.; Andjelkovic, M.; Jacobs, G.; Voorspoels, S.; Covaci, A.; Van Loco, J. *Occurrence of HBCDs, TBBPA, Brominated Phenols and Their Derivative in Foodstuffs in Belgium*; BFR: Halem, Germany, 2017; pp. 1–3.
18. Wang, J.; Hao, Z.; Shi, F.; Yin, Y.; Cao, D.; Yao, Z.; Liu, J. Characterization of brominated disinfection by-products formed during the chlorination of aquaculture seawater. *Environ. Sci. Technol.* **2018**, *52*, 5662–5670. [CrossRef] [PubMed]
19. Feng, C.; Xu, Q.; Jin, Y.; Lin, Y.; Qui, X.; Lu, D.; Wang, G. Determination of urinary bromophenols (BrPs) as potential biomarkers for human exposure to polybrominated diphenyl ethers (PBDEs) using gas chromatography-tandem mass spectrometry (GC–MS/MS). *J. Chromat. B* **2016**, *1022*, 70–74. [CrossRef] [PubMed]
20. Dufour, P.; Pirard, C.; Charlier, C. Determination of phenolic organohalogens in human serum from a Belgian population and assessment of parameters affecting the human contamination. *Sci. Total Environ.* **2017**, *599*, 1856–1866. [CrossRef] [PubMed]
21. Gutierrez, M.; Becerra, J.; Godoy, J.; Barra, R. Occupational and environmental exposure to tribromophenol used for wood surface protection in sawmills. *Int. J. Environ. Health Res.* **2005**, *15*, 171–179. [CrossRef] [PubMed]
22. Smeds, A.; Saukko, P. Brominated flame retardants and phenolic endocrine disrupters in Finnish human adipose tissue. *Chemosphere* **2003**, *53*, 1123–1130. [CrossRef]
23. Gao, S.; Wan, Y.; Zheng, G.; Luo, K.; Kannan, K.; Giesy, J.P.; Lam, M.H.W.; Hu, J. Organobromine compound profiling in human adipose: Assessment of sources of bromophenol. *Environ. Pollut.* **2015**, *204*, 81–89. [CrossRef] [PubMed]
24. Leonetti, C.; Butt, C.M.; Hoffman, K.; Hammel, S.C.; Miranda, M.L.; Stapleton, H.M. Brominated flame retardants in placental tissues: Associations with infant sex and thyroid hormone endpoints. *Environ. Health* **2016**, *15*, 113. [CrossRef] [PubMed]
25. Meerts, I.A.T.M.; van Zanden, J.J.; Luijks, E.A.C.; van Leeuwen-Bol, I.; Marsh, G.; Jakobsson, E.; Bergman, A.; Brouwer, A. Potent competitive interactions of some brominated flame retardants and related compounds with human transthyretin in vitro. *Toxicol. Sci.* **2000**, *56*, 95–104. [CrossRef] [PubMed]

26. Leonetti, C.P.; Butt, C.M.; Stapleton, H.M. Disruption of thyroid hormone sulfotransferase activity by brominated flame retardant chemicals in the human choriocarcinoma placenta cell line, BeWo. *Chemosphere* **2018**, *197*, 81–88. [CrossRef] [PubMed]
27. Deng, J.; Liu, C.; Yu, L.; Zhou, B. Chronic exposure to environmental levels of tribromophenol impairs zebrafish reproduction. *Toxicol. Appl. Pharmacol.* **2010**, *243*, 87–95. [CrossRef] [PubMed]
28. Hassenklöver, T.; Predehl, S.; Pilli, J.; Ledwolorz, J.; Assmann, M.; Bickmeyer, U. Bromophenols, both present in marine organisms and in industrial flame retardants, disturb cellular $Ca^{2+}$ signalling in neuroendocrine cells (PC12). *Aquat. Toxicol.* **2006**, *76*, 37–45. [CrossRef]
29. Chen, C.-L.; Yang, P.; Kao, Y.; Chen, P.; Chung, C.-L.; Wang, S. Pentabromophenol suppresses TGF-β signaling by accelerating degradation of type II TGF-β receptors via caveolae-mediated endocytosis. *Sci. Rep.* **2017**, *7*, 43206. [CrossRef]
30. Roane, B.M.; Arend, R.C.; Birrer, M.J. Review: Targeting the transforming growth factor-beta pathway in ovarian cancer. *Cancers* **2019**, *11*, 668. [CrossRef]
31. Jarosiewicz, M.; Duchnowicz, P.; Włuka, A.; Bukowska, B. Evaluation of the effect of brominated flame retardants on haemoglobin oxidation and hemolysis in human erythrocytes. *Food Chem. Toxicol.* **2017**, *109*, 264–271. [CrossRef]
32. Jarosiewicz, M.; Krokosz, A.; Marczak, A.; Bukowska, B. Changes in the activities of antioxidant enzymes and reduced glutathione level in human erythrocytes exposed to selected brominated flame retardants. *Chemosphere* **2019**, *227*, 93–99. [CrossRef]
33. Włuka, A.; Woźniak, A.; Woźniak, E.; Michałowicz, J. Tetrabromobisphenol A, terabromobisphenol S and other bromophenolic flame retardants cause cytotoxic effects and induce oxidative stress in human peripheral blood mononuclear cells (in vitro study). *Chemosphere* **2020**, *261*, 127705. [CrossRef]
34. Barańska, A.; Woźniak, A.; Mokra, K.; Michałowicz, J. Genotoxic mechanism of action of TBBPA, TBBPS and selected bromophenols in human peripheral blood mononuclear cells. *Front. Immunol.* **2022**, *13*, 869741. [CrossRef] [PubMed]
35. Elmore, S. Apoptosis: A review of programmed cell death. *Toxicol. Pathol.* **2007**, *35*, 495–516. [CrossRef] [PubMed]
36. Pallardy, M.; Biola, A.; Lebrec, H.; Breard, J. Assessment of apoptosis in xenobiotic-induced immunotoxicity. *Methods* **1999**, *19*, 36–47. [CrossRef] [PubMed]
37. Jarosiewicz, M.; Michałowicz, J.; Bukowska, B. In vitro assessment of eryptotic potential of tetrabromobisphenol A and other bromophenolic flame retardants. *Chemosphere* **2019**, *215*, 404–412. [CrossRef]
38. Montalbano, A.M.; Albano, G.D.; Anzalone, G.; Moscato, M.; Gagliardo, R.; Di Sano, C.; Bonnano, A.; Ruggieri, S.; Cibella, F.; Profita, M. Cytotoxic and genotoxic effects of the flame retardants (PBDE-47, PBDE-99 and PBDE-209) in human bronchial epithelial cells. *Chemosphere* **2020**, *245*, 125600. [CrossRef]
39. Dong, H.; Wang, L.; Guo, M.; Stagos, D.; Giakountis, A.; Trachana, V.; Lin, X.; Liu, Y.; Liu, M. Antioxidant and anticancer activities of synthesized methylated and acetylated derivatives of natural bromophenols. *Antioxidants* **2022**, *11*, 786. [CrossRef]
40. LaRosa, F.D.; Orange, S.J. Lymphocytes. *J. Allergy Clin. Immunol.* **2008**, *121*, 364–369. [CrossRef]
41. Weinberg, A.; Jesser, R.; Edelstein, C.; Billy, J.; Wohl, D. Excess apoptosis of mononuclear cells contributes to the depressed cytomegalovirus-specific immunity in HIV-infected patients on HAART. *Virology* **2004**, *330*, 313–321. [CrossRef]
42. Ratomski, K.; Skotnicka, B.; Kasprzycka, E.; Żelazowska-Rutkowska, B.; Wysocka, J.; Anisimowicz, S. Evaluation of percentage of the $CD19^+$ $CD5^+$ lymphocytes in hypertrophied adenoids at children with otitis media with effusion. *Otolaryngol. Pol.* **2007**, *61*, 962–966. [CrossRef]
43. Zhou, C.-X.; Zhou, D.-H.; Liu, G.-X.; Suo, X.; Zhu, X.-Q. Transcriptomic analysis of porcine PBMCs infected with Toxoplasma gondii RH strain. *Acta Trop.* **2016**, *154*, 82–88. [CrossRef]
44. Halit, S.; Salameh, P. Exposure to toxins during pregnancy and childhood and asthma in children: A pilot study. *J. Epidemiol. Glob. Health* **2017**, *7*, 147–154. [CrossRef]
45. Sen, P.; Kemppainen, E.; Orešič, M. Perspectives on Systems Modeling of Human Peripheral Blood Mononuclear Cells. *Front. Mol. Biosci.* **2017**, *4*, 96. [CrossRef] [PubMed]
46. Xie, Y.; Jiang, L.; Qiu, J.; Wang, Y. A comparative evaluation of the immunotoxicity and immunomodulatory effects on macrophages exposed to aromatic trihalogenated DBPs. *Immunopharmacol. Immunotoxicol.* **2019**, *41*, 319–326. [CrossRef] [PubMed]
47. Bowen, C.; Childers, G.; Perry, C.; Martin, N.; McPherson, C.; Lauten, T.; Santos, J.; Harry, G.J. Mitochondrial-related effects of pentabromophenol, tetrabromobisphenol A, and triphenyl phosphate on murine BV-2 microglia cells. *Chemosphere* **2020**, *255*, 126919. [CrossRef]
48. Ríos, J.C.; Repetto, G.; Jos, A.; del Peso, A.; Salguero, M.; Camean, A.; Repetto, M. Tribromophenol induces the differentiation of SH-SY5Y human neuroblastoma 378 cells in vitro. *Toxicol. Vitr.* **2003**, *17*, 635–641. [CrossRef]
49. Rutkowska, J. Zjawisko apoptozy w chorobach alergicznych. *Med. News* **2007**, *76*, 48–54.
50. Wang, Y.; Zhang, G.-Y.; Han, Q.-L.; Suriguga, J.W.; Li, Y.; Yu, C.-H.; Li, Y.-R.; Yi, Z.-C. Phenolic metabolites of benzene induced caspase-dependent cytotoxicities to K562 cells accompanied with decrease in cell surface sialic acids. *Environ. Toxicol.* **2014**, *29*, 1437–1451. [CrossRef] [PubMed]
51. Michałowicz, J.; Sicińska, P. Chlorophenols and chlorocatechols induce apoptosis in human lymphocytes (in vitro). *Toxicol. Lett.* **2009**, *191*, 246–252. [CrossRef] [PubMed]
52. Wispriyono, B.; Matsuoka, M.; Igisu, H. Effects of pentachlorophenol and tetrachlorohydroquinone on mitogen-activated protein kinase pathways in Jurkat T cells. *Environ. Health Perspect.* **2002**, *110*, 139–143. [CrossRef]

53. Chen, J.; Jiang, J.; Zhang, F.; Yu, H.; Zhang, J. Cytotoxic effects of environmentally relevant chlorophenols on L929 cells and their mechanisms. *Cell Biol. Toxicol.* **2004**, *20*, 183–196. [CrossRef]
54. Hajnoczky, G.; Davies, E.; Madesh, M. Calcium signaling and apoptosis. *Biochem. Biophys. Res. Commun.* **2003**, *304*, 445–454. [CrossRef]
55. Kaufman, R.J.; Scheuner, D.; Schröder, M. The unfolded protein response in nutrient sensing and differentiation. *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 411–421. [CrossRef] [PubMed]
56. Kroemer, G.; Reed, J.C. Mitochondrial control of cell heath. *Nat. Med.* **2000**, *6*, 513–519. [CrossRef]
57. Mokra, K.; Kocia, M.; Michałowicz, J. Bisphenol A and its analogs exhibit different apoptotic potential in peripheral blood mononuclear cells (in vitro study). *Food Chem. Toxicol.* **2015**, *84*, 79–88. [CrossRef] [PubMed]
58. Yan, C.; Huang, D.; Zhang, Y. The involvement of ROS overproduction and mitochondrial dysfunction in PBDE-47-induced apoptosis on Jurkat cells. *Exp. Toxicol. Pathol.* **2011**, *63*, 413–417. [CrossRef] [PubMed]
59. Mashimo, M.; Onishi, M.; Uno, A.; Tanimichi, A.; Nobeyama, A.; Mori, M.; Yamada, S.; Negi, S.; Bu, X.; Kato, J.; et al. The 89-kDa PARP1 cleavage fragment serves as a cytoplasmic PAR carrier to induce AIF-mediated apoptosis. *J. Biol. Chem.* **2021**, *296*, 100046. [CrossRef] [PubMed]
60. Wang, X.; Li, F.; Liu, J.; Li, Q.; Ji, C.; Wu, H. New insights into the mechanism of hepatocyte apoptosis induced by typical organophosphate ester: An integrated in vitro and in silico approach. *Ecotoxicol. Environ. Saf.* **2021**, *219*, 112342. [CrossRef] [PubMed]
61. Chmielewski, M.; Linke, K.; Zabel, M. Metody wykrywania zjawiska apoptozy w komórkach wątrobowych in situ. *Med. News* **2008**, *77*, 223–226.
62. Luo, J.; Wu, N.; Jiang, B.; Wang, L.; Wang, S.; Li, X.; Wang, B.; Wang, C.; Shi, D. Marine bromophenol derivative 3,4-dibromo-5-(2-bromo-3,4-dihydroxy-6-isopropoxymethyl benzyl) benzene-1,2-diol protects hepatocytes from lipid-Induced cell damage and insulin resistance via PTP1B inhibition. *Mar. Drugs* **2015**, *13*, 4452–4469. [CrossRef] [PubMed]
63. Rogalska, A.; Gajek, A.; Szwed, M.; Jóźwiak, Z.; Marczak, A. The role of reactive oxygen species in WP 631-induced death of human ovarian cancer cells: A comparison with the effect of doxorubicin. *Toxicol. Vitr.* **2011**, *25*, 1712–1720. [CrossRef] [PubMed]
64. Watała, C. *Biostatystyka-Wykorzystanie Metod Statystycznych W Pracy Badawczej W Naukach Biomedycznych*, 2nd ed.; Alfa-Medica Press: Bielsko-Biała, Poland, 2002; pp. 54–68.

molecules

MDPI

*Article*

# Determination of Apoptotic Mechanism of Action of Tetrabromobisphenol A and Tetrabromobisphenol S in Human Peripheral Blood Mononuclear Cells: A Comparative Study

**Anna Barańska, Bożena Bukowska and Jaromir Michałowicz ***

Department of Biophysics of Environmental Pollution, Faculty of Biology and Environmental Protection, University of Lodz, Pomorska Str. 141/143, 90-236 Lodz, Poland
* Correspondence: jaromir.michalowicz@biol.uni.lodz.pl; Tel.: +48-42-6354453

**Abstract:** Background: Tetrabromobisphenol A (TBBPA) is the most commonly used brominated flame retardant (BFR) in the industry. TBBPA has been determined in environmental samples, food, tap water, dust as well as outdoor and indoor air and in the human body. Studies have also shown the toxic potential of this substance. In search of a better and less toxic BFR, tetrabromobisphenol S (TBBPS) has been developed in order to replace TBBPA in the industry. There is a lack of data on the toxic effects of TBBPS, while no study has explored apoptotic mechanism of action of TBBPA and TBBPS in human leukocytes. Methods: The cells were separated from leucocyte-platelet buffy coat and were incubated with studied compounds in concentrations ranging from 0.01 to 50 μg/mL for 24 h. In order to explore the apoptotic mechanism of action of tested BFRs, phosphatidylserine externalization at cellular membrane (the number of apoptotic cells), cytosolic calcium ion and transmembrane mitochondrial potential levels, caspase-8, -9 and -3 activation, as well as PARP-1 cleavage, DNA fragmentation and chromatin condensation in PBMCs were determined. Results: TBBPA and TBBPS triggered apoptosis in human PBMCs as they changed all tested parameters in the incubated cells. It was also observed that the mitochondrial pathway was mainly involved in the apoptotic action of studied compounds. Conclusions: It was found that TBBPS, and more strongly TBBPA, triggered apoptosis in human PBMCs. Generally, the mitochondrial pathway was involved in the apoptotic action of tested compounds; nevertheless, TBBPS more strongly than TBBPA caused intrinsic pathway activation.

**Keywords:** tetrabromobisphenol A; tetrabromobisphenol S; peripheral blood mononuclear cells; apoptosis; cytosolic calcium ion level; transmembrane mitochondrial potential; caspase activation; PARP-1 cleavage; chromatin condensation



**Citation:** Barańska, A.; Bukowska, B.; Michałowicz, J. Determination of Apoptotic Mechanism of Action of Tetrabromobisphenol A and Tetrabromobisphenol S in Human Peripheral Blood Mononuclear Cells: A Comparative Study. *Molecules* **2022**, 27, 6052. https://doi.org/10.3390/molecules27186052

Academic Editors: Ester Heath, Ana Kovačič and Bojana Žegura

Received: 19 August 2022
Accepted: 12 September 2022
Published: 16 September 2022



## 1. Introduction

Brominated flame retardants (BFRs), including brominated bisphenols, are utilized in the production of various everyday products in order to reduce their flammability [1,2].

The synthesis of tetrabromobisphenol A (TBBPA) accounts for 60% of the total global market for BFRs, making it the most widely used flame retardant worldwide [3]. This substance is utilized in the synthesis of polymers (polycarbonates, epoxy resins) and production of electronic and electric equipment, as well as textiles and furniture [4,5].

Due to the widespread use of TBBPA, this substance has been repeatedly found in the environment [6–8], dust of residential and office spaces [9], as well as ambient and indoor air [7,10,11].

TBBPA has also been determined in the human organism. Cariou et al. [12] found TBBPA in samples of mothers milk and serum, as well as fetuses serum in the range of concentrations from 0.06 to 37.34 ng/g of fat, while Fujii et al. [13] detected TBBPA in the plasma of Japanese men in an average concentration of 950 pg/g fresh weight. Some research works have documented the occurrence of significant amounts of TBBPA in the

human body. For instance, Lankova et al. [14] determined TBBPA in the concentration of up to 16.2 μg/L in samples of women breast milk.

The ubiquitous presence of TBPPA in the environment and the human body prompted the scientists to assess toxic potential of this substance. The conducted studies have shown pro-oxidative, pro-inflammatory, neurotoxic, nephrotoxic, hepatotoxic, genotoxic and endocrine disrupting potential of TBBPA [15–23].

In search of a better and less toxic BFR, tetrabromobisphenol S (TBBPS) has been developed in order to potentially replace TBBPA in the industry [24]. TBBPS is commonly utilized in the synthesis of polycarbonates and epoxy resins, as well as in the production of textiles and electronic devices [25–27]. Although TBBPS is still less commonly utilized in the industry than TBBPA, it has been found in the environment, food and indoor air [1,27–31]. Recently, TBBPS was determined in an average concentration of 0.593 μg/L of serum samples collected from pregnant Chinese women [32].

Limited data exist on the effect of TBBPS on living organisms. Ding et al. [33] noticed that TBBPS changed the circadian rhythm network in zebrafish and induced developmental changes in zebrafish embryos. These scientists have considered that TBBPS may not have exhibited lower toxicity, in comparison to TBBPA in humans. Liang et al. [34] noticed that TBBPA and TBBPS revealed comparable toxic effects on human embryonic stem cells. They found that tested substances altered the development of neural ectoderm, influenced the growth of axons and the transmission of neurons, as well as disturbed the WNT and AHR signaling pathways.

Apoptosis is a regulated process that allows the removal of old and damaged cells without inflammatory reactions. However, it has been shown that different stimuli, including toxicants, accelerate apoptosis that may contribute to development of various pathological states [35]. Two main apoptotic pathways are involved in apoptosis of lymphocytes. The intrinsic (mitochondrial) cell death pathway is triggered by various apoptotic stimuli, such as genomic instability, cytokine withdrawal or toxicants. Intrinsic cell death signals generally focus on the cell at the outer membrane of mitochondria, which leads to a loss of mitochondrial membrane integrity, and the subsequent induction of apoptotic pathways, in which various proteases, including essential initiator caspase-9 are activated. The major mediators in the intrinsic cell death pathway are Bcl-2 family proteins, which control mitochondrial membrane integrity [36].

On the other hand, death receptors are involved in the initiation of the extrinsic (receptor) death pathway. All death receptors contain a death domain (DD) in their cytoplasmic tail. There are two major DD-containing adaptor proteins implicated in death receptor signaling, such as Fas-associated death domain (FADD) and tumor necrosis factor (TNF) receptor-associated death domain (TRADD). When ligands, including reactive oxygen species (ROS) and xenobiotics, bind to death receptors, the DD mediates the interaction with other DD-containing adaptor proteins, which activates the extrinsic pathway, in which the induction of other important proteins, such as initiator caspase 8 occurs [37].

PBMCs are directly exposed to toxicants entering the human organism. Those cells play a crucial role in the human body as they are involved in antibodies production, killing virus-infected and cancer cells, as well as regulating the immune system response [38].

It was shown that accelerated apoptosis of PBMCs may be associated with adverse changes in the immune system, such as depleted human immunity [39], which, in consequence, may lead to the development of autoimmune diseases (type 1 diabetes, asthma, allergy) and cancer [40–43].

Some research works have revealed that TBBPA can disturb the function of the immune system. Feiterio et al. [44] proved that TBBPA altered the tumor killing function of NK cells and changed cytokines production, such as interferon gamma (IFNγ), interleukin-1β (IL-1β) and tumor necrosis factor (TNF). In another work, Hall et al. [45] noticed that, in rats, TBBPA caused the downregulation of genes involved in the immune response, which could have led to estrogen-mediated immunosuppression in studied animals.

According to our best knowledge, no research work has been performed to assess the mechanism of apoptotic action of TBBPA in human leucocytes, and only Cato et al. [46] noticed that TBBPA activated caspase-3 and mitogen-activated protein kinases (MAPKs) in human NK cells. Moreover, the effect of TBBPS on apoptosis induction has not been assessed in any cell type. Our earlier research works have revealed that TBBPA and TBBPS caused ROS formation and induced damage to lipids, proteins and DNA in human PBMCs [22,23], while it is well known that ROS induction and DNA damage can trigger apoptotic cell death [47].

Taking the above into consideration, we compared apoptotic potential of TBBPA and TBBPS in human PBMCs and determined the mechanism underlying the action of these substances by assessing changes in phosphatidylserine translocation in cellular membrane, alterations in cytosolic calcium ion and transmembrane mitochondrial potential levels, and caspase-8, -9 and -3 activation. Moreover, the effect of tested compounds on the cleavage of PARP-1, DNA fragmentation and condensation of chromatin was evaluated.

The investigations were conducted in the range of concentrations of tested compounds that referred to their levels detected in humans environmentally or/and occupationally exposed. If no effects were observed at the above-mentioned BFRs levels, higher concentrations of TBBPA and TBBPS were used.

## 2. Results

### *2.1. Quantitative Analysis of Apoptosis*

TBBPA and TBBPS increased PS translocation in peripheral blood mononuclear cells (PBMCs), which was assessed by annexin V-FITC and PI staining. After 24 h of treatment, TBBPA at 5 µg/mL, 25 µg/mL and 50 µg/mL caused a concentration-dependent increase in the number of apoptotic cells. TBBPS induced substantially lower alterations in examined parameters, increasing the number of apoptotic cells only at 50 µg/mL (Figure 1A,B).

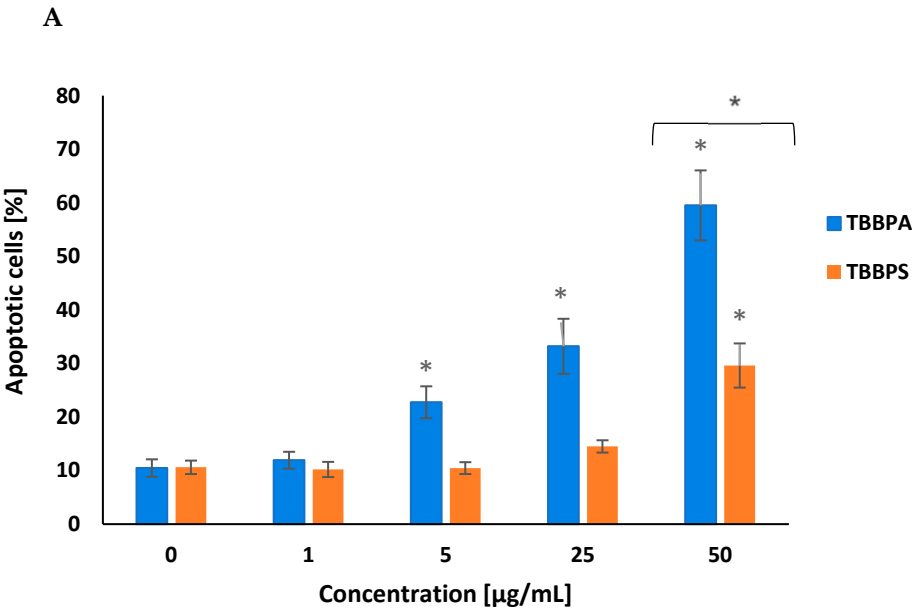



**Figure 1.** *Cont.*

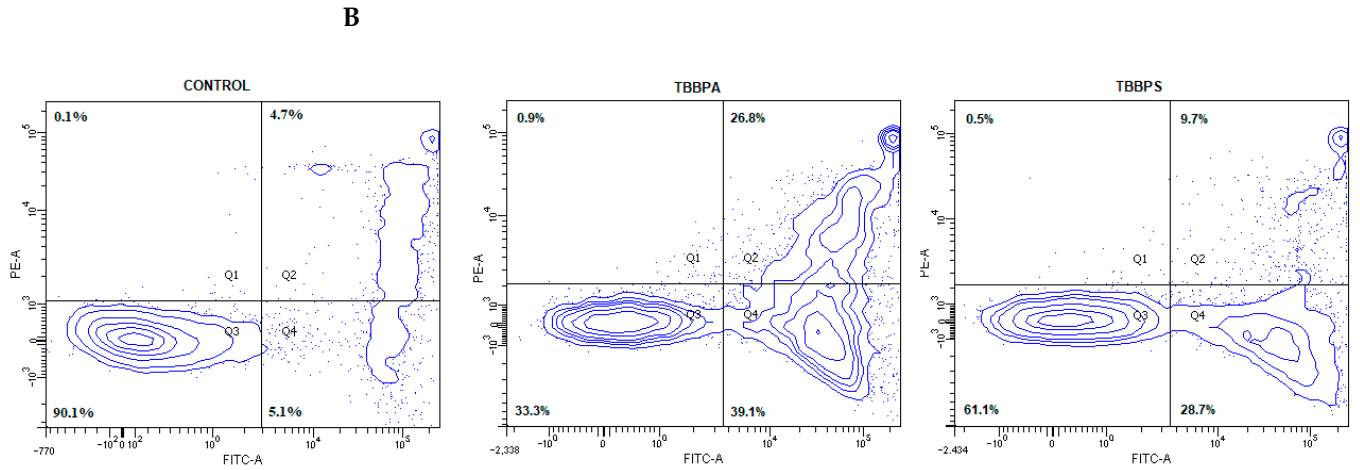



**Figure 1.** Apoptotic alterations in human PBMCs treated with TBBPA and TBBPS in the range from 1 to 50 µg/mL (n = 4) for 24 h (**A**). The cells were stained with Annexin-FITC and PI. Exemplary dot plots showing apoptotic alterations in human PBMCs unexposed (control) and exposed to TBBPA and TBBPS at 50 µg/mL for 24 h, Q3—live cells, Q2 + Q4—apoptotic cells (**B**). Statistically different from negative control at * $p < 0.05$. Statistical analysis was conducted using one-way ANOVA and a posteriori Tukey test.

### 2.2. Cytosolic Calcium Ions Level

Alteration in the intracellular calcium ion level were determined using Fluo-3/AM, which is hydrolyzed by membrane esterases to Fluo-3. Fluo-3 fluorescence intensity increases with rise of cytosolic $Ca^{2+}$ level. A concentration-dependent increase in cytosolic $Ca^{2+}$ level was noted in PBMCs incubated with TBBPA at 0.1 µg/mL, 1 µg/mL and 5 µg/mL for 24 h. TBBPS at the same concentrations caused smaller changes in the studied parameter (Figure 2).

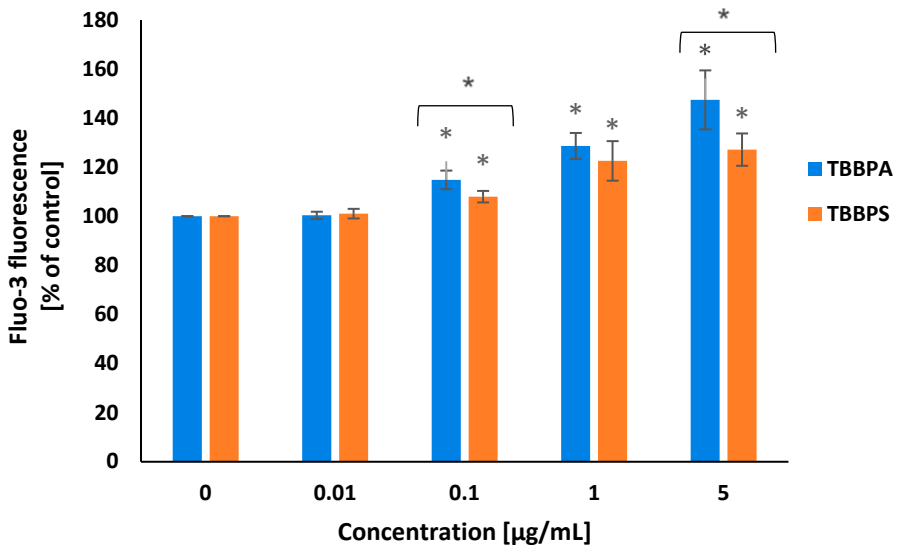



**Figure 2.** Alterations in cytosolic calcium ion level in human PBMCs treated with TBBPA and TBBPS in the range from 0.01 to 5 µg/mL (n = 4) for 24 h. Statistically different from negative control at * $p < 0.05$. Statistical analysis was conducted using one-way ANOVA and a posteriori Tukey test.

### 2.3. Transmembrane Mitochondrial Potential

Transmembrane mitochondrial potential ($\Delta\Psi m$) was evaluated using MitoLite Red CMXRos probe. Alterations in the intensity of marker fluorescence were associated with changes in $\Delta\Psi m$ level. It was revealed that after 24 h of treatment, TBBPA and TBBPS in a concentration-dependent manner reduced $\Delta\Psi m$ in the incubated cells. TBBPA at 1 µg/mL

and 5 µg/mL, and, particularly at 25 µg/mL, substantially reduced the tested parameter, while TBBPS at 5 µg/mL and 25 µg/mL induced a smaller depletion of $\Delta\Psi m$ in PBMCs (Figure 3).

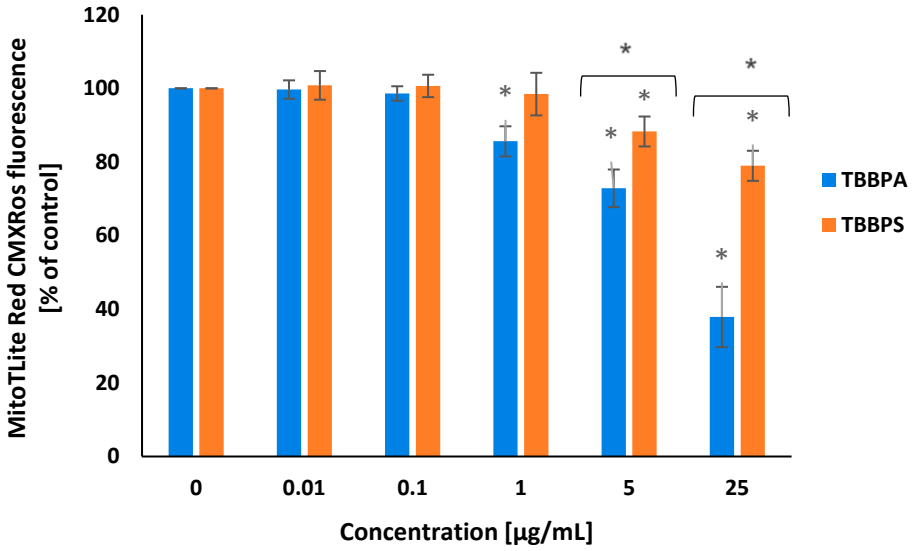



**Figure 3.** Alterations in transmembrane mitochondrial potential ($\Delta\Psi m$) in PBMCs treated with TBBPA and TBBPS in the range from 0.01 to 25 µg/mL (n = 4) for 24 h. Statistically different from negative control at * $p < 0.05$. Statistical analysis was conducted using one-way ANOVA and a posteriori Tukey test.

### *2.4. Caspase-8, -9 and -3 Activation*

Caspase-8 and caspase-9 belong to the initiator enzymes of programmed cell death. A small rise in caspase-8 activation was shown in tested cells treated for 24 h with TBBPA and TBBPS at 5 µg/mL and 25 µg/mL, whereas TBBPS caused slightly higher caspase-8 activation than TBBPA in tested cells (Figure 4A).

After 24 h of treatment, TBBPA and TBBPS at 5 µg/mL and 25 µg/mL caused a substantial increase in caspase-9 activation. It was observed that TBBPA, more strongly (particularly at its highest concentration) than TBBPS, increased caspase-9 activation in tested cells (Figure 4B). Caspase-3 is an executory enzyme of apoptosis. It was noticed that after 24 h of incubation, both studied BFRs, and more strongly TBBPA at 5 µg/mL and 25 µg/mL raised caspase-3 activation in PBMCs (Figure 4C).

Caspase-8, caspase-9 and caspase-3 inhibitors were also used in these experiments. The preincubation of the cells with appropriate caspase inhibitor depleted caspase activity up to control value (data not shown).

### *2.5. PARP-1 Cleavage*

PARP-1 is a substrate for caspase-3. The cleavage of PARP-1 and its inactivation helps cells undergoing apoptosis. To determine PARP1 cleavage, a concentration of 25 µg/mL of TBBPA and TBBPS was selected, which caused the strongest caspase-3 activation in PBMCs.

It was found that after 24 h of treatment, TBBPS and more strongly TBBPA at 25 µg/mL induced PARP-1 cleavage in tested cells (Figure 5).

### *2.6. APO-BrdU TUNEL Assay*

APO-BrdU TUNEL assay was used for labeling DNA strand breaks along with total cellular DNA to detect apoptotic cells. It was noticed that after 24 h of treatment, TBBPA and less strongly TBBPS at 25 µg/mL induced substantial DNA fragmentation in PBMCs (Figure 6).

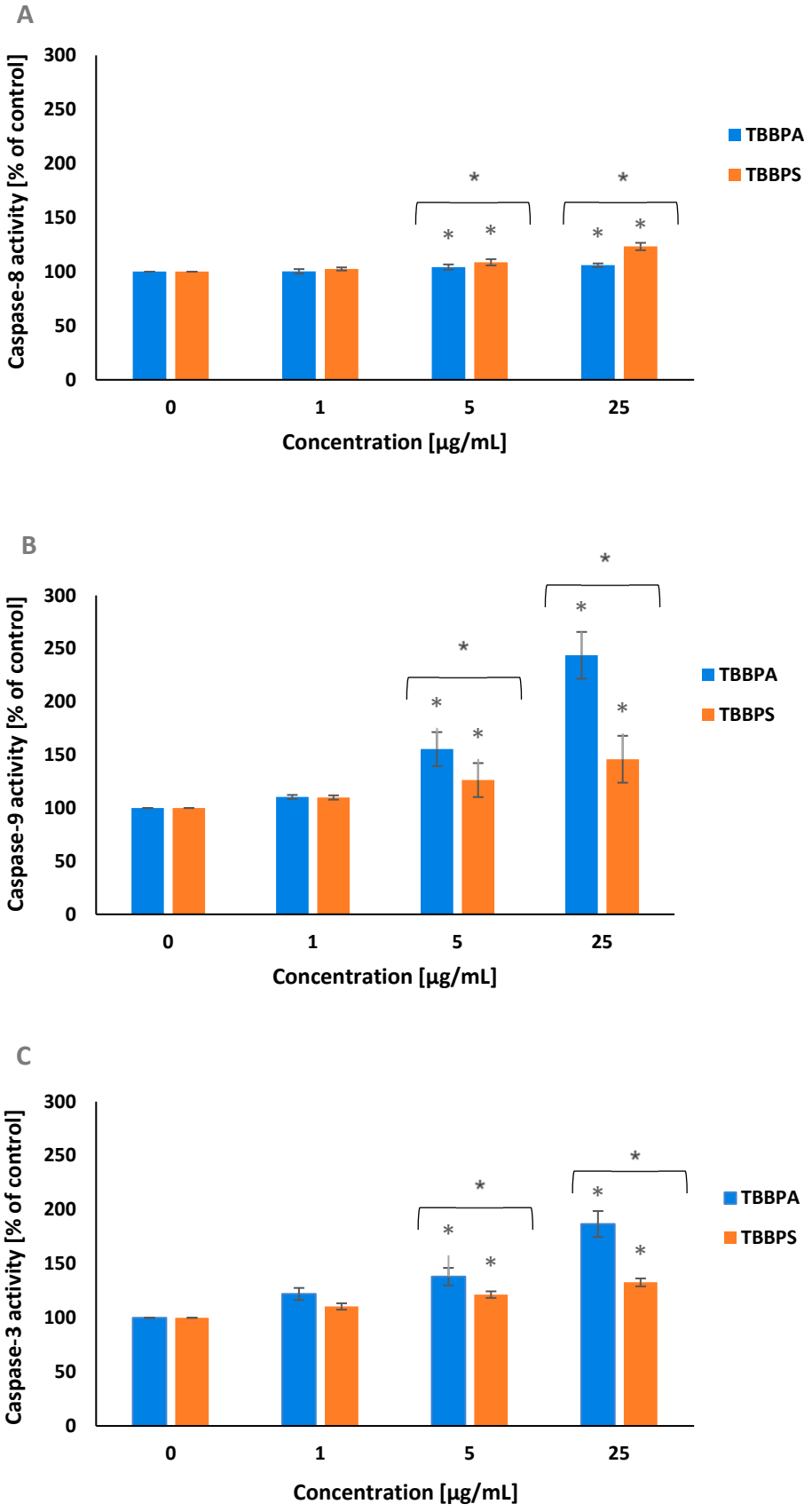



**Figure 4.** Alterations in caspase-8 (**A**), caspase-9 (**B**) and caspase-3 (**C**) activity in PBMCs treated with TBBPA and TBBPS in the range from 1 to 25 µg/mL (n = 4) for 24 h. Statistically different from negative control at * $p < 0.05$. Statistical analysis was conducted using one-way ANOVA and a posteriori Tukey test.

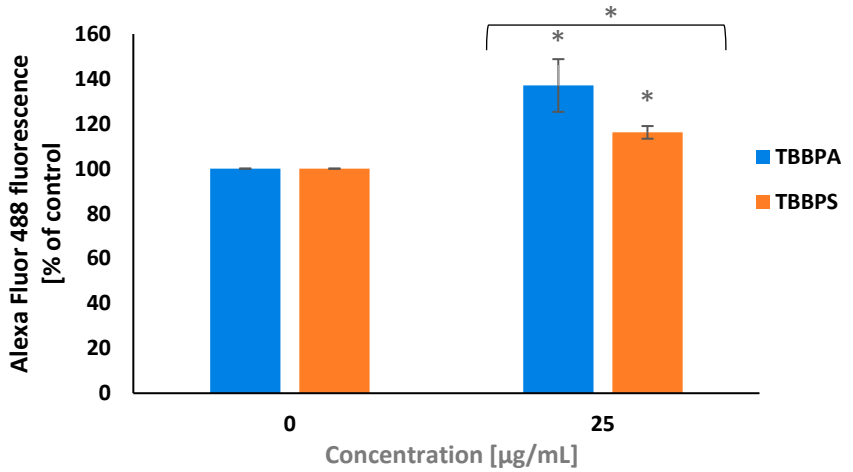



**Figure 5.** Changes in the level of 85 kDa PARP1 fragments in PBMCs treated with TBBPA and TBBPS at 25 µg/mL (n = 3) for 24 h. Statistically different from negative control at * $p < 0.05$. Statistical analysis was conducted using Welch's test.

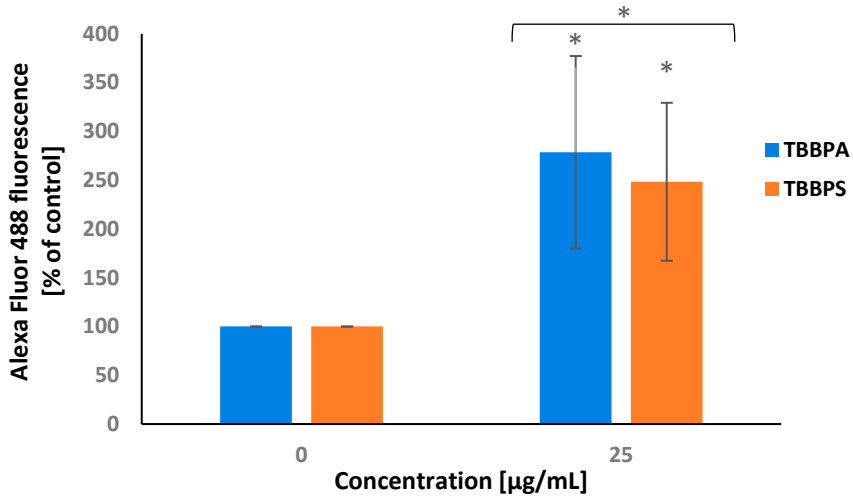



**Figure 6.** Alterations in DNA fragmentation in PBMCs incubated with TBBPA and TBBPS at 25 µg/mL (n = 3) for 24 h. Statistically different from negative control at * $p < 0.05$. Statistical analysis was conducted using Welch's test.

### *2.7. Apoptosis Detection by Fluorescence Microscopy*

Figure 7 presents representative photomicrographs of PBMCs stained with Hoechst 33324/PI. Before staining, the cells had been incubated for 24 h with DMSO at 0.2% (control), as well as with TBBPA or TBBPS at 5 µg/mL and 50 µg/mL. In control samples, only viable PBMCs were noticed. In probes incubated with TBBPA at 5 µg/mL mainly viable, as well as single early apoptotic cells were observed, while in the probes treated with TBBPS at 5 µg/mL, mainly viable PBMCs were found. Probes treated with TBBPA at 50 µg/mL contained mostly late apoptotic and necrotic cells, whereas the probes incubated with TBBPS at 50 µg/mL consisted of mainly viable, as well as some early apoptotic and late apoptotic PBMCs.

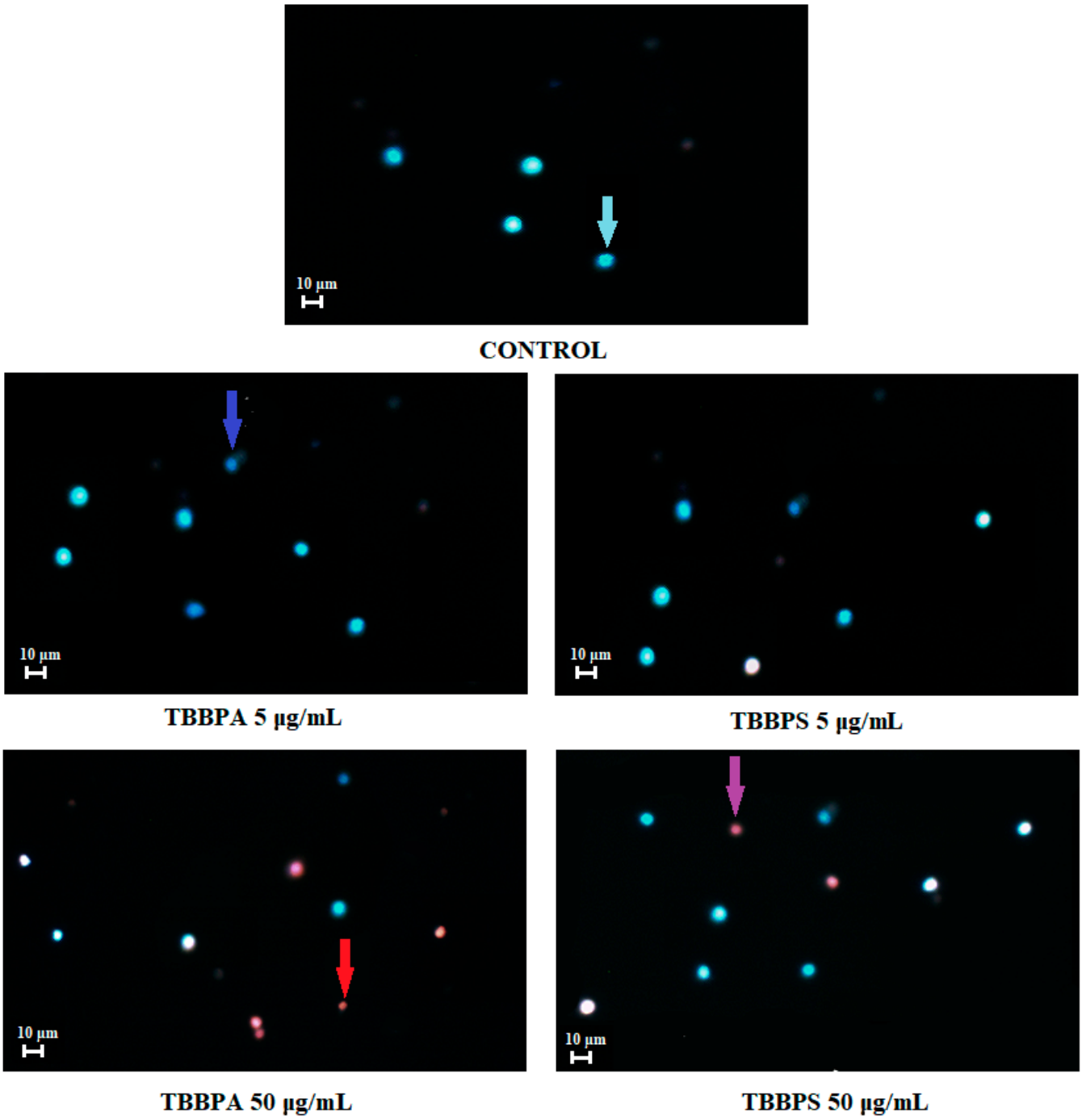



**Figure 7.** Apoptotic alterations in PBMCs treated with 0.2% DMSO (negative control), as well as with TBBPA and TBBPS at 5 µg/mL and 50 µg/mL. PBMCs were stained with Hoechst 33342 and PI. Viable cells (blue fluorescence), early apoptotic cells (intensive bright blue fluorescence), late apoptotic cells (blue/violet fluorescence) and necrotic cells (red fluorescence) [48].

## 3. Discussion

Apoptosis is a regulated process, which enables the removal of unwanted (old, damaged) cells from organisms without inflammatory reactions. It has been shown that various xenobiotics can trigger apoptosis [35], while excessive apoptosis may contribute to the development of infections, heart failure, neurodegenerative diseases or myocardial ischemia.

Peripheral blood mononuclear cells (PBMCs) are key cells of the immune system and are directly exposed to toxicants entering the human body. The accelerated apoptosis

of lymphocytes (main population of PBMCs) caused by action of xenobiotics, such as BFRs, may lead to disorders in the immune system function and possibly be involved in cancer development. Additionally, excessive apoptosis of PBMCs can contribute to the development of allergy and asthma [49].

In this research work, apoptotic potential and mechanism of action of TBBPA and TBBPS in human PBMCs was assessed by analyzing phosphatidylserine (PS) translocation in cellular membrane, determining cytosolic calcium ion and transmembrane mitochondrial potential levels, as well as evaluation of caspase-8, -9 and -3 activation, PARP-1 degradation, chromatin condensation and DNA fragmentation.

Our study revealed that tested compounds induced apoptosis of PBMCs but exhibited different apoptotic potential in studied cells. Both examined compounds increased the number of cells showing PS exposure, while TBBPA caused greater alteration in this parameter, when compared with TBBPS. It was observed that TBBPA from 5 μg/mL induced apoptosis of PBMCs, whereas TBBPS, only at 50 μg/mL, triggered apoptotic cell death (Figure 1). Similar findings were achieved by Mokra et al. [50] who assessed the apoptotic potential of debrominated analogs of TBBPA and TBBPS in PBMCs. They observed that bisphenol A (BPA) exhibited stronger apoptotic potential than bisphenol S (BPS) in this cell type. Cho et al. [51] proved that TBBPA (25–200 μM) after 24 h of incubation induced apoptosis in mouse astrocytes and neural stem cells. The authors reported that TBBPA increased ROS formation, which caused mitochondrial dysfunction and ultimately led to apoptosis through c-Jun N-terminal kinase-p53 pathway activation. In another study, Zhang et al. [52] reported a substantial rise in the number of apoptotic human liver L02 cells treated for 48 h with TBBPA in the range from 5 to 40 μM. TBBPA at 20 μM was also shown to induce significant apoptosis of insulinoma RIN-m5F rat cells after 48 h of incubation [53], while at a low concentration of 81 nM, this substance increased the number of apoptotic HepG2 cells by increasing ROS formation, decreasing $\Delta\Psi m$ and activating Ras signaling pathway [54]. Moreover, Wu et al. [55] observed an increased number of apoptotic cells in the heart and brain of embryos and larvae of Zebrafish exposed to TBBPA.

A crucial point in the apoptosis is the increase in cytosolic calcium ions level. Calcium ions released from endoplasmic reticulum into cytosol accumulate in mitochondria, which may lead to the depletion of $\Delta\Psi m$ and, finally, to the induction of apoptosis [56–58].

We observed that tested substances increased the level of cytosolic calcium ions and depleted $\Delta\Psi m$ level from 0.1 μg/mL and 1 μg/mL, respectively, while TBBPA induced greater changes than TBBPS (Figures 2 and 3). Ogunbayo et al. [59] noticed that TBBPA at 30 μM raised $Ca^{2+}$ level and reduced $\Delta\Psi m$ in TM4 mouse Sertoli cells. Similarly, Cho et al. [51] reported that TBBPA from 25 μg/mL depleted $\Delta\Psi m$ in mouse astrocytes and neural stem cells, whereas Lu et al. [54] noticed that this substance at 81 nM depleted $\Delta\Psi m$ in HepG2 cells. Moreover, Mokra et al. [50] observed that BPA and BPS were capable of increasing $Ca^{2+}$ level and decreasing $\Delta\Psi m$ in human PBMCs.

The intrinsic pathway, the so-called mitochondrial pathway, is activated as a result of oxidative stress and DNA damage, as well as by increased cytosolic calcium ion level and disturbances of $\Delta\Psi m$ [60,61]. The main component of the intrinsic pathway is the mitochondria. It has been shown that through the reactions involving Bcl-2 family proteins, mitochondrial permeability transition pores (MTP) are formed that release cytochrome c into mitochondrial intermembrane space. Then, cytochrome c together with procaspase-9, ATP and the cytosolic protein Apaf-1 forms an apoptosome in the cytoplasm that activates initiator caspase-9 [61–63]. On the other hand, the extrinsic (receptor) pathway may be activated by ROS, which involves the interactions of ligands with transmembrane receptors leading to the activation of initiator caspase-8 [47,62]. Although, the formation of ROS has not been assessed in this study, our previous work showed that TBBPA and TBBPS at low concentrations increased ROS level in human PBMCs [22].

Generally, our study showed that caspase-9 was more strongly activated than caspase-8 in PBMCs exposed to TBBPA and TBBPS at 5 μg/mL and 25 μg/mL. Moreover, it was

observed that TBBPA induced a stronger increase (than TBBPS) in caspase-9 activity, while in the case of caspase-8 activation, the results were opposite (Figure 4A,B). The stronger activation of caspase-9 suggests that mitochondrial pathway was mainly implicated in apoptosis induction of PBMCs exposed to TBBPS and, in particular, TBBPA.

It has been proven that both caspase-8 and caspase-9 can activate caspase-3, which is the executioner enzyme of apoptosis.

Obtained results revealed a substantial rise in caspase-3 activity, which was stronger in cells incubated with TBBPA (Figure 4C). A rise in caspase-8, -9, and -3 activities in human PBMCs exposed to debrominated analogs of tested BFRs, such as BPA and BPS was reported by Mokra et al. [50]. In other studies, Szychowski and Wójtowicz [64] observed that TBBPA (100 nM–100 µM, 6 h of incubation) increased caspase-3 activity in mouse hippocampal neuronal cells, while Al-Mousa and Michelangeli [17] showed that TBBPA in the concentrations from 1 to 30 µM activated caspase-3 in SH-SY5Y neuroblastoma cell line. Caspase-3 activation was also reported by Wu et al. [65] in A549 cells (human lung carcinoma cell line) exposed for 48 h to TBBPA in the concentrations range from 8 to 64 µg/mL. Moreover, Jarosiewicz et al. [66] observed apoptotic changes in human erythrocytes exposed to bromophenolic flame retardants. They demonstrated that TBBPS, and more strongly TBBPA, caused PS externalization (increased the number of apoptotic erythrocytes) and induced caspase-3 activation.

Caspase-3 causes PARP-1 cleavage into fragments of 89 kDa and 24 kDa. Fragments of 24 kDa bind to DNA breaks. In contrast, fragments of 89 kDa with attached PAR polymers are moved from nucleus into cytoplasm and interact with apoptosis-inducing factors (AIF), which, as a consequence, leads to the shrinking of the cell nucleus and apoptosis [67].

In order to determine PARP-1 cleavage, a concentration of 25 µg/mL of TBBPA and TBBPS was chosen, which the most strongly activated caspase-3 in tested cells. It was revealed that TBBPA and less strongly TBBPS at 25 µg/mL caused PARP-1 cleavage in human PBMCs (Figure 5). Similarly, Mokra et al. [50] observed PARP-1 fragmentation in human PBMCs treated with BPA and BPS.

TUNEL method allows the detection of apoptotic DNA fragmentation and is one of the most widely used assays to detect programmed cell death [68]. We used this method to confirm that tested compounds were capable of inducing apoptosis in studied cells.

Our study revealed that TBBPS, and more strongly TBBPA at 25 µg/mL, increased the number of TUNEL-positive cells (Figure 6). Park et al. [69] reported that TBBPA at 125 µg/mL (24 h of incubation) caused caspase-3 activation and increased the number of TUNEL-positive HEI-OC1 cells (mouse auditory cell line). Moreover, an increase in TUNEL-positive cells was noticed by Zatecka et al. [70] in mouse spermatozoa, treated with TBBPA at 200 µg/L dissolved in drinking water.

Staining PBMCs with Hoechst33342/PI and using fluorescence microscopy allowed to visualize differences in apoptotic/necrotic changes induced by tested compounds. We noted that TBBPA, particularly at higher concentration of 50 µg/mL induced greater alterations than TBBPS in studied cells. The samples treated with TBBPA contained mainly late apoptotic and necrotic cells, while the probes exposed to TBBPS showed the presence of mostly early apoptotic and viable PBMCs (Figure 7).

It must be underlined that the determination of the mechanism of proapoptotic action of toxicants, such as TBBPA and TBBPS, is crucial for the recognition of cellular targets, which are affected by studied compounds. It must also be taken into account that alterations in some apoptotic parameters, such as calcium ion level or $\Delta\Psi m$ (that usually occur at relatively low toxicant concentrations), are linked to other cellular processes (e.g., cell signaling, energy state of the cell, etc.), and their disturbance may change cell function before apoptosis occurs.

Summing up, this research work represents a mechanistic approach elucidating the effect of tested BFRs on nucleated blood cells, which brings us closer to understanding the action of these substances in the human organism.

## 4. Conclusions

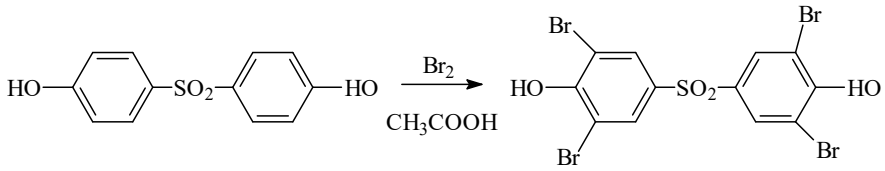
In conclusion, (1) TBBPA and TBBPS exhibited different apoptotic potential in human PBMCs. (2) Tested compounds triggered apoptosis by PS externalization on cellular membrane, increasing cytosolic $Ca^{2+}$ level, decreasing transmembrane mitochondrial potential, activating caspase-8, -9 and -3, as well as increasing PARP-1 cleavage, DNA fragmentation and chromatin condensation. (3) Tested BFRs induced apoptosis mainly by the involvement of mitochondrial pathway; although TBBPA and TBBPS more strongly activated intrinsic and extrinsic mitochondrial pathways, respectively. (4) Similarly, as in our previous studies on prooxidative and genotoxic potential, stronger apoptotic effects were provoked by TBBPA, in comparison to TBBPS.

## 5. Materials and Methods

### *5.1. Chemicals*

Tetrabromobisphenol A (99%, 2,2-bis[3,5-dibromo-4-hydroxyphenyl]propane) was bought from LGC Standards (Teddington, UK). Tetrabromobisphenol S (4,4′-sulfonylbis[2,6-dibromo-phenol]) (98.8%) was synthetized in the Institute of Industrial Organic Chemistry in Warsaw (Warsaw, Poland). Caspase-3 fluorimetric assay kit, HBSS solution, pluronic F-127, Hoechst 33342, propidium iodide and valinomycin were bought in Sigma-Aldrich (St. Louis, MO, USA). Perm/Wash Buffer and FITC Annexin V Apoptosis Detection Kit were obtained from Becton Dickinson (Franklin Lakes, NJ, USA). MitoLite Red CMXRos was bought in AAT Bioquest (Sunnyvale, CA, USA). Fluo-3 AM was obtained in PromoCell (Heidelberg, Germany). Caspase-8 fluorimetric assay kit and caspase-9 chromogenic substrate and caspase-9 inhibitor were bought in BioVision (San Francisco, CA, USA). PARP1 (cleaved Asp214) Monoclonal Antibody (HLNC4) and APO-BrdU TUNEL Assay Kit were obtained from Thermo-Fisher (Waltham, MA, USA). Separation medium (LSM) (1.077 g/cm$^3$) and RPMI medium with L-glutamine were purchased from Cytogen (Seoul, South Korea). Camptothecin was bought from Pol-Aura (Dywity, Poland). Ionomycin was obtained from Biokom (Janki, Poland). Other chemicals were obtained from Pol-Aura (Dywity, Poland) and Roth (Karlsruhe, Germany).

TBBPS was synthesized in a reaction of bisphenol S bromination with liquid bromide in concentrated acetic acid at 80 °C [71], according to Scheme 1:




**Scheme 1.** TBBPS synthesized in a reaction of bisphenol S bromination with liquid bromide in concentrated acetic acid at 80 °C.

Crude residue was rinsed with concentrated acetic acid, dissolved in tetrahydrofuran and precipitated out of the solution using hexane. As a result, TBBPS was obtained, which was a colorless solid with melting temperature of 293.3 °C and HPLC purity of 98.8% (internal standardization), efficiency of the reaction was 35%.

Analysis of TBBPS using mass spectrometry - (EI, 70 eV, *m/z*, int[%]): 570(19), 569(9), 568(67), 567(14), 566(100, M), 565(100, 564(69), 562(18), 502(8), 315(14), 302(120, 301(35), 300(23), 299(64), 298(12), 297(33), 270(18), 269(13), 268(38), 267(22), 266(19), 265(11), 253(10), 252(17), 251(25), 250(14), 249(10), 223(10), 221(10), 172(24), 171(16), 170(25), 169(10), 143(14), 141(13), 91(30), 90(27), 63(33), 62(43), 61(13), 53(13).

### *5.2. Methods*

#### 5.2.1. Cell Isolation and Treatment

The method of peripheral blood mononuclear cells (PBMCs) isolation was described in detail by Włuka et al. [22]. PBMCs were isolated from the leukocyte-platelet buffy

coat, which was separated from whole blood in the Blood Bank in Lodz, Poland. Blood was taken from healthy, non-smoking volunteers (aged 18–45) who showed no signs of infection disease symptoms. The study was approved by Bioethical Commission of Scientific Research at the University of Lodz, no. 1/KBBN-UŁ/II/2017.

Tested compounds were dissolved in DMSO. Final concentration of DMSO in untreated samples (negative control) and samples incubated with TBBPA or TBBPS was 0.2%. The above concentration of DMSO was not toxic for PBMCs, as evaluated by all studied parameters.

The cells were exposed to tested substances in the range of concentrations from 0.01 to 50 μg/mL (depending on the method used) for 24 h at 37 °C in 5% $CO_2$ atmosphere in total darkness. The final PBMCs density used in the experiments (after addition of BFR solution) was $4 \times 10^6$ cells/mL for caspase analysis and $1 \times 10^6$ cells/mL for assessment of other tested parameters. The viability of PBMCs in negative control was over 95%.

The lowest tested BFRs concentration used in this study corresponded to TBBPA concentration determined in humans environmentally exposed to this substance [14]. If no effects were observed at the above-mentioned BFRs levels, higher TBBPA and TBBPS concentrations were used.

#### 5.2.2. Quantitative Determination of Apoptosis (Annexin V-FITC/PI Staining)

During apoptosis phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane. The method is based on the ability of annexin V (labelled with fluorescein isothiocyanate—FITC) to bind to PS located on the outer monolayer of the cellular membrane. Propidium iodide (PI) is used in order to detect necrotic cells as it enters the cell through damaged membrane and binds to DNA. The experiment was conducted according to manufacturer's procedure, as described by Jarosiewicz et al. [66].

PBMCs were incubated with TBBPA or TBBPS in the range from 1 to 50 μg/mL for 24 h at 37 °C in total darkness. After incubation, the cells were centrifuged (300× *g*) for 5 min at 4 °C and suspended in RPMI medium. Then, PBMCs were stained with the mixture of Annexin V-FITC and PI (1 μM each) dissolved in Annexin V-binding buffer and incubated for 20 min at room temperature in total darkness. In the cells, apoptosis was induced with camptothecin at 10 μM (positive control). The samples were detected by flow cytometry (LSR II, Becton Dickinson, Franklin Lakes, NJ, USA) (excitation/emission maxima: 488/525 nm for annexin V and 530/620 nm for PI, respectively). FMC gate on PBMCs was established and the data were recorded for a total of 10,000 cells per sample.

#### 5.2.3. Cytosolic Calcium Ion Level

One of the primary parameter of apoptosis is the rise in the level of cytosolic $Ca^{2+}$, which can be measured using stain Fluo-3/AM. Fluo-3/AM shows very low fluorescence, but after its hydrolysis by membrane esterases (Fluo-3 formation) and complexation with calcium ions, it exhibits about 100-fold increase in green fluorescence intensity. The analysis of calcium ion level in human PBMCs was previously described in detail by Mokra et al. and Barańska et al. [50,72].

The cells were incubated with TBBPA or TBBPS in the range from 0.01 to 5 μg/mL for 24 h at 37 °C in total darkness. Then, PBMCs were centrifuged (300× *g*) for 5 min at 4 °C, resuspended in solution of Fluo-3 AM (1 μM), and incubated for 20 min at 37 °C in total darkness. In the next step, HBSS consisting of 1% BSA was added to the cells, which were incubated for 40 min at 37 °C in total darkness. PBMCs were rinsed twice using HEPES buffer, and then centrifuged (300× *g*) for 5 min at 4 °C. Finally, the cells were resuspended in HEPES buffer, and incubated for 10 min at 37 °C in total darkness. Positive control contained the cells treated with ionomycin at 1 μM (calcium ionophore). The samples were detected using flow cytometry (LSR II, Becton Dickinson, Franklin Lakes, NJ, USA) (excitation/emission maxima: 488/525 nm for Fluo-3). FMC gate on PBMCs was established, and the data were recorded for a total of 10,000 cells per sample.

#### 5.2.4. Mitochondrial Transmembrane Potential

A characteristic sign of early apoptosis is a reduction in transmembrane mitochondrial potential ($\Delta\Psi m$), which can be determined by changes in the level of fluorescence of MitoLite Red CMXRos (excitation/emission maxima: 579/599 nm). This fluorescent stain is a cationic dye that readily penetrates living cells and accumulates in mitochondria, depending on the value of $\Delta\Psi m$. Due to the presence of thiol-reactive chloromethyl moieties, this stain is retained in the mitochondria [50].

PBMCs were treated with TBBPA or TBBPS in the range of the concentrations from 0.01 to 25 µg/mL for 24 h at 37 °C in total darkness. Nigericin and valinomycin (1 µM), which are capable of increasing and decreasing $\Delta\Psi m$, respectively, were used as positive controls. After the probes had been incubated, they were centrifuged (300× *g*) for 5 min at 4 °C. The supernatant was discarded, and the cells were resuspended in PBS solution. PBMCs were stained with MitoLite CMXRos at 1 µM, and then incubated for 20 min at 37 °C in total darkness. The probes were determined in 96-well plates using a microplate reader (Cary Eclipse, Varian, Waltham, MA, USA).

#### 5.2.5. Caspase-3, -8, -9 Activation

The process of apoptosis is regulated directly and indirectly by caspases. Fluorimetric analysis of caspase-3 and caspase-8 activation was conducted according to the manufacturers' protocols with slight modifications [72].

The methods of caspase-3 and caspase-8 detection are based on the hydrolysis of peptide substrates acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) and acetyl-Ile-GluThr-Asp-7-amino-4-methylcoumarin (Ac-IETD-AMC), respectively. Hydrolysis of the substrates results in the release of fluorescent 7-amino-4-methylcoumarin (AMC) (excitation/emission maxima: 360/460 nm). Colorimetric determination of caspase-9 activity was associated with hydrolysis of the substrate Acetyl-Leu-Glu-His-Asp-p-nitroaniline (Ac-LEHD-pNA), which resulted in a release of p-nitroaniline (pNA) (absorption at 405 nm). In all experiments, positive controls were employed that contained cells suspension treated with camptothecin (10 µM). Preincubation with caspases inhibitors was also executed for all experiments. Determination of caspase-3 and caspase-8 activity was performed using a fluorescent microplate reader (Fluoroskan Ascent FL, Labsystem, Thermo-Fisher, Waltham, MA, USA), whereas detection of caspase-9 activity was done using an absorbance microplate reader (BioTek ELx808, Bio-Tek, Santa Clara, CA, USA).

#### 5.2.6. PARP-1 Cleavage

Human poly (ADP-ribose) polymerase (PARP1) is a 116 kDa nuclear enzyme implicated in DNA repair. During apoptosis, caspase-3 cleaves PARP1 between Asp214 and Gly215, generating two fragments of 85 kDa and 25 kDa. The HLNC4 antibody (conjugated with Alexa fluor 488) added to the sample specifically recognizes the 85 kDa PARP1 fragment formed after the enzyme cleavage.

PBMCs were incubated with TBBPA or TBBPS at 25 ug/mL for 24 h at 37 °C in total darkness. Then, PBMCs were washed and suspended in 1% paraformaldehyde (dissolved in PBS). Then, HLNC4 antibody conjugated with Alexa fluor 488 was added to the probes, which were incubated for 30 min at 37 °C in total darkness. In the cells, apoptosis was induced by camptothecin at 10 µM (positive control). Cytometric analysis of the samples was conducted (LSR II, Becton Dickinson, Franklin Lakes, NJ, USA) at excitation/emission maxima of 494/519 nm for Alexa Fluor 488. FMC gate on PBMCs was established for data acquisition, and the data were recorded for 10,000 cells per sample.

#### 5.2.7. APO-BrdU TUNEL Assay

The TUNEL method detects apoptosis by labelling the 3′-OH ends of single- and double-stranded DNA fragments with labelled Br-dUTP (brominated deoxyuridine triphosphate nucleotides). The reaction is catalyzed by terminal deoxynucleotidyl transferase (TdT) [73].

The cells were incubated with TBBPA or TBBPS at 25 μg/mL for 24 h at 37 °C in total darkness. Then, PBMCs were fixed in 1% paraformaldehyde. The samples were incubated (1 h at 37 °C in total darkness) in DNA labelling solution containing BrdUTP and TdT. Then, anti BrdUTP antibody was added to the probes, which were incubated for 30 min at room temperature in total darkness. In the cells, apoptosis was induced by camptothecin at 10 μM (positive control). The probes were determined by flow cytometry (LSR II, Becton Dickinson, Franklin Lakes, NJ, USA). Maxima of excitation and emission for Alexa Fluor 488 were 494 nm and 519 nm, respectively. FMC gate on PBMCs was established for data acquisition, and the data were recorded for 10,000 cells per sample.

#### 5.2.8. Apoptosis Determination by Fluorescence Microscopy

Apoptotic changes in tested cells incubated with TBBPA or TBBPS were evaluated by their staining with Hoechst 33342 and PI and analyzing by fluorescence microscopy. The method was described by Rogalska et al. [48], while preparation of the samples (PBMCs) was presented in detail by Mokra et al. and Barańska et al. [50,72].

#### 5.2.9. Statistical Analysis

Data are shown as average values with standard deviation. ANOVA (one-way analysis of variance) test and Tukey's post-hoc test or Welch's test (analysis of PARP1 cleavage and DNA fragmentation) were employed in order to evaluate statistical significance between examined probes [74]. Statistical significance was $p < 0.05$. All analyses were done using STATISTICA 13 software (StatSoft, Inc., Tulusa, OK, USA). The experiments were done on blood taken from 4 donors, while for each individual experiment (one blood donor), an experimental point was a mean value of 2–3 replications.

**Author Contributions:** Conceptualization, J.M.; methodology A.B.; validation, A.B.; investigation, A.B. and B.B.; writing—original draft preparation, A.B. and J.M., writing—review and editing, J.M. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was carried out under the research project PRELUDIUM 17 no. 2019/33/N/NZ7/01202 awarded by the National Science Centre, Poland, and the grant for Young Scientists no. B1911000002126.02 awarded by the Dean of the Faculty of Biology and Environmental Protection, University of Lodz.

**Institutional Review Board Statement:** The study was approved by Bioethical Commission of Scientific Research at the University of Lodz, no. 1/KBBN-UŁ/II/2017.

**Informed Consent Statement:** PBMCs were isolated from the leucocyte-buffy coat separated from blood bought in the Regional Centre of Blood Donation and Blood Treatment in Lodz, Poland. All the procedures related to blood donation were executed at the Regional Centre of Blood Donation and Blood Treatment in Lodz, Poland. Blood donors recruitment was completed at the Centre, according to national legal procedures and European Union regulations (incl. the regulation (EU) 2016/679 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 27 April 2016 on the protection of natural persons with regard to the processing of personal data and on the free movement of such data).

**Data Availability Statement:** The raw data supporting the conclusions of this paper are deposited in the Department of Biophysics of Environmental Pollution, University of Lodz and will be made available by the authors, without undue reservation.

**Conflicts of Interest:** The authors declare no conflict of interest.

**Sample Availability:** Samples of the compounds are not available from authors.

## References


1. Liu, A.; Shi, J.; Shen, Z.; Lin, Y.; Qu, G.; Zhao, Z.; Jiang, G. Identification of unknown brominated bisphenol S congeners in contaminated soils as the transformation products of tetrabromobisphenol S derivatives. *Environ. Sci. Technol.* **2018**, *52*, 10480–10489. [CrossRef] [PubMed]
2. Michałowicz, J.; Włuka, A.; Bukowska, B. A review of environmental occurrence, toxic effects and transformation of man-made bromophenols. *Sci. Total Environ.* **2022**, *811*, 152289. [CrossRef] [PubMed]
3. Yang, H.; Luo, X.-J.; Zeng, Y.-H.; Mai, B.-X. Determination of tetrabromobisphenol-A/S and their eight derivatives in abiotic (soil/dust) samples using ultra-high performance liquid chromatography-tandem mass spectrometry. *J. Chromat. A* **2021**, *1647*, 462152. [CrossRef]
4. Kacew, S.; Hayes, A. Absence of neurotoxicity and lack of neurobehavioral consequences due to exposure to tetrabromobisphenol A (TBBPA) exposure in humans, animals and zebrafish. *Arch. Toxicol.* **2019**, *94*, 59–66. [CrossRef] [PubMed]
5. Macêdo, W.; Sánchez, F.; Zaiat, M. What drives tetrabromobisphenol A degradation in biotreatment systems? *Rev. Environ. Sci. Bio/Technol.* **2021**, *20*, 729–750. [CrossRef]
6. Xiong, J.K.; An, T.C.; Zhang, C.S.; Li, G.Y. Pollution profiles and risk assessment of PBDEs and phenolic brominated flame retardants in water environments within a typical electronic waste dismantling region. *Environ. Geochem. Health* **2015**, *37*, 457–473. [CrossRef]
7. Liu, K.; Li, J.; Yan, S.; Zghang, W.; Li, Y.; Han, D. A review of status of tetrabromobisphenol A (TBBPA) in China. *Chemosphere* **2016**, *148*, 8–20. [CrossRef]
8. Rothenbacher, K.; Pecquet, A. Summary of historical terrestrial toxicity data for the brominated flame retardant tetrabromobisphenol A (TBBPA): Effects on soil microorganisms, earthworms, and seedling emergence. *Environ. Sci. Pollut. Res.* **2018**, *25*, 17268–17277. [CrossRef]
9. Abafe, O.A.; Martincigh, B.S. Determination and human exposure assessment of polybrominated diphenyl ethers and tetrabromobisphenol A in indoor dust in South Africa. *Environ. Sci. Pollut. Res.* **2016**, *23*, 7038–7049. [CrossRef]
10. Xie, Z.; Ebinghaus, R.; Lohmann, R.; Heemken, O.; Caba, A.; Püttmann, W. Trace determination of the flame retardant tetrabromobisphenol A in the atmosphere by gas chromatography–mass spectrometry. *Anal. Chim. Acta* **2007**, *584*, 333–342. [CrossRef]
11. Abdallah, M.A.E.; Harrad, S.; Covaci, A. Hexabromocyclododecanes and tetrabromobisphenol-A in indoor air and dust in Birmingham, UK: Implications for human exposure. *Environ. Sci. Technol.* **2008**, *42*, 6855–6861. [CrossRef] [PubMed]
12. Cariou, R.; Antignac, J.P.; Zalko, D.; Berrebi, A.; Cravedi, J.P.; Maume, D.; Marchand, P.; Montenau, F.; Riu, A.; Andre, F.; et al. Exposure assessment of French women and their newborns to tetrabromobisphenol-A: Occurrence measurements in maternal adipose tissue, serum, breast milk and cord serum. *Chemosphere* **2008**, *73*, 1036–1041. [CrossRef] [PubMed]
13. Fujii, Y.; Harada, K.H.; Hitomi, T.; Kobayashi, H.; Koizumi, A.; Haraguchi, K. Temporal trend and age-dependent serum concentration of phenolic organohalogen contaminants in Japanese men during 1989–2010. *Environ. Pollut.* **2014**, *185*, 228–233. [CrossRef]
14. Lankova, D.; Lacina, O.; Pulkrabova, J.; Majslova, J. The determination of perfluoroalkyl substances, brominated flame retardants and their metabolites in human breast milk and infant formula. *Talanta* **2013**, *117*, 318–325. [CrossRef] [PubMed]
15. Fukuda, N.; Ito, Y.; Yamaguchi, M.; Mitumori, K.; Koizumi, M.; Hasegawa, R.; Kamata, E.; Ema, M. Unexpected nephrotoxicity induced by tetrabromobisphenol A in newborn rats. *Toxicol. Lett.* **2004**, *150*, 145–155. [CrossRef]
16. Choi, E.; Suh, K.; Rhee, S.; Oh, S.; Kim, S.; Pak, Y.; Choe, W.; Ha, J.; Chon, S. Exposure to tetrabromobisphenol A induces cellular dysfunction in osteoblastic MC3T3-E1 cells. *J. Environ. Sci. Health Part A* **2017**, *52*, 561–570. [CrossRef]
17. Al-Mousa, F.; Michelangel, F.; Vavvas, D. Some commonly used brominated flame retardants cause $Ca^{2+}$-ATPase inhibition, beta-amyloid peptide release and apoptosis in SH-SY5Y neuronal cells. *PLoS ONE* **2012**, *7*, e33059. [CrossRef]
18. Koike, E.; Yanagisawa, R.; Takano, H. Brominated flame retardants, hexabromocyclododecane and tetrabromobisphenol A, affect proinflammatory protein expression in human bronchial epithelial cells via disruption of intracellular signalling. *Toxicol. In Vitro* **2016**, *32*, 212–219. [CrossRef]
19. Sanders, J.; Coulter, S.; Knudsen, G.; Dunnick, J.; Kissling, G.; Birnbaum, L. Disruption of estrogen homeostasis as a mechanism for uterine toxicity in Wistar Han rats treated with tetrabromobisphenol A. *Toxicol. Appl. Pharmacol.* **2016**, *298*, 31–39. [CrossRef]
20. Yasmin, S.; Whalen, M. Flame retardants, hexabromocyclododecane (HCBD) and tetrabromobisphenol a (TBBPA), alter secretion of tumor necrosis factor alpha (TNFα) from human immune cells. *Arch. Toxicol.* **2018**, *92*, 1483–1494. [CrossRef]
21. Sharma, P.; Chadha, P.; Saini, H. Tetrabromobisphenol A induced oxidative stress and genotoxicity in fish Channa punctatus. *Drug Chem. Toxicol.* **2019**, *42*, 559–564. [CrossRef] [PubMed]
22. Włuka, A.; Woźniak, A.; Woźniak, E.; Michałowicz, J. Tetrabromobisphenol A, terabromobisphenol S and other bromophenolic flame retardants cause cytotoxic effects and induce oxidative stress in human peripheral blood mononuclear cells (in vitro study). *Chemosphere* **2020**, *261*, 127705. [CrossRef] [PubMed]
23. Barańska, A.; Woźniak, A.; Mokra, K.; Michałowicz, J. Genotoxic mechanism of action of TBBPA, TBBPS and selected bromophenols in human peripheral blood mononuclear cells. *Front. Immunol.* **2022**, *13*, 869741. [CrossRef] [PubMed]
24. Xu, H.; Li, Y.; Lu, J.; Lu, J.; Zhou, L.; Chovelo, J.M.; Ji, Y. Aqueous photodecomposition of the emerging brominated flame retardant tetrabromobisphenol S (TBBPS). *Environ. Pollut.* **2021**, *271*, 116406. [CrossRef]

25. Letcher, R.; Chu, S. High-sensitivity method for determination of tetrabromobisphenol-S and tetrabromobisphenol-A derivative flame retardants in Great Lakes Herring Gull eggs by liquid chromatography−atmospheric pressure photoionization−tandem mass spectrometry. *Environ. Sci. Technol.* **2010**, *44*, 8615–8621. [CrossRef] [PubMed]
26. Liu, K.; Lu, J.; Ji, Y. Formation of brominated disinfection by-products and bromate in cobalt catalyzed peroxymonosulfate oxidation of phenol. *Water Res.* **2015**, *84*, 1–7. [CrossRef] [PubMed]
27. Qu, G.; Liu, A.; Hu, A.; Liu, S.; Shi, J.; Jiang, G. Recent advances in the analysis of TBBPA/TBBPS, TBBPA/TBBPS derivatives and their transformation products. *TrAC Trends Anal. Chem.* **2016**, *83*, 14–24. [CrossRef]
28. Wang, X.L.; Luan, J.; Lu, Q.L.; Lin, H.G.; Xu, C. Three new metal–organic complexes derived from N,N′-bis(3-pyridinecarboxamide)-1,2-ethane and polycarboxylate ligands: Synthesis, fluorescent and electrochemical properties. *J. Organometal. Chem.* **2012**, *719*, 1–7. [CrossRef]
29. Song, S.; Song, M.; Zeng, L.; Wang, T.; Liu, R.; Ruan, T.; Jiang, G. Occurrence and profiles of bisphenol analogues in municipal sewage sludge in China. *Environ. Pollut.* **2014**, *186*, 14–19. [CrossRef]
30. Yang, B.; Ying, G.G.; Chen, Z.F.; Zhao, J.L.; Peng, F.Q.; Chen, X.W. Ferrate(VI) oxidation of tetrabromobisphenol A in comparison with bisphenol A. *Water Res.* **2014**, *62*, 211–219. [CrossRef]
31. Malysheva, S.; Goscinny, S.; Malarvannan, G.; Poma, G.; Andjelkovic, M. Development and validation of a quantitative UHPLC-MS/MS method for selected brominated flame retardants in food. *Food Addit. Contam. Part A* **2018**, *35*, 293–304. [CrossRef] [PubMed]
32. Li, A.; Zhuang, T.; Shi, W.; Liang, Y.; Liao, C.; Song, M.; Jiang, G. Serum concentration of bisphenol analogues in pregnant women in China. *Sci. Total Environ.* **2020**, *707*, 136100. [CrossRef] [PubMed]
33. Ding, Y.; Dong, X.; Feng, W.; Mao, G.; Chen, Y.; Oiu, X.; Chen, K.; Xu, H. Tetrabromobisphenol S alters the circadian rhythm network in the early life stages of zebrafish. *Sci. Total Environ.* **2022**, *806*, 150543. [CrossRef] [PubMed]
34. Liang, S.; Liang, S.; Yin, N.; Hu, B.; Faiola, F. Toxicogenomic analyses of the effects of BDE-47/209, TBBPA/S and TCBPA on early neural development with a human embryonic stem cell *in vitro* differentiation system. *Toxicol. Appl. Pharmacol.* **2019**, *379*, 114685. [CrossRef] [PubMed]
35. Pallardy, M.; Biola, A.; Lebrec, H.; Breard, J. Assessment of apoptosis in xenobiotic-induced immunotoxicity. *Methods* **1999**, *19*, 36–47. [CrossRef] [PubMed]
36. Zhang, N.; Hartig, M.; Dzhagalov, I.; Draper, D.; He, Y.W. The role of apoptosis in the development and function of T lymphocytes. *Cell Res.* **2005**, *15*, 749–769. [CrossRef] [PubMed]
37. Karuma, M.M.; Tan, N.Y.; Bennett, M.R. Death receptors and their ligands in atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **2008**, *28*, 1694–1702.
38. LaRosa, F.D.; Orange, S.J. Lymphocytes. *J. Allergy Clin. Immunol.* **2008**, *121*, 364–369. [CrossRef]
39. Weinberg, A.; Jesser, R.; Edelstein, C.; Billy, J.; Wohl, D. Excess apoptosis of mononuclear cells contributes to the depressed cytomegalovirus-specific immunity in HIV-infected patients on HAART. *Virology* **2004**, *330*, 313–321. [CrossRef]
40. Ratomski, K.; Skotnicka, B.; Kasprzycka, E.; Żelazowska-Rutkowska, B.; Wysocka, J.; Anisimowicz, S. Evaluation of percentage of the $CD19^+$ $CD5^+$ lymphocytes in hypertrophied adenoids at children with otitis media with effusion. *Otolaryngol. Pol.* **2007**, *61*, 962–966. [CrossRef]
41. Zhou, C.-X.; Zhou, D.-H.; Liu, G.-X.; Suo, X.; Zhu, X.-Q. Transcriptomic analysis of porcine PBMCs infected with Toxoplasma gondii RH strain. *Acta Tropica.* **2016**, *154*, 82–88. [CrossRef] [PubMed]
42. Halit, S.; Salameh, P. Exposure to toxins during pregnancy and childhood and asthma in children: A pilot study. *J. Epidemiol. Global Health* **2017**, *7*, 147–154. [CrossRef] [PubMed]
43. Sen, P.; Kemppainen, E.; Orešič, M. Perspectives on Systems Modeling of Human Peripheral Blood Mononuclear Cells. *Front. Mol. Biosci.* **2017**, *4*, 96. [CrossRef] [PubMed]
44. Feiterio, J.; Mariana, M.; Cairrao, E. Health toxicity effects of brominated flame retardants: From environmental to human exposure. *Environ. Pollut.* **2021**, *285*, 117475. [CrossRef] [PubMed]
45. Hall, S.M.; Coulter, S.J.; Knudsen, G.A.; Sanders, J.M.; Birnbaum, L.S. Gene expression changes in immune response pathways following oral administration of tetrabromobisphenol A (TBBPA) in female Wistar Han rats. *Toxicol. Lett.* **2017**, *272*, 68–74. [CrossRef]
46. Cato, A.; Celada, L.; Kibakaya, E.; Simmons, N.; Whalen, M. Brominated flame retardants, tetrabromobisphenol A and hexabromocyclododecane, activate mitogen-activated protein kinases (MAPKs) in human natural killer cells. *Cell. Biol. Toxicol.* **2014**, *30*, 345–360. [CrossRef]
47. Elmore, S. Apoptosis: A review of programmed cell death. *Toxicol. Pathol.* **2007**, *35*, 495–516. [CrossRef]
48. Rogalska, A.; Gajek, A.; Szwed, M.; Jóźwiak, Z.; Marczak, A. The role of reactive oxygen species in WP 631-induced death of human ovarian cancer cells: A comparison with the effect of doxorubicin. *Toxicol. Vitr.* **2011**, *25*, 1712–1720. [CrossRef]
49. Rutkowska, J. Zjawisko apoptozy w chorobach alergicznych. *Now. Lek.* **2007**, *76*, 48–54.
50. Mokra, K.; Kocia, M.; Michałowicz, J. Bisphenol A and its analogs exhibit different apoptotic potential in peripheral blood mononuclear cells (in vitro study). *Food Chem. Toxicol.* **2015**, *84*, 79–88. [CrossRef]
51. Cho, J.-H.; Lee, S.; Jeon, H.; Kim, A.; Lee, W.; Lee, Y.; Yang, S.; Yun, J.; Jung, Y.-S.; Lee, J. Tetrabromobisphenol A-induced apoptosis in neural stem cells through oxidative stress and mitochondrial dysfunction. *Neurotox. Res.* **2020**, *38*, 74–85. [CrossRef] [PubMed]

52. Zhang, Y.; Wang, X.; Chen, C.; An, J.; Shang, Y.; Li, H.; Xa, H.; Yu, J.; Wang, C.; Liu, Y.; et al. Regulation of TBBPA-induced oxidative stress on mitochondrial apoptosis in L02 cells through the Nrf2 signaling pathway. *Chemosphere* **2019**, *226*, 463–471. [CrossRef] [PubMed]
53. Suh, K.; Choi, E.; Rhee, S.; Oh, S.; Kim, S.; Pak, Y.; Choe, W.; Ha, J.; Chon, S. Tetrabromobisphenol A induces cellular damages in pancreatic β-cells in vitro. *J. Environ. Sci. Health Part A* **2017**, *52*, 624–631. [CrossRef] [PubMed]
54. Lu, L.; Hu, J.; Li, G.; An, T. Low concentration Tetrabromobisphenol A (TBBPA) elevating overall metabolism by inducing activation of the Ras signalling pathway. *J. Hazard. Mat.* **2021**, *416*, 125797. [CrossRef]
55. Wu, S.; Ji, G.; Liu, J.; Zhang, S.; Gong, Y.; Shi, L. TBBPA induces developmental toxicity, oxidative stress, and apoptosis in embryos and Zebrafish Larvae (*Danio rerio*). *Environ. Toxicol.* **2016**, *31*, 1241–1249. [CrossRef]
56. Hajnoczky, G.; Davies, E.; Madesh, M. Calcium signalling and apoptosis. *Biochem. Biophys. Res. Commun.* **2003**, *304*, 445–454. [CrossRef]
57. Kaufman, R.J.; Scheuner, D.; Schröder, M. The unfolded protein response in nutrient sensing and differentiation. *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 411–421. [CrossRef]
58. Kroemer, G.; Reed, J.C. Mitochondrial control of cell death. *Nat. Med.* **2000**, *6*, 513–519. [CrossRef]
59. Ogunbayo, O.; Lai, P.; Conolly, T.; Michelangeli, A. Tetrabromobisphenol A (TBBPA), induces cell death in TM4 Sertoli cells by modulating $Ca^{2+}$ transport proteins and causing dysregulation of $Ca^{2+}$ homeostasis. *Toxicol. Vitr.* **2008**, *22*, 943–952. [CrossRef]
60. Łabędzka, K.; Grzanka, A.; Izdebska, M. Mitochondrium a śmierć komórki. *Post. Hig. Med. Dośw.* **2006**, *60*, 439–446.
61. Hongmei, Z. Extrinsic and intrinsic apoptosis signal pathway review. In *Apoptosis and Medicine*; IntechOpen: London, UK, 2012; Volume 16, pp. 1–22.
62. Stępień, A.; Izdebska, M.; Grzanka, A. Rodzaje śmierci komórki. *Post. Hig. Med. Dośw.* **2007**, *61*, 420–428.
63. Pihán, P.; Carreras-Sureda, A.; Hetz, C. BCL-2 family: Integrating stress responses at the ER to control cell demise. *Cell Death Different.* **2017**, *24*, 1478–1487. [CrossRef] [PubMed]
64. Szychowski, K.; Wójtowicz, A. TBBPA causes neurotoxic and the apoptotic responses in cultured mouse hippocampal neurons in vitro. *Pharmacol. Rep.* **2015**, *68*, 20–26. [CrossRef] [PubMed]
65. Wu, S.; Wu, M.; Qi, M.; Zhong, L.; Qiu, L. Effects of novel brominated flame retardant TBBPA on human airway epithelial cell (A549) in vitro and proteome profiling. *Environ. Toxicol.* **2018**, *33*, 1245–1253. [CrossRef]
66. Jarosiewicz, M.; Michałowicz, J.; Bukowska, B. In vitro assessment of eryptotic potential of tetrabromobisphenol A and other bromophenolic flame retardants. *Chemosphere* **2019**, *215*, 404–412. [CrossRef]
67. Mashimo, M.; Onishi, M.; Uno, A.; Tanimichi, A.; Nobeyama, A.; Mori, M.; Yamada, S.; Negi, S.; Bu, X.; Kato, J.; et al. The 89-kDa PARP1 cleavage fragment serves as a cytoplasmic PAR carrier to induce AIF-mediated apoptosis. *J. Biol. Chem.* **2021**, *296*, 100046. [CrossRef]
68. Chmielewski, M.; Linke, K.; Zabel, M. Metody wykrywania zjawiska apoptozy w komórkach wątrobowych in situ. *Now. Lek.* **2008**, *77*, 223–226.
69. Park, C.; Se-Jin, K.; Lee, W.K.; Moon, S.K.; Kwak, S.; Choe, S.K.; Park, R. Tetrabromobisphenol-A induces apoptotic death of auditory cells and hearing loss. *Biochem. Biophys. Res. Commun.* **2016**, *478*, 1667–1673. [CrossRef]
70. Zatecka, E.; Castillo, J.; Elzeinova, F.; Kubatova, A.; Ded, J.; Peknicova, J.; Oliva, R. The effect of tetrabromobisphenol A on protamine content and DNA integrity in mouse spermatozoa. *Andrology* **2014**, *2*, 910–917. [CrossRef]
71. Lue, J.; Han, L.-W.; Lin, J.-X.; Cao, R. Organic complexes built by halogenated molecules: Unexpected in situ C_N bond formation in metal-free solvothermal conditions. *Cryst. Growth Des.* **2010**, *10*, 4217–4220.
72. Barańska, A.; Sicińska, P.; Michałowicz, J. Apoptosis-inducing potential of selected bromophenolic flame retardants 2,4,6-tribromophenol and pentabromophenol in human peripheral blood mononuclear cells. *Molecules* **2022**, *27*, 5056. [CrossRef] [PubMed]
73. Gralewska, P.; Gajek, A.; Marczak, A.; Rogalska, A. Metformin affects olaparib sensitivity through induction of apoptosis in epithelial ovarian cancer cell lines. *Int. J. Mol. Sci.* **2021**, *22*, 10557. [CrossRef] [PubMed]
74. Watała, C. *Biostatystyka—Wykorzystanie Metod Statystycznych w Pracy Badawczej w Naukach Biomedycznych*, 2nd ed.; Alfa-Medica Press: Bielsko-Biała, Poland, 2002; pp. 54–68.

Science of the Total Environment 811 (2022) 152289

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Review

# A review on environmental occurrence, toxic effects and transformation of man-made bromophenols

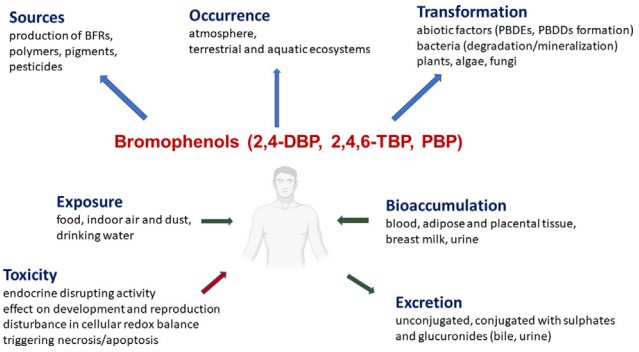
Jaromir Michałowicz *, Anna Włuka, Bożena Bukowska

*University of Lodz, Faculty of Biology and Environmental Protection, Department of Biophysics of Environmental Pollution, Pomorska Str. 141/143, 90-236 Lodz, Poland*

## HIGHLIGHTS

- Synthetic bromophenols (BPs), such as 2,4-DBP, 2,4,6-TBP and PBP are widespread in the environment.
- Man is exposed to BPs mainly by food, and to a lower extent by dust and drinking water.
- BPs exhibit necrotic and apoptotic potential and have endocrine activities.
- BPs influence cell signalling and cellular redox balance and alter enzymes activities.
- BPs are transformed in human, animals and plants and degraded by bacteria.

## GRAPHICAL ABSTRACT




## ARTICLE INFO

*Article history:*
Received 17 September 2021
Received in revised form 18 November 2021
Accepted 5 December 2021
Available online 10 December 2021

Editor: Adrian Covaci

*Keywords:*
Bromophenols
Estrogenicity
Toxicity
Transformation
Polybrominated dioxins

## ABSTRACT

Brominated phenols (BPs) of anthropogenic origin are aromatic substances widely used in the industry as flame retardants (FRs) and pesticides as well as the components of FRs and polymers. In this review, we have focused on describing 2,4-dibromophenol (2,4-DBP), 2,4,6-tribromophenol (2,4,6-TBP) and pentabromophenol (PBP), which are the most commonly used in the industry and are the most often detected in the air, aquatic and terrestrial ecosystems and the human body. This review describes human-related sources of these BPs that influence their occurrence in the environment (atmosphere, surface water, sediment, soil, biota), indoor air and dust, food, drinking water and the human organism. Data from *in vitro* and *in vivo* studies showing 2,4-DBP, 2,4,6-TBP and PBP toxicity, including their estrogenic activity, effects on development and reproduction, perturbations of cellular redox balance and cytotoxic action have been described. Moreover, the processes of BPs transformation that occur in human and other mammals, plants and bacteria have been discussed. Finally, the effect of abiotic factors (e.g. UV irradiation and temperature) on BPs conversion to highly toxic brominated dioxins and brominated furans as well as polybrominated biphenyls and polybrominated diphenyl ethers has been presented.

## Contents

1. Introduction . . . 2
2. Physicochemical properties, production and use . . . 3
3. Occurrence in the environment . . . 4
   3.1. Atmosphere . . . 4

* Corresponding author at: University of Lodz, Faculty of Biology and Environmental Protection, Department of Biophysics of Environmental Pollution, Pomorska 141/143 St., 90-236 Lodz, Poland.
*E-mail address:* jaromir.michalowicz@biol.uni.lodz.pl (J. Michałowicz).

http://dx.doi.org/10.1016/j.scitotenv.2021.152289

3.2. Aquatic ecosystems . . . 4
3.2.1. Surface water . . . 4
3.2.2. Sediments . . . 4
3.3. Sites contaminated with BPs . . . 4
3.4. Biota . . . 4
4. Transformation . . . 6
4.1. Impact of abiotic factors – PBDEs, PBDFs and PBDDs formation . . . 6
4.2. Degradation by bacteria . . . 6
4.3. Transformation by plants and other organisms . . . 7
5. Human exposure . . . 7
5.1. Indoor air and dust . . . 7
5.2. Food . . . 7
5.3. Drinking water . . . 8
6. Human biomonitoring . . . 8
7. Metabolism in humans and other mammals . . . 9
8. Toxicity . . . 9
8.1. Acute toxicity . . . 9
8.2. Endocrine disrupting potential . . . 9
8.3. Effect on development and reproduction . . . 9
8.4. Effect on cell signalling . . . 11
8.5. Disturbance of cellular redox balance . . . 11
8.6. Necrotic and apoptotic potential . . . 11
8.7. Effect on selected enzymes . . . 12
8.8. Impact on aquatic organisms . . . 12
9. Conclusions . . . 12
Funding . . . 13
CRediT authorship contribution statement . . . 13
Declaration of competing interest . . . 13
Acknowledgements . . . 13
References . . . 13

## 1. Introduction

Bromophenols (BPs) are aromatic pollutants commonly occurring in the environment and human surrounding due to their wide and large amount of usage. BPs are used in the synthesis of brominated flame retardants (BFRs), pigments, resorcinol, herbicides, germicides and antifungal agents and are produced during combustion of leaded petrol (Howe et al., 2005; Ezechiáš et al., 2014; Zhu et al., 2014; Yu et al., 2016; Mizukawa et al., 2017a, b; Koch and Sures, 2018). Moreover, BPs are released through transformation of tetrabromobisphenol A (TBBPA), polybrominated diphenyl ethers (PBDEs), and other BFRs through UV photolysis, thermal treatment and biological processes (Bendig and Vetter, 2013; Ortuno et al., 2014; Wang et al., 2015; Liu et al., 2017).

4-Bromophenol (4-BP) was synthetised as the first BP in the 1920s (Adams and Marvel, 1921), while first biological studies on BPs were published at the end of the 1930s (Stekol and Dash, 1939). Nowadays, several BPs have been synthetised, including 2-bromophenol (2-BP), 3-bromophenol (3-BP), 4-BP, 2,4-dibromophenol (2,4-DBP), 2,5-dibromophenol (2,5-DBP), 2,6-dibromophenol (2,6-DBP), 3,5-dibromophenol (3,5-DBP), 2,4,6-tribromophenol (2,4,6-TBP), 2,3,4,6-tetrabromophenol and pentabromophenol; however, most of them have minor environmental importance, while some of them like 3,5-DBP and 2,3,4,6-TeBP appear to exist only in the laboratory (Howe et al., 2005).

Among BPs, 2,4-DBP, 2,4,6-TBP and PBP are the most commonly used in the industry (European Food Safety Authority, 2012; Zhu et al., 2014; Yu et al., 2016). These BPs have also been most often detected in the air, aquatic and terrestrial ecosystems and the human body (European Food Safety Authority, 2012; Koch and Sures, 2018). For instance, Gao et al. (2015) assessed the presence of six isomers of TBP and three isomers of TeBP in adipose tissue collected from people from New York City, USA and found only 2,4,6-TBP in tested samples.

First studies on the occurrence of BPs in the environment and the human body were published in 1960s. Bracha and Bonard (1966) detected 2,5-dichloro-4-bromophenol (product of transformation of insecticide used for mosquito control) in soil and urine of inhabitants of the West Africa. Up to now, BPs have been detected in various compartments of the environment and different human cohorts. 2,4-DBP and 2,4,6-TBP have been determined in low concentrations of pg/$m^3$ in the air of Arctic regions and urban areas (Schlabach et al., 2011; Bidleman et al., 2019), while 2,4,6-TBP was detected in huge amount of mg/$m^3$ in exhaust gases from waste incineration plants (Öberg et al., 2002). 2,4-DBP, 2,4,6-TBP and PBP have been found in freshwater and marine water usually in the concentrations of ng/L (Xiong et al., 2016b; Chi et al., 2017); however contaminated river water, and particularly sewage water contain high amounts of these substances ranging from μg to mg/L (Nomani et al., 1996; Chi et al., 2017). High concentrations of discussed BPs have also been detected in sediments (μg - mg/kg) and soil adjacent to wastes of electrical and electronic equipment (e-wastes) (μg/kg) (Tolosa et al., 1991; Remberger et al., 2002; Han et al., 2013).

The widespread occurrence of synthetic BPs in fresh water and marine ecosystems leads to bioaccumulation of these substances in aquatic organisms. It must be noted that some naturally-produced BPs, i.e. 2,4-DBP and 2,4,6-TBP, which are formed as secondary metabolites by diverse marine organisms are also accumulated in food chain (Gribble, 2010; Haldén et al., 2010; Haraguchi et al., 2010; Dong et al., 2020). Fish that consume algae and a variety of invertebrates accumulate BPs in the concentrations range from a few to few hundreds of μg/kg. The amounts of 2,4-DBP and 2,4,6-TBP detected in marine fish have been shown to be higher than in freshwater fish (Oliveira et al., 2009; European Food Safety Authority, 2012), which may be due to much higher content of bromine in ocean water in comparison to inland waters, which is a precursor for bromophenols formation in aquatic ecosystems, including fish and invertebrates that live in them (Wang et al., 2016).

Man is exposed to BPs from various sources, while food is considered to be the main source of the exposure of human to BPs (Harrad et al., 2010). 2,4-DBP and 2,4,6-TBP have been detected in significant concentrations of up to few hundreds of μg/kg of edible clams and fish (Aznar-Alemany et al., 2017) and in much lower amounts (ng/kg) in animals fats, instant foods (Poma et al., 2018) and alcoholic beverages (Bendig et al., 2014). 2,4-DBP and 2,4,6-TBP have been determined in low concentrations of

ng/m$^3$ in indoor air (Sha et al., 2018) and in significant amounts (up to few hundreds of μg/kg) in indoor dust (Takigami et al., 2009). Some studies have also shown the presence of 2,4-DBP and 2,4,6-TBP (ng/L) in drinking water (Howe et al., 2005).

The common occurrence of BPs in the environment and human surrounding leads to accumulation of these substances in the human body. 2,4-DBP, PBP, and particularly 2,4,6-TBP have been detected in blood serum in the concentrations ranging from ng to few hundreds of μg/kg of lipids (Fujii et al., 2014), adipose tissue (Gao et al., 2015), placental tissue (Leonetti et al., 2016), breast milk (Ohta et al., 2004) and urine of environmentally exposed (a few μg/L) (Feng et al., 2016) and occupationally exposed (a few mg/L) individuals (Gutierrez et al., 2005).

Numerous *in vitro* and *in vivo* studies have shown that BPs exhibits toxic effects in animals and human. 2,4,6-TBP and PBP due to structural similarity to thyroxine (Meerts et al., 2000) are capable of binding to nuclear receptors causing disturbance in thyroid hormone metabolism and transport, which in consequence may result in changes in foetus development (Leonetti et al., 2018). 2,4-DBP and 2,4,6-TBP have also been shown to bind to estrogen and androgen receptors (*in vitro*) and change the levels of testosterone and estradiol in animals (Deng et al., 2010). The scientists have also observed that endocrine disrupting activity and other adverse effects provoked by 2,4,6-TBP and PBP, which may be associated with carcinogenic potential of these substances (e.g. PBP) (Roane et al., 2019) are related with their ability to alter some transduction pathways, including cellular calcium ion or transforming growth factor (TGF-β) signalling (Hassenklöver et al., 2006; Chen et al., 2017).

Moreover, BPs have been shown to disturb cellular redox balance both in isolated cells and entire organism. Jarosiewicz et al. (2019a) observed that 2,4-DBP, 2,4,6-TBP and PBP (50–100 μg/mL) decreased the activity of antioxidant enzymes, i.e. superoxide dismutase (SOD) and catalase (CAT) in human erythrocytes, while Włuka et al. (2020) noticed that 2,4,6-TBP and PBP (0.0001–20 μg/mL) increased reactive oxygen species (ROS) level and induced lipid and protein oxidation in human peripheral blood mononuclear cells (PBMCs).

Prooxidative properties of BPs are the most probably responsible for their cytotoxic potential, i.e. triggering of necrosis/hemolysis and apoptosis/eryptosis. Jarosiewicz et al. (2017) observed that 2,4-DBP, 2,4,6-TBP and PBP (50–100 μg/mL) caused hemolysis in human red blood cells. In another study, Jarosiewicz et al. (2019a) noticed that the above-mentioned BPs at lower concentrations range (10–50 μg/mL) induced eryptosis in human erythrocytes. Moreover, Włuka et al. (2020) showed that 2,4,6-TBP and PBP (25–100 μg/mL) exhibited necrotic potential and depleted ATP level in human PBMCs.

Except the effect of BPs on the enzymes of antioxidant system, these substances can influence other enzymes activity, which may have detrimental effects on living organisms. Trexler et al. (2019) showed that in rats 2,4,6-TBP at 0.4 μmol/L reduced transport activity of P-glycoprotein (P-gp), which is a blood-brain barrier transporter and suggested that this could have an adverse impact on elimination of xenobiotics from the brain. Wang et al. (2020) showed that 2,4,6-TBP (2.85 to 31 μM) inhibited the activity of UDP-glucuronosyltransferase isoforms that could influence faster excretion of various substances, i.e. drugs, bile acids, androgens and estrogens from the body. In another work, Ríos et al. (2003) observed that 2,4,6-TBP at low concentration of 0.1 μM increased the activity of acetylcholinesterase (AChE) in human neuroblastoma cell cultures.

BPs have also been shown to adversely influence aquatic organisms. For instance, Deng et al. (2010) observed that 2,4,6-TBP caused an increase in testosterone and estradiol levels in males and a decrease in the levels of these hormones in females of zebrafish, while Lu et al. (2018) reported that 2,4-DBP strongly increased the activity of glutathione S-transferase (GST) in goldfish (*Carassius auratus*).

BPs have been shown to be transformed by animals and plants, efficiently degraded by bacteria as well as converted under influence of abiotic factors usually to highly toxic and highly persistent substances.

Ho et al. (2015) observed that 2,4-DBP and 2,4,6-TBP were readily conjugated to glucuronides and sulphates in humans. In another study, Knudsen et al. (2019) observed that in rodents 2,4,6-TBP was readily adsorbed and rapidly excreted primarily *via* urine, in which a mixture of 2,4,6-TBP, 2,4,6-TBP-glucuronide and 2,4,6-TBP-sulphate was detected. BPs are degraded by bacteria in both aerobic and anaerobic conditions; however the presence of oxygen positively influence BPs degradation efficiency (Yadu et al., 2016). In aerobic conditions, BPs may be converted to respective hydroquinones, and then hydroxyquinoles, which are subsequently degraded to maleylacetate and beta-ketoadipate (Sanchez and Gonzalez, 2007). Similarly to chlorophenols, BPs may be converted to catechol derivatives (Golan et al., 2019), which are then efficiently degraded. In anaerobic conditions, BPs are transformed by reductive dehalogenation. For instance, Kunze et al. (2017) showed that 2,4-DBP and 2,4,6-TBP were debrominated to 4-BP and 2,4-DBP, respectively, which were finally dehalogenated to phenol.

Plants are also capable of metabolizing of BPs, although their degrading abilities are limited. Zhang et al. (2019) showed that 2,4,6-TBP was debrominated, hydroxylated, methylated and conjugated with sulphates in rice. Moreover, they observed that some persistent and toxic hydroxylated products, including hydroxylated polybrominated diphenyl ethers (OH-PBDEs) and polybrominated dibenzo(*p*)dioxins (PBDDs) were formed as a result of dimeric reactions of 2,4,6-TBP.

Abiotic transformation of BPs usually leads to the formation of products exhibiting high toxicity and high environmental persistence. Zhao et al. (2017) showed that UV phototransformation of 2,4,6-TBP led to the formation of dihydroxylated dibromobenzene, OH-PBDEs and dihydroxylated polybrominated biphenyls (di-OH-PBBs). Similarly, combustion of BPs results in the formation of various products possessing high toxicity. It was shown that polybrominated dibenzo-p-dioxins and polybrominated dibenzofurans (PBDFs) were formed during combustion processes of e-wastes containing BFRs, including 2,4,6-TBP (Dopico and Gomez, 2015).

## 2. Physicochemical properties, production and use

2,4-DBP, 2,4,6-TBP and PBP are the most commonly used BPs in the manufacture. The selected physicochemical properties of these substances are presented in Table 1.

2,4,6-TBP is the most widely synthesized brominated phenol with worldwide production volume of approximately 9500 tons per year estimated in 2001, while its production volume in Japan was estimated at 3600 tons in 2001 (Watanabe and Sakai, 2003; Okon et al., 2020). Manufacturing of 2,4,6-TBP is being gradually increased as it is intensively used in the production of BFRs. Production of 2,4,6-TBP only in United States was estimated to be 4500 to <23,000 tons in 2006, and this substance is considered a high production volume chemical (HPV) in the EU (Watanabe and Sakai, 2003; Covaci et al., 2011; Okon et al., 2020). Production volume of 2,4-DBP is not publicly available (Okon et al., 2020).

Industrial production of PBP involves the reaction of 2,4,6-TBP with anhydrous bromine with the participation of ferric bromide as a catalyst. The quantities of PBP production are not known. A total of 55 sources, among which some are for authentic reference purposes only, are identified by Scifinder (European Food Safety Authority, 2012). The primary commercial sources are located in the USA and China (European Food Safety Authority, 2012).

2,4-DBP, 2,4,6-TBP and PBP are mainly used as FRs and are also utilized as intermediates for the production of 2,4,6-TBP allyl ether (2,4,6-TBP-AE), PBP allyl ether (PBP-AE) and 2,4,6-TBP-2,3-dibromopropyl ether (2,4,6-TBP-DBPE) (European Food Safety Authority, 2012; Zhu et al., 2014). Bromophenolic based FRs are used to reduce flammability or the burning rate of various everyday products, including plastics, textiles, paints as well as furniture and electric and electronic devices (Zhu et al., 2014; Yu et al., 2016). 2,4,6-TBP is also a part of brominated epoxy oligomers, including TBBPA epoxy oligomer endcapped with 2,4,6-TBP. These oligomers are used in housings for business machinery and electrical/electronic parts based upon polycarbonate, polybutylene terephthalate-alloys, polybutylene terephthalate and thermosetting resins (Covaci et al.,

**Table 1**
Comparison of physical and chemical properties of 2,4-dibromophenol, 2,4,6-tribromophenol and pentabromophenol.

| | 2,4-Dibromophenol (2,4-DBP) | 2,4,6-Tribromophenol (2,4,6-TBP) | Pentabromophenol (PBP) |
|---|---|---|---|
| Molecular formula | $C_6H_4Br_2O$ | $C_6H_3Br_3O$ | $C_6HBr_5O$ |
| Molecular weight | 251.9 g/mol | 330.8 g/mol | 488.6 g/mol |
| Density | 2.07 g/cm$^3$ | 2.55 g/cm$^3$ | 2.89 g/cm$^3$ |
| Melting point | 38 °C–40 °C | 87 °C–97 °C | 223 °C–228 °C |
| Boiling point | 238.5 °C | 286 °C | 352.3 °C |
| Solubility | Very soluble in carbon disulfide, ethanol, ether, benzene. Slightly soluble in water (1.9 g/L at 15 °C). | Very soluble in ethanol. Soluble in chloroform, ether, benzene, carbon tetrachloride. Poorly soluble in water (70 mg/L at 15 °C) | Soluble in carbon disulfide, ethanol, benzene. Slightly soluble in ether. Almost insoluble in water (2 mg/L at 25 °C) |
| Physical properties | Solid with needle-like crystals | Soft, long, white crystals with a bromine odour | Light brown powder |

2009). 2,4,6-TBP is also utilized as a wood preservative. Nichkova et al. (2008) found very high concentration of 2,4,6-TBP (2 mg/kg) in the contaminated wood materials used in the food industry. Moreover, 2,4,6-TBP is registered as a fungicide.

PBP was synthesized at the beginning of 1950s as a molluscicide to be used in the prevention of bilharziasis (Halawani et al., 1951). Now, PBP is still used as a molluscicide as well as bactericide and chemical intermediate for synthesis of pentabromophenoxy compounds (WHO, 2005; Mizukawa et al., 2017a, 2017b).

2,4-DBP, 2,4,6-TBP and PBP are also used in the production of vinyl-aromatic polymers and as intermediates in the synthesis of epoxy-phenolic polymers (European Food Safety Authority, 2012).

## 3. Occurrence in the environment

### 3.1. Atmosphere

In general, BPs have been detected in low concentrations in the unpolluted and urban atmosphere with similar level reported for 2,4-DBP and 2,4,6-TBP and much lower for PBP. In Arctic regions BPs were detected in ambient air and deposition at Nordic stations with the concentrations not exceeding 1 pg/m$^3$ and 10 pg/m$^3$ for 2,4-DBP and 2,4,6-TBP, respectively (Bidleman et al., 2019). Low concentrations of 2,4-DBP and 2,4,6-TBP (0.3–27 pg/m$^3$) and particularly PBP (0.3–1.5 pg/m$^3$) were determined in urban areas of Norway (European Food Safety Authority, 2012). In the air of Sweden and Denmark 2,4-DBP and 2,4,6-TBP were detected at similar concentrations from 8 to 30 pg/m$^3$, while PBP was determined in the concentration of below 0.5 pg/m$^3$ (Schlabach et al., 2011). In opposite, huge concentrations of 2,4,6-TBP from 32 to 530 mg/m$^3$ were detected in the raw exhaust gas in the Swedish (Norrtorp) hazardous waste incineration plant (Öberg et al., 2002). Significant concentrations of 2,4-DPB from 18 ng to 210 ng/m$^3$ were also determined in flue gas from a hazardous waste incinerator (European Food Safety Authority, 2012) (Table 2).

### 3.2. Aquatic ecosystems

#### 3.2.1. Surface water

2,4,6-TBP is the most commonly detected BP in aquatic ecosystems, although its concentration in surface water seems to be lower in comparison to 2,4-DBP. In sediments 2,4,6-TBP has been determined in much higher concentrations than in water, while 2,4-DBP and PBP have been seldom detected. In Ganges River (India) 2,4-DBP and 2,4,6-TBP were found in significant concentrations of 40 μg/L and 0.3 μg/L, respectively (Nomani et al., 1996). Differently, in the Beijiang River water (South China) 2,4,6-TBP and PBP were determined in very low concentrations of 0.40 ng/L and 0.15 ng/L, respectively (Xiong et al., 2016b). The content of BPs in see water is comparable to their concentrations in freshwater. Remberger et al. (2002) analyzed samples collected from the Baltic Sea, where the concentration of 2,4-DBP ranged from 5 ng to 35 ng/L. In another study, 2,4-DBP and 2,4,6-TBP were detected in the mean concentrations of 103.7 ng/L and 68.4 ng/L, respectively in surface water samples derived from Bohai Sea (Dalian, China) (Chi et al., 2017). Similar concentration of 2,4,6-TBP (mean - 10 ng/L) was determined by Albaladejo et al. (2012) in Spanish Mediterranean hydrographic basins.

#### 3.2.2. Sediments

The studies have shown the occurrence of BPs, mostly 2,4,6-TBP, in freshwater sediments at varying levels. Xiong et al. (2016b) assessed the presence of 2,4,6-TBP and PBP in sediments of Beijiang River water (South China). They detected low concentrations of 2,4,6-TBP from detection limit (DL) to 410 ng/kg, while PBP was not found. In another study, much higher concentrations of 2,4,6-TBP (0.2–36 μg/kg) were determined in the sediments collected from freshwater ecosystems of Osaka Prefecture, Japan (Watanabe et al., 1985). The highest concentrations of 2,4,6-TBP ranging from 26 μg/kg to 3.69 mg/kg were detected by Tolosa et al. (1991) in the sediments of the Rhone estuary (France). Marine sediments may also contain significant concentrations of BPs. For instance, Remberger et al. (2002) detected 2,4-DBP in the concentrations range from 5 to 13 μg/kg in the sediments of the Baltic Sea. The study of Manasfi et al. (2019) showed that sediment samples collected from various locations of Gulf of Fos (France) contained 2,4,6-TBP (only 2 of 24 samples) in the concentrations of 2.1 μg/kg and 1.5 μg/kg. Similar concentrations of 2,4,6-TBP (1.6–9 μg/kg) were detected by Sim et al. (2009) in marine sediments collected from South Korea.

### 3.3. Sites contaminated with BPs

Recycling e-waste is a global problem due to toxic compounds arising during the recovery (Evangelopoulos et al., 2019). BPs are usually found in water, soil and sediments contaminated by waste recycling sites (Pivnenko et al., 2017; Kumar, 2018; Kapustka et al., 2018). For instance, in an e-waste recycling sites in Guangdong Province (China) the concentrations of 2,4,6-TBP and PBP in water ranged from < DL to 320 ng/L and from < DL to 37 ng/L, respectively, while in sediments 2,4,6-TBP and PBP were detected in significant concentrations from <DL to 47 μg/kg and from <DL to 25 μg/kg, respectively (Xiong et al., 2015). In another study, Han et al. (2013) showed that soil adjacent to e-wastes was contaminated with 2,4-DBP (0.17 to 2.10 μg/kg) and 2,4,6-TBP (0.64 to 2.64 μg/kg). Sewage usually contains high concentrations of BPs. For instance, high mean concentrations of 2,4-DBP (1.69 μg/L) and 2,4,6-TBP (7063 μg/L) were determined in discharge sewage of factory producing FRs in China (Chi et al., 2017).

### 3.4. Biota

It has been shown that marine fish and some marine invertebrates, such as clams and polychaetes accumulate the highest concentrations of BPs, and particularly 2,4,6-TBP. Some BPs, like 2,4-DBP and 2,4,6-TBP are naturally occurring because they are produced as secondary metabolites by diverse marine organisms such as algae, polychaetes, sponge and hemichordates (Goerke and Weber, 1991; Gribble, 2010). For instance, marine sponges are natural sources of brominated organic compounds, including BPs that may comprise up to 12% of the sponge dry weight. It is suggested that

**Table 2**
Occurrence of bromophenols in the environment.

| | | | |
|---|---|---|---|
| **Atmosphere** | | | |
| Arctic regions | 2,4-DBP | 1 pg/m$^3$ | Bidleman et al., 2019 |
| | 2,4,6-TBP | 10 pg/m$^3$ | |
| Urban areas (Norway) | 2,4-DBP/2,4,6-TBP/PBP | 0.3–27 pg/m$^3$ | European Food Safety Authority, 2012 |
| Sweden/Denmark | 2,4-DBP/2,4,6-TBP | 8–30 pg/m$^3$ | Schlabach et al., 2011 |
| | PBP | <0.5 pg/m$^3$ | |
| Treated exhaust gas (waste incinerator) | 2,4-DPB | 18–210 ng/m$^3$ | European Food Safety Authority, 2012 |
| Raw exhaust gas (incineration plant) | 2,4,6-TBP | 32–530 mg/m$^3$ | Öberg et al., 2002 |
| **Water and sewage** | | | |
| Ganges River (India) | 2,4-DBP | 40 μg/L | Nomani et al., 1996 |
| | 2,4,6-TBP | 0.30 μg/L | |
| Beijiang River (China) | 2,4,6-TBP | 0.40 ng/L | Xiong et al., 2016b |
| | PBP | 0.15 ng/L | |
| Bohai Sea (China) | 2,4-DBP | 103.7 ng/L | Chi et al., 2017 |
| | 2,4,6-TBP | 68.4 ng/L | |
| Baltic Sea | 2,4-DBP | 5–35 ng/L | Remberger et al., 2002 |
| Mediterranean hydrographic basins (Spain) | 2,4,6-TBP | 10 ng/L | Albaladejo et al., 2012 |
| Surface water contaminated with e-waste recycling sites (Guangdong Province, China) | 2,4,6-TBP | <DL - 320 ng/L | Xiong et al., 2015 |
| | PBP | <DL - 37 ng/L | |
| Sewage water (brominated flame retardants factory, China) | 2,4-DBP | 1.69 μg/L | Chi et al., 2017 |
| | 2,4,6-TBP | 7.06 mg/L | |
| **Sediment and soil** | | | |
| Beijing River (China) | 2,4,6-TBP | DL - 410 ng/kg | Xiong et al., 2016b |
| Osaka Prefecture (Japan) | 2,4,6-TBP | 0.2–36 μg/kg | Watanabe et al., 1985 |
| Rhone estuary (France) | 2,4,6-TBP | 26 μg/kg–3.69 mg/kg | Tolosa et al., 1991 |
| Baltic Sea | 2,4-DBP | 5–13 μg/kg | Remberger et al., 2002 |
| South Korea marine environments | 2,4,6-TBP | 1.6–9 μg/kg | Sim et al., 2009 |
| Gulf of Fos (France) | 2,4,6-TBP | 1.5–2.1 μg/kg | Manasfi et al., 2019 |
| Sediments of freshwater ecosystems contaminated with e-waste recycling sites (Guangdong Province, China) | 2,4,6-TBP | <DL - 47 μg/kg | Xiong et al., 2015 |
| | PBP | <DL - 25 μg/kg | |
| Soil adjacent to e-waste sites | 2,4-DBP | 0.17–2.1 μg/kg | Han et al., 2013 |
| | 2,4,6-TBP | 0.64–2.64 μg/kg | |
| **Algae and invertebrates** | | | |
| Macroalgae | 2,4,6-TBP | 0.5–107 μg/kg | Haraguchi et al., 2010 |
| Sponges | 2,4,6-TBP | 0.2–240 μg/kg | Boyle et al., 1992 |
| Hydroid | 2,4,6-TBP | 29 μg/kg | |
| Antarctic krill | 2,4,6-TBP | 0.057–0.40 μg/kg | Bidleman et al., 2019 |
| Clams | 2,4,6-TBP | 2.36 mg/kg | Chung et al., 2003 |
| Marine polychaetes | 2,4,6-TBP | 8.3 g/kg | Haldén et al., 2010 |
| **Fish and birds** | | | |
| Perch (freshwater fish) | 2,4,6-TBP | 0.013–3.5 μg/kg | European Food Safety Authority, 2012 |
| Lane snapper and Yellowtail snapper (marine fish) | 2,4-DBP | 7–372 μg/kg | Oliveira et al., 2009 |
| | 2,4,6-TBP | 3–171 μg/kg | |
| Atlantic cod (marine fish) | 2,4-DBP | 0.47–7 μg/kg | Lopez et al., 2018 |
| Guillemot and Black guillemot eggs | PBP | 0.12–0.40 μg/kg | European Food Safety Authority, 2012 |
| European shag and European herring gull eggs | 2,4-DBP | 2.9 μg/kg | Huber et al., 2015 |
| | 2,4,6-TBP | 0.34 μg/kg | |
| | PBP | 0.38 μg/kg | |
| Falcon eggs | 2,4,6-TBP | 1.7–15 μg/kg lipids | Vetter et al., 2017 |

2,4-DBP – 2,4-dibromophenol, 2,4,6-TBP – 2,4,6-tribromophenol, PBP – pentabromophenol, DL – detection limit.

those compounds serve as a chemical defence against predators and biofouling (Whitfield et al., 1999; Kicklighter et al., 2004). Seasonal growth of seaweed synthesizing BPs affects the total content of these compounds in fish, oyster crabs and shrimps, which varies depending on the season (Chung et al., 2003). For instance, Haldén et al. (2010) observed that 2,4,6-TBP level in the tissue of marine organisms was associated with their diet.

The concentration of 2,4,6-TBP was measured, among others in macroalgae (0.5–107 μg/kg), sponges (0.2–240 μg/kg) and hydroid (29 μg/kg) (Boyle et al., 1992; Haraguchi et al., 2010). The concentration of 2,4,6-TBP in marine invertebrates is very different. Bidleman et al. (2019) detected 2,4,6-TBP in Antarctic krill (*Euphausia superba*) in low concentrations from 57 to 398 ng/kg, while Chung et al. (2003) found high concentrations of 2,4,6-TBP (mean – 2.36 mg/kg) in clams from Hong Kong. In another study Haldén et al. (2010) found huge concentration of 2,4,6-TBP of 8.3 g/kg in marine polychaetes.

BPs have been shown to accumulate in fish. 2,4-DBP was detected in Atlantic cod (*Gadus morhua*) from Norway, Faroe Islands and Ireland in the concentrations ranging from 0.47 to 7 μg/kg (Lopez et al., 2018). High concentrations of 2,4-DBP and 2,4,6-TBP in fish tissues (stomach and muscles) have been determined in Lane snapper (*Lutjanus synagris*) and Yellowtail snapper (*Ocyurus chrysurus*). Their contents in stomach tissues and muscles were respectively 7–372 μg/kg (2,4-DBP) and 3–104 μg/kg (2,4,6-TBP). In muscle tissues, the content was respectively 7–158 μg/kg (2,4-DBP) and 6–171 μg/kg (2,4,6-TBP) (Oliveira et al., 2009). High concentrations of 2,4,6-TBP (from <limit of quantification to 299 μg/kg) were detected in snapper from Brazil (European Food Safety Authority, 2012). In European fish, 2,4,6-TBP predominates among BPs,

with low concentrations in fish meat ranging from <0.013 μg/kg in capelin, up to 3.5 μg/kg in perch (European Food Safety Authority, 2012).

It was shown that Guillemot (*Uria aalge*) eggs from the Baltic Sea contained PBP at approx. 0.4 μg/kg, while Black guillemot (*Ceppus grille*) eggs from the Faroe Islands contained PBP between 0.12 and 0.18 μg/kg (European Food Safety Authority, 2012). Eggs of three species of sea birds, i.e.: Common eider (*Somateria mollisima*), European shag (*Phalacrocorax aristotelis*), and European herring gull (*Larus argentatus*) from Norway have been tested for exposure to various chemical mixtures, including BRFs. Among the compounds tested, 2,4,6-TBP was detected in the highest mean concentration of 11.5 μg/kg in Common eider in Sklinna (Huber et al., 2015). In European shag and European herring gull eggs the concentration of 2,4,6-TBP was lower than 0.34 μg/kg (most commonly detected), while 2,4-DBP and PBP were determined in 28% (>2.9 μg/kg) and 6% samples (0.38 μg/kg), respectively (Huber et al., 2015). In Germany, 11 falcon eggs and eggs from 6 other species were tested for BFRs. 2,4,6-TBP was detected only in falcon eggs in the concentrations range from 1.7 to 15 μg/kg lipids (Vetter et al., 2017) (Table 2).

In another study, Mizukawa et al. (2017a, 2017b) determined 2,4,6-TBP and PBP in blood collected from dogs and cats in Japan. In addition, they analyzed dry and wet dog and cat food samples where significant amounts of 2,4,6-TBP were detected. This study suggested that diet is an important route of exposure of pets to BPs.

## 4. Transformation

### 4.1. Impact of abiotic factors – PBDEs, PBDFs and PBDDs formation

BPs may be formed during transformation of various aromatic compounds exposed to abiotic factors. Lin et al. (2016) reported formation of 4-BP, 2,4-DBP and 2,4,6-TBP from natural manganese oxides-catalyzed oxidation of phenol in the presence of bromine ions at ambient temperature condition. In another study, Bendig and Vetter (2013) revealed that 2,4-DBP and 2,4,6-TBP were formed as a result of UV irradiation (simulated sunlight and natural sunlight irradiation) of polybrominated diphenyl ethers (PBDEs) dissolved in methanol/water mixture that was considered to be a good compromise to stimulate reactions in the aqueous phase of the aquatic ecosystems. Similarly, Christiansson et al. (2009) observed that DecaBDE present in water and exposed to UV light was converted to various brominated products, including PBP.

Nevertheless, abiotic reactions most often lead to conversion of BPs to products exhibiting higher toxicity and environmental persistence, including PBDEs, polybrominated dibenzofurans (PBDFs) and polybrominated dibenzo-p-dioxins (PBDDs). Goto et al. (2017) reported that BPs were converted to PBDDs and PBDFs during photolysis reaction occurring below the surface of see water. In another study, Zhao et al. (2017) assessed UV phototransformation of 2,4,6-TBP in aqueous solution. They observed that dihydroxylated dibromobenzene products were mostly formed, which indicated that 2,4,6-TBP was mainly debrominated. Moreover, the products possessing higher toxicity than 2,4,6-TBP, like hydroxylated PBDEs (OH-PBDEs) and di-OH-PBBs were determined, which indicated on photodimerization reactions of 2,4,6-TBP. The process of dimerization of BPs in the environment under biotic and abiotic conditions is preceded by the formation of their respective phenoxy radicals. For instance, it has been shown that 2,4-DBP and 2,4,6-TBP are transformed under the influence of birnessite and $MnO_2$ (Lin et al., 2014a) as well as bromoperoxidase (Lin et al., 2014b) to form 2,4-dibromophenoxy and 2,4,6-tribromophenoxy radical, respectively as the intermediates of the reactions.

Unintentional processes of combustion of e-wastes containing BFRs (e.g. 2,4,6-TBP) also lead to formation of PBDDs and PBDFs (Dopico and Gomez, 2015; Sindiku et al., 2015). The formation of 75 PBDDs and 135 PBDFs congeners is possible, which depends on the position at which the bromine atoms are attached during combustion process (Bjurlid, 2018). The amount of dioxins emissions into the environment is measured using the TEQ (Toxic Equivalency) value. This value refers to parameters resulting from the multiplication of single concentration of a given PBDDs/PBDFs (PBDDs/Fs) congener by the corresponding Toxicity Equivalent Factor (TEF) for that congener (Yanxiao and Jinsong, 2018).

There are two main mechanisms of formation of PBDDs/Fs during combustion. First is associated with the formation of these substances through thermal processes such as: pyrolysis, thermal stress (combustion imbalance), debromination and hydrogenation. The main and the fastest conversion precursors in thermal processes are PBDEs. PBDFs are formed by elimination of Br or H atom in *ortho*- position, and then closing the aromatic ring. Closure of the aromatic ring in the case of PBDDs involves the insertion of an oxygen atom between the PBDEs molecules. The second mechanism, which is connected with de novo PBDDs/Fs synthesis, is complicated, long and not efficient (Yanxiao and Jinsong, 2018). In this mechanism, the reaction temperature is very important, because it determines the fate of PBDDs precursors (Na et al., 2007). The formation of PBDDs/Fs may also appear during photolysis reaction of BPs occurring below the surface of sea water (Goto et al., 2017).

PBDDs/Fs have been detected in various samples collected from groundwater and soil situated near wastewater plants and waste recycling sites, respectively (Sindiku et al., 2015; Jiang et al., 2018), sewage sludges (Jiang et al., 2018), dust (Tue et al., 2013; Wong et al., 2017) biota, including shellfish, fish, eggs of birds, whales (Bjurlid et al., 2018; Orisakwe et al., 2019) and human (Pratt et al., 2013).

### 4.2. Degradation by bacteria

Among the organisms that transform BPs, only bacteria have significant ability to degrade these substances. Those organisms due to various enzymes activities, particularly dioxygenases may cleave aromatic ring, and therefore degrade (mineralize) BPs to inorganic compounds, including water, carbon dioxide and bromine atoms (Chen et al., 2019). BPs are transformed by bacteria in both aerobic and anaerobic conditions; however the presence of oxygen may positively influence BPs degradation efficiency. For instance, microbial consortium removed 15 mg/L of 4-BP with efficiency of 98.99%, 97% and 90.5% in aerobic, anaerobic and anoxic reactor, respectively (Yadu et al., 2016). Sanchez and Gonzalez (2007) proposed a catabolic pathway of 2,4,6-TBP in aerobic environment. The pathway was initiated by the conversion of 2,4,6-TBP to 2,6-dibromo-*p*-hydroquinone (2,6-DBHQ), and then to 6-bromohydroxyquinol (6-BHQ); both steps were catalyzed by flavin adenine dinucleotide (FAD)-dependent 2,4,6-TCP monooxygenase (TCP-MO). In the next steps, 6-BHQ was degraded to 2-bromomaleylacetate (2-BMA) by 6-chlorohydroxyquinol 1,2-dioxygenase (HQDO), which was then degraded to beta-ketoadipate by NADH-dependent maleylacetate reductase (MAR). Similarly to chlorophenols, BPs may also be converted to catechol derivatives. For instance, Golan et al. (2019) showed that an aerobic strain *Ochrobactrum* sp. HI1 isolated from soils in the vicinity of a bromophenol (BP) production plant converted BP to bromocatechol and benzenetriol, which were then efficiently degraded.

In anaerobic environment BPs are degraded during the process of reductive dehalogenation. Organohalide-respiring microbe *Sulfurospirillum multivorans* was capable of debrominating monobromophenols, 2,4-DBP and 2,4,6-TBP to phenol, 4-BP and 2,4-DBP, respectively, which were finally dehalogenated to phenol. The bacteria used cobamide-containing reductive dehalogenase, which served as terminal reductase in this process (Kunze et al., 2017). In another study, anaerobic mineralization of 2,4,6-TBP to $CO_2$ and $H_2O$ occurred as a result of degrading activity of anaerobe community consisting of *Dehalobacter* sp., *Clostridium* sp., and *Desulfatiglans* sp. strains (Li et al., 2015). An interesting results were obtained by Ahn et al. (2003) who showed that marine sponge *Aplysina aerophoba* debrominated 2,4,6-TBP and 2,6-DBP to 2-BP. As the presence of homologous reductive dehalogenase gene motifs in the sponge-associated microorganisms was found, it was suggested that reductive dehalogenation might have been coupled to dehalorespiring bacteria inhabited this invertebrate species.

Some microorganisms are potent in degradation of BPs, and therefore may be utilized in the removal of these substances from polluted

environment. Sahoo (2020) observed a complete biodegradation of BP (200 mg/L) by an actinomycetes strain - *Arthrobacter chlorophenolicus* A6. Another bacteria *Bacillus* sp. GZT isolated from river sludge in e-waste dismantling area had also remarkable degradation ability of 2,4,6-TBP (Xiong et al., 2016a). Lang et al. (2019) expressed and functionally characterized the genes and enzymes responsible for 2,4,6-TBP degradation by *Bacillus* sp. GZT and observed that cytochrome P450 reductase encoded by *tbpA* gene significantly increased the efficiency of removal of TBP (98.8%) as compared to wild-type strain GZT (93.2%). During 2,4,6-TBP debromination, the intermediates, like 2,4-DBP and 2-BP were formed that were further significantly transformed to phenol by halophenol dehalogenases. Surprising results were obtained by Bernstein et al. (2013) in an experiment using microbial consortium enriched from a contaminated site of the northern Negev (Israel). They observed that the increase in the number of bromine atoms in BPs was positively correlated with their biodegradation efficacy as after 3 days of incubation, the concentration of 2,4,6-TBP, 2,4-DBP and 4-BP decreased to an average of 4, 16, and 88% of the initial concentrations, respectively. The authors also observed that 2,4,6-TBP was converted to phenol, while 2,4-DBP and 4-BP were determined as the intermediates of the reaction.

### 4.3. Transformation by plants and other organisms

Plants are capable of metabolizing of BPs, although their degrading abilities are limited. Zhang et al. (2019) observed that 99.2% of 2,4,6-TBP (4 mg/L) was metabolized by rice. Many metabolites were detected in rice root, while ten of them were translocated and determined in rice stems or leaves. Based on the kind of metabolites found, the authors suggested that 2,4,6-TBP was debrominated, hydroxylated and methylated. Moreover, the processes of coupling and conjugation, i.e. sulfation and glycosylation of 2,4,6-TBP were noted. Which is disturbing, seven persistent and toxic hydroxylated products, including OH-PBDEs and PBDDs were detected, which indicated that biotic dimeric reactions of 2,4,6-TBP occurred in the rice plants. In another study, it was shown that carrot cell cultures hydroxylated 2,4-DBP to dihydroxy bromophenol derivative (Sun et al., 2018). Transformation of 2,4-DBP and 2,4,6-TBP to OH-PBDEs was also reported at presence of laccase produced by fungus *Trametes versicolor* (Lin et al., 2015). The production of OH-PBDEs was associated with the coupling of bromophenoxy radicals, generated from the laccase-catalyzed oxidation of both tested BRs. In another study, efficient (65–85%) transformation of BPs, including 2,4-DBP and 2,4,6-TBP by laccase from *T. versicolor* was noted (Uhnakova et al., 2009).

The formation of BPs may also occur in the environment as a result of the activity of various enzymes synthesized in fungi and algae. An example is chloroperoxidase being a hybrid of two different families of oxidative enzymes (peroxidases and P450s) that is capable of transforming phenol at presence of bromide to 2,4-DBP and 2,4,6-TBP (Wanga et al., 2019). Another enzyme is bromoperoxidase produced by macroalgae that is able to produce 2,4-DBP and 2,4,6-TBP from various substrates, such as phenol, 4-hydoxybenzoic acid and 4-hydroxybenzyl alcohol (Bidleman et al., 2019).

## 5. Human exposure

### 5.1. Indoor air and dust

By burning of contaminated wastes, fuel and peat, people are strongly exposed to BPs. The highest amounts of 2,4,6-TBP emitted from car exhausts and incinerated wastes were of 4.5 μg/m$^3$ and 0.38 μg/m$^3$, respectively, while in the case of peat combustion, 2,4,6-TBP emission was estimated for 0.29 μg/m$^3$ (WHO, 2005). The content of 2,4,6-TBP in the indoor air was found to be low and ranged from <2.0 to 6.8 ng/m$^3$ for residential rooms and from <2.0 to 2.8 ng/m$^3$ for office rooms, while the concentrations of PBP in offices was from <1.1 to 1.7 ng/m$^3$ (Japan) (Saito et al., 2007). More recent study by Sha et al. (2018) conducted at three Ultuna campus buildings from Sweden showed the presence of 2,4,6-TBP at mean concentrations of 0.31 ng/m$^3$, 0.23 ng/m$^3$ and 0.12 ng/m$^3$ in office rooms, dining rooms and homes, respectively.

BPs have been found in significant concentrations in indoor dust. Takigami et al. (2009) determined 2,4,6-TBP in the concentrations range from 16 to 130 μg/kg in house dust and from 27 to 620 μg/kg in office dust of buildings in Japan (Table 3).

### 5.2. Food

Ingestion is the major route of human exposure to BPs (Harrad et al., 2010). The presence of BPs has been shown in fauna and flora being a part of the human diet. For instance, high content of 2,4,6-TBP (7.8–97 μg/kg) was detected in Endeavor prawns (*Metapenaeus endeavouri*) originating from Australia (Whitfield et al., 1992). In the years 1993–1996 (east coast of Australia), 30 samples of nine shrimps species were collected,

**Table 3**
Occurrence of bromophenols in indoor air, dust, food and drinking water.

| | | | |
|---|---|---|---|
| Air and dust | | | |
| Indoor air (home, office), Sweden | 2,4,6-TBP | 0.12–0.31 ng/m$^3$ | Sha et al., 2018 |
| Indoor air | 2,4,6-TBP | < 2.0–6.8 ng/m$^3$ | Saito et al., 2007 |
| | PBP | <1.1–1.7 ng/m$^3$ | |
| Car exhausts | 2,4,6-TBP | 4.5 μg/m$^3$ | WHO, 2005 |
| Incinerated wastes exhaust | 2,4,6-TBP | 0.38 μg/m$^3$ | |
| Dust (home, office), Japan | 2,4,6-TBP | 16–620 μg/kg | Takigami et al., 2009 |
| Food | | | |
| Fish | 2,4-DBP | <0.05–158 μg/kg | Mota da Silva et al., 2005 |
| | 2,4,6-TBP | <0.05–299 μg/kg | Fuller et al., 2008 |
| Clams (European markets) | 2,4,6-TBP | 23.6–324 μg/kg lipid | Aznar-Alemany et al., 2017 |
| Biscuits | 2,4,6-TBP | 27 ng/kg | Poma et al., 2018 |
| Paprika chips | | 156 ng/kg | |
| Animal and vegetable fat | | 318 ng/kg | |
| Wine | 2,4,6-TBP | 392.6 ng/L | Chatonnet et al., 2004 |
| Scotch whiskey | 2,4-DBP | 31–44 ng/L | |
| | 2,4,6-TBP | 24–112 ng/L | Bendig et al., 2014 |
| Drinking water | | | |
| Canada | 2,4-DBP | 0.6–1.2 ng/L | Sithole et al., 1986 |
| | 2,4,6-TBP | 0.2–20 ng/L | Howe et al., 2005 |

2,4-DBP – 2,4-dibromophenol, 2,4,6-TBP – 2,4,6-tribromophenol, PBP – pentabromophenol.

in which 2,4,6-TBP was determined in the concentrations range from 0.01 to 170 μg/kg. Analysis of different seafood species available on European markets has shown the presence of 2,4,6-TBP in all clam samples in the concentrations ranging from 23.6 to 324 μg/kg lipids (Aznar-Alemany et al., 2017). Fuller et al. (2008) analyzed the presence of 2,4-DBP and 2,4,6-TBP in edible fish Barramundi (Australia) where the content of these substances was from <0.05 to 0.14 μg/kg and from <0.05 to 0.16 μg/kg, respectively. Another study showed that Lane snapper (*Lutjanus synagris*) and yellowtail snapper (*Ocyurus chrysurus*) (prized for their tasty meat) caught in Atlantic contained significant concentrations of 2,4-DBP (from 6.6 to 158 μg/kg) and 2,4,6-TBP (from 15.2 to 299 μg/kg) (Mota da Silva et al., 2005). For other examples, see section: '*Biota*'.

EFSA has delivered an opinion contributing to protecting consumers against the threat of 2,4,6-TBP. According to EFSA, daily exposure to 2,4,6-TBP through diet is 39 ng/kg body weight for consumers of fish, molluscs and crustaceans.

Poma et al. (2018) analyzed the content of BFRs in Belgian food. The most frequently detected compound was 2,4,6-TBP, which was present in 63% of tested samples (baby biscuits: 27 ng/kg, paprika chips 156 ng/kg, animal and vegetable fat up to 318 ng/kg). 2,4,6-TBP and 2,4-DBP have been shown most commonly detected (among all BPs) in fish products and dairy products in Belgian foodstuffs, while these substances have not been determined in cereal products, potatoes as well as animal and vegetable fats (Malysheva et al., 2017).

Alcoholic beverages, including wine are also the potential sources of human exposure to BPs, and particularly 2,4,6-TBP because they are produced in wooden barrels impregnated with this compound. The concentration of 2,4,6-TBP determined in wine by Chatonnet et al. (2004) was up to 392.6 ng/L. Bendig et al. (2014) studied Scotch whiskey produced on the island of Islay, which has an iodine-like taste associated with the marine environment. The scientists proved that in two types of whiskey: Lagavulin and Laphroagin there were 2,4-DBP (44 ng/L and 31 ng/L, respectively) and 2,4,6-TBP (112 ng/L and 24 ng/L, respectively) (Table 3).

### 5.3. Drinking water

BPs are formed in treated water during reactions between dissolved organic matter or xenobiotics (e.g. phenolic ions) and bromide ions at presence of disinfectants (chlorine, ozone). 2,4,6-TBP is one of the main products formed as a result of disinfection of aquaculture seawater *via* chlorination (Wang et al., 2018). Moreover, low concentrations of 2,4-DBP and 2,4,6-TBP have been determined in drinking chlorinated waters derived from surface freshwaters or sewages containing significant level of bromide ions (Watanabe et al., 1984; Sharma et al., 2014). BPs formation in water was also observed during oxidation of phenol with manganese dioxides (Lin et al., 2016).

Drinking water can be a source of human exposure to BPs. In Canada, 2,4,6-TBP was detected in the concentrations up to 20 ng/L in tap water (Howe et al., 2005). In another study, Sithole et al. (1986) analyzed samples of raw water and treated water from 40 drinking water treatment plants from 39 cities in Canada. The average concentrations of detected BPs in treated water were 0.6–1.2 ng/L for 2.4-DBP and 0.2–0.6 ng/L for 2,4,6-TBP (Table 3).

## 6. Human biomonitoring

2,4,6-TBP was determined in the concentrations range from 0.077 to 81 μg/kg lipids in serum of people from Norway (Thomsen et al., 2001). In another study, Thomsen et al. (2002a) analyzed serum samples collected from 8 groups of males of 40–50 year-old (Norway) and found 2,4,6-TBP in the concentrations from 0.08 to 26 μg/kg lipids, while PBP was not determined. Fujii et al. (2014) analyzed Japanese serum samples in 1989–2010 and detected much lower 2,4,6-TBP concentrations ranged from 46 to 960 ng/kg lipids. Comparable concentration of 2,4,6-TBP (mean 360 ng/kg lipids) was detected in serum samples of people living near the coast of India (Eguchi et al., 2012). More recently, Dufour et al. (2017) determined 2,4,6-TBP in the concentrations from trace to 1.28 μg/L of blood of the general population of Belgium. 2,4,6-TBP was detected by Smeds and Saukko (2003) in adipose tissue (2.16–53.8 μg/kg lipids) from people in Finland, while Gao et al. (2015) found this compound in the concentrations up to 54.3 μg/kg lipids of adipose tissue in people from New York City, USA. 2,4,6-TBP was also found in placental tissue taken from 95 women who delivered term (>37 weeks) infants in Durham (USA). The geometric concentration of this compound was 15.4 μg/kg lipids (range 1.31–316 μg/kg lipids) and was higher in male infants than in female infants (Leonetti et al., 2016). The authors suggested that it is important to continue research to determine whether this relationship has an adverse effect on health in infants and children. 2,4,6-TBP at mean concentration of 33 ng/kg was detected in the umbilical cord of Japanese mothers (Kawashiro et al., 2008). Although the level of 2,4,6-TBP was low, it could cross the placental barrier and affected the foetus. In another study, Thomsen et al. (2002b) determined 2,4,6-TBP in a mean concentration of 0.6 μg/kg lipids in human breast milk samples (Norway). Similarly, Ohta et al. (2004) analyzed breast milk samples collected from 4 female primipare and 5 female multiparae (Japan) and detected 2,4,6-TBP in the concentrations range from 0.81 to 3.90 μg/kg lipids.

Feng et al. (2016) determined 2,4,6-TBP at the concentration of 5.57 ± 4.05 μg/L in the urine of the general population of China. In people from Hong Kong (China) high urinary concentrations of sulphates, and particularly glucuronides of 2,4-DBP and 2,4,6-TBP (0.08–106.49 μg/g creatinine) were found (Ho et al., 2015). 2,4-DBP and 2,4,6-TBP were also detected in human urine samples in workers occupationally exposed to PBDEs and other organohalogenated substances (Feng et al., 2016). Workers of sawmills where 2,4,6-TBP is used in order to protect lumber against microorganisms, have been shown to be heavily exposed to this substance. For instance, 2,4,6-TBP was determined in the urine of Chilean sawmills workers in a mean concentration of 6.9 mg/g creatinine (approx. 6.9 mg per 1 L of urine) (Gutierrez et al., 2005) (Table 4).

**Table 4**
Occurrence of bromophenols in the human body.

| | | | |
|---|---|---|---|
| **Blood** | | | |
| Norway | 2,4,6-TBP | 0.077–81 μg/kg lipids | Thomsen et al., 2001 |
| India | 2,4,6-TBP | 0.360 μg/kg lipids (mean) | Eguchi et al., 2012 |
| Japan | 2,4,6-TBP | 0.046–0.96 μg/kg lipids | Fujii et al., 2014 |
| Belgium | 2,4,6-TBP | Trace - 1.28 μg/L serum | Dufour et al., 2017 |
| **Adipose tissue** | | | |
| Finland | 2,4,6-TBP | 2.16–53.8 μg/kg lipids | Smeds and Saukko, 2003 |
| USA (New York) | 2,4,6-TBP | trace - 54.3 μg/kg lipids | Gao et al., 2015 |
| **Placental tissue** | | | |
| USA | 2,4,6-TBP | 1.31–316 μg/kg lipids | Leonetti et al., 2016 |
| **Breast milk** | | | |
| Norway | 2,4,6-TBP | 0.6 μg/kg lipids (mean) | Thomsen et al., 2002b |
| Japan | 2,4,6-TBP | 0.81–3.9 μg/kg lipids | Ohta et al., 2004 |
| **Urine** | | | |
| China (general population) | 2,4,6-TBP | 5.57 ± 4.05 μg/L | Feng et al., 2016 |
| Hong Kong (China) (general population) | 2,4-DBP-glucuronide/sulphate 2,4,6-TBP-glucuronide/sulphate | 0.08–106.49 μg/g creatinine (≈L) | Ho et al., 2015 |
| Chile (occupational cohort, sawmill workers) | 2,4,6-TBP | 6.9 mg/g creatinine (≈L) (mean) | Gutierrez et al., 2005 |

2,4-DBP – 2,4-dibromophenol, 2,4,6-TBP – 2,4,6-tribromophenol.

## 7. Metabolism in humans and other mammals

In humans BPs are readily conjugated to glucuronides and sulphates. Ho et al. (2015) found glucuronides and sulphates of 2,4-DBP and 2,4,6-TBP in the urine of people from Hong Kong, China. They observed that 2,4,6-TBP-glucuronide level was higher in comparison to 2,4,6-TBP-sulphate level.

In female rats 2,4,6-TBP was readily adsorbed independently on route (oral, dermal) tested. It was rapidly excreted primarily *via* urine with approximately 61% of the dose recovered after 4 h, and 89%–94% after 24 h; 5% was recovered in faces and 1%–2% in blood/solid tissues. Urine contained a mixture of 2,4,6-TBP, 2,4,6-TBP-glucuronide, and 2,4,6-TBP-sulphate. Faecal extracts contained only parent 2,4,6-TBP, whereas bile contained only 2,4,6-TBP-glucuronide. 2,4,6-TBP did not appear to bioaccumulate or alter its own metabolism after repeated administration (Knudsen et al., 2019).

It has been shown that BPs can be formed in mammals as a result of biotransformation

of other brominated contaminants, like bromobenzenes and PBDEs in the reactions catalyzed by cytochrome P450 (CYP). For instance, 2,4-DBP and TBP were detected as metabolites in the human liver tissue after *in vitro* exposure to pentabromodiphenyl ether (BDE-99), while the major role of CYP2B6 was shown in this process (Erratico et al., 2012). In another study dog and cat liver microsomes were incubated with PBDEs mixtures (BDE-47, BDE-99 and BDE-209). As a result, TBP was found in dog liver microsomes but not in cat liver microsomes, implying species-specific metabolic capacities for PBDEs (Mizukawa et al., 2017a, 2017b).

## 8. Toxicity

### 8.1. Acute toxicity

Acute toxicity of BPs towards rodents increases along with increasing number of bromines in phenol molecule. $LD_{50}$ for 2,4-DBP given to guinea pig was evaluated for 3000 mg/kg b.w. The value of $LD_{50}$ for 2,4,6-TBP given orally to rats (both sexes) was estimated for 1500 mg/kg b.w, while for PBP (given orally) ranged from 250 to 300 mg/kg b.w. Acute oral exposure of rats to 2,4,6-TBP included convulsion, hypoactivity, lacrimation, prostration, decreased motor activity and nasal discharge (Fujishima and Fujiwara, 1999). $LC_{50}$ in rats exposed to 2,4,6-TBP in the concentrations >50,000 mg/$m^3$ by inhalation caused reduced motor activity, shortness of breath, erythema and diarrhoea, but no deaths or weight gains were found (International Research Development Corporation, 1974). Tanaka et al. (1999) conducted a study during which they administered 2,4,6-TBP orally at doses of 0, 100, 300 or 1000 mg/kg b.w. The study group consisted of 12 male and 12 female Sprague-Dawley rats. The administration period was 48 days for males and 41–48 days for females. A dose of 100 mg/kg b.w. did not cause any side effects in any of the genders. A dose of 300 mg/kg b.w. resulted in drooling in both sexes and in males of creatinine level, while a dose of 1000 mg/kg b.w. caused drooling in both sexes, as well as reduced appetite, increased renal and liver mass. Oral PBP poisoning in rats was characterized by general body tremors, increased respiratory and amplitude, sporadic convulsions and death (Clayton and Clayton, 1993). Moreover, Anon (1992) observed pulmonary haemorrhage and hepatic vein thrombosis during necropsy of rats exposed to PBP orally.

### 8.2. Endocrine disrupting potential

BPs have the ability to interrupt thyroid function because of their structural similarity to thyroid hormones. 2,4,6-TBP can also disrupt thyroid hormone homeostasis and influence thyroid actions as it is able to affect regulation of gene expression.

Biological effects of thyroid hormones, like triiodothyronine (T3) and thyroxine (T4) are mediated through interactions with thyroid hormone receptors (TRs), including TRs, TRα, and TRβ (Saponaro et al., 2020). TRs are members of the nuclear receptor superfamily of ligand-activated transcription factors, and serve as a link between thyroid hormones and target gene expression (Aranda et al., 2013).

Because BPs have some structural similarity to T4, Meerts et al. (2000) suggested that they may interfere with thyroid hormone metabolism and transport, e.g., by competition with T4 on transthyretin (TTR). That is why, they investigated the possible interaction of several BPs with T4 binding to TTR in an *in vitro* competitive binding assay, using human TTR and 125 I-T4 as the displaceable radioligand. They showed very potent competition binding for PBP (7.1-fold stronger than the natural ligand T4). These results indicated that some BPs are very potent competitors for T4 binding to human transthyretin *in vitro*. High binding affinity of PBP for the thyroid hormones transporter TTR has been recently confirmed by Collet et al. (2020). Similarly, Kollitz et al. (2018) showed the affinity of 2,4,6-TBP and PBP for human and zebrafish thyroid receptor beta (TRβ). They observed that the degree of binding affinity was dependent on the number of bromines of studied compound. In another study, 2,4-DBP, 2,4,6-TBP and PBP inhibited binding of triiodothyronine (T3) to the thyroid hormone receptor in human breast cancer cell line (MCF7) (Kitamura et al., 2008). Sulfation is an important pathway of thyroid hormone metabolism that facilitates the degradation of the hormone by deiodinase 1 (Dio1). The scientists used the BeWo cell line (line from human chorionic cancer having similar structure to human uterine trophoblasts) to determine the effect of 2,4,6-TBP on thyroid sulfotransferase (TH SULT) activity. The experiment showed that 2,4,6-TBP strongly inhibited TH SULT activity, which had a main effect on the intracellular decrease of thyroid hormones levels. TH SULT activity has been recognized as an important element of the thyroid and placental metabolic pathway and its inhibition can cause changes in the thyroid gland and the development of foetus (Leonetti et al., 2018). In *in vivo* study, Lee et al. (2016) observed depleted thyroid activity and decreased levels of free T3 and T4 in serum of mice exposed to 2,4,6-TBP. Moreover, Dio1 and thyroid hormone receptor β isoform 2 (Thrβ2) levels decreased, while deiodinase 2 (Dio2), growth hormone (Gh) mRNA and the relative mRNA level of thyroid stimulating hormone β (Tshβ) increased in the pituitary gland of the animals studied. Based on 2,4,6-TBP affinity for TTR, the authors also showed that 2,4,6-TBP could compete with endogenous thyroid hormone (Fig. 1).

Leonetti et al. (2016) investigated associations between placental BPs (including 2,4,6-TBP) concentration and TH level as well as Dio3 and TH SULT activities in placenta tissue samples collected from 95 women who delivered term (>37 weeks) infants. 2,4,6-TBP concentration was positively associated with T3 level in female infants. Moreover, Dio3 activity was significantly higher in placental tissues from male infants compared to females, while TH SULT was significantly higher in placental tissues from females compared to males. Obtained results showed that BPs can accumulate in placenta and may affect TH function in a sex-specific manner.

BPs have also been shown to bind to estrogen receptors and probably alter sex hormones activities. Olsen et al. (2002) assessed 2,4-DBP and 2,4,6-TBP for estrogen-like effects on the MCF-7 cell line (human breast cancer cell line). Tested compounds exhibited the ability to bind to the estrogen receptor, but they did not stimulate cell growth nor increased the regulatory levels of estrogen proteins and progesterone receptor. In another study, Ezechiáš et al. (2012) used yeast reporter-gene assays and showed that 2,4,6-TBP reduced transcriptional activity of human estrogen and androgen receptors. The authors suggested that 2,4,6-TBP was able to bind to the active site of receptors, and thus inhibited receptor activity.

The scientists have suggested that changes in $Ca^{2+}$ homeostasis may be involved in endocrine disrupting effect of BPs. Hassenklöver et al. (2006) reported that di- and tribromophenols disturbed cellular $Ca^{2+}$ signalling in the endocrine cells (PC 12) depending on the number and the position of bromine, while 2,4,6-TBP, and particularly 2,4-DBP exhibited the strongest effect.

### 8.3. Effect on development and reproduction

BPs exhibit endocrine disrupting effects, which may be potentially associated with adverse alterations in reproduction and development of animals

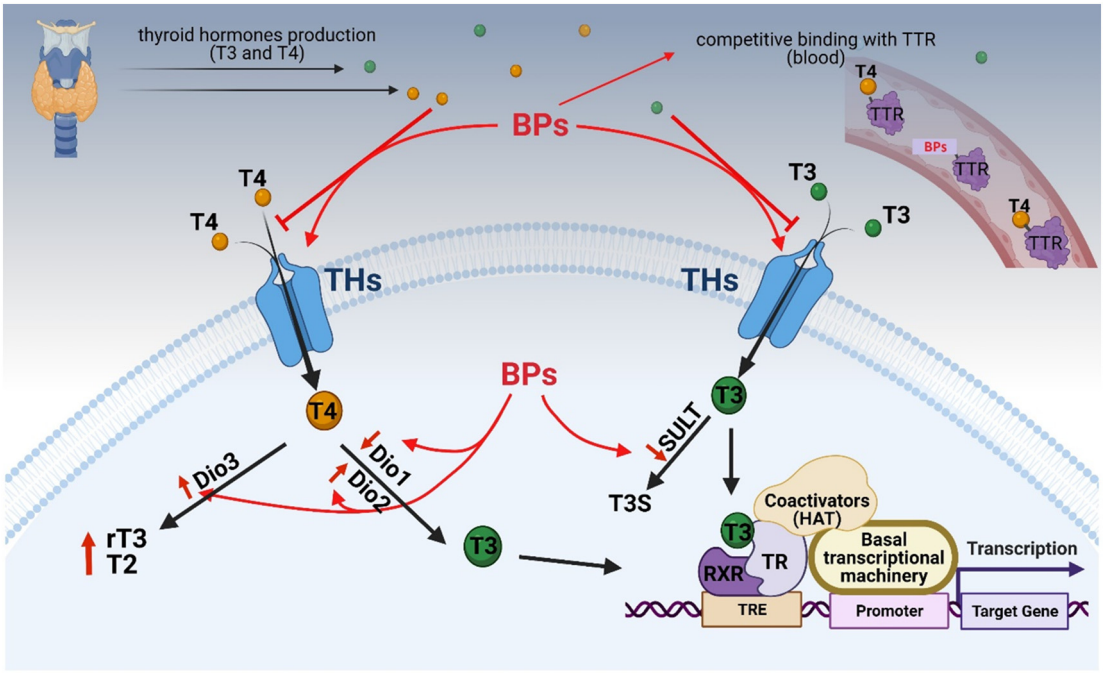



**Fig. 1.** Potential effects of BPs (Bowen et al., 2020) on distribution and conversion of thyroid hormones (FT3, FT4) and activation of gene expression in animals. General model for thyroid hormone triiodothyronine (T3) formation and action in nucleus: thyroxine (T4), the prevalent form of thyroid hormone (TH) produced by the thyroid gland is converted to T3 by deiodinases 1 and 2 (Dio1, Dio2), while Dio3 converts T4 to inactive form rT3, additionally T3 is blocked by sulfotransferase, thyroid hormone receptors (TRs) act as transcriptional factors regulating a wide variety of genes (Kudo et al., 2006; Kitamura et al., 2008; Lee et al., 2016; Leonetti et al., 2016; Collet et al., 2020; Saponaro et al., 2020). BPs block transporters of thyroid hormones, disturb Dio1 and Dio2 activities and decrease sulfotransferase activity. BPs have also the ability to bind to human transthyretin (TTR) in plasma, thus they block the reaction (binding) of T4 with this transporter. Created with BioRender.com.

and human. Aromatase (CYP19) is the enzyme that mediates the conversion of androgens to estrogens through a bioconversion process known as aromatization. In humans, the activity of this enzyme is expressed in various tissues as well as breast, testes, ovaries, placenta, foetal adrenals and brain (Pezzi et al., 2003), while CYP19 is involved in development, reproduction, behaviour, and estrogen-dependent carcinogenesis (Simpson et al., 2002).

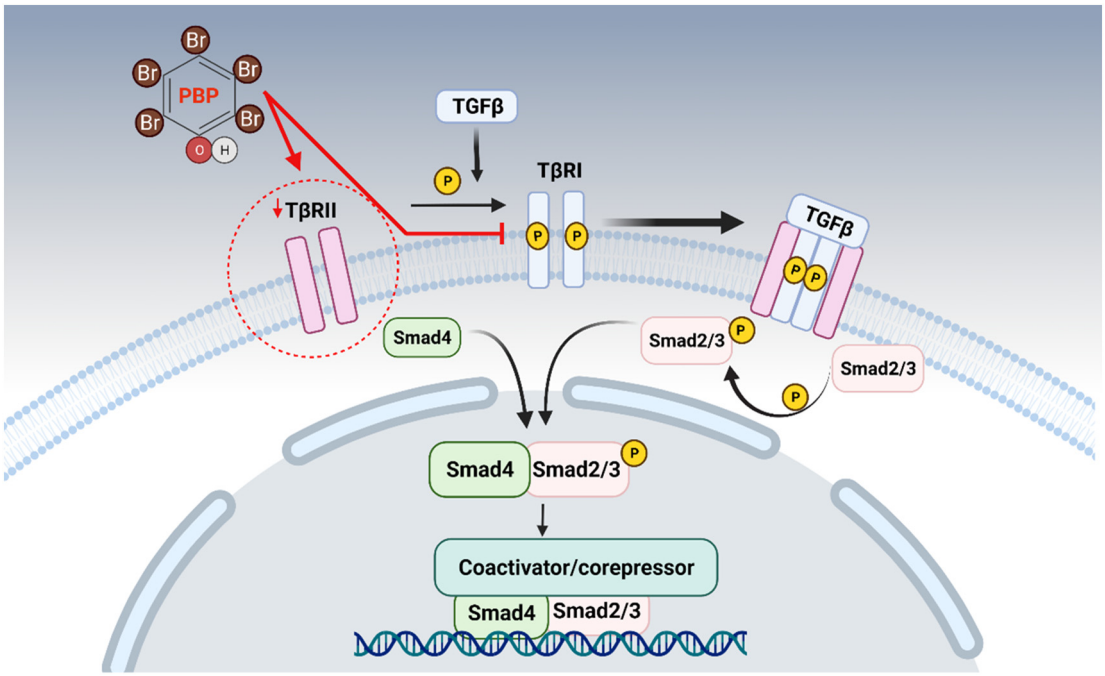



**Fig. 2.** PBP inhibits transforming growth factor beta (TGF-β) signalling by increasing the clearance rate of receptor protein serine/threonine kinase (TβRII) from the cell surface, and by accelerating its turnover. It promotes caveolae-mediated endocytosis of cell surface TβRII, resulting in the degradation of TβRII and subsequent termination of the signalling of TGF-β. PBP also inhibits all TGF-β responses, including Smad2/3 and Smad4 phosphorylation, serine protease inhibitor (PAI-1) promoter activation, epithelial-mesenchymal transition (EMT), and cell migration, based on results obtained by Chen et al. (2017). Created with BioRender.com.

It was shown that 2,4,6-TBP at high level (maximum of 3.8-fold induction at 7.5 mM) caused a concentration-dependent induction of aromatase activity in the human adrenal cell line (H295R) (Cantón et al., 2005). Similarly, Lyubimov et al. (1998) noticed that 2,4,6-TBP given by inhalation (0.03–1 mg/m$^3$) to pregnant Wistar rats caused changes in the preimplantation and post-implantation period of the embryos and altered foetal body weight. Recently, in the study of Sheller-Miller et al. (2020) placental explants were treated with 2,4,6-TBP. In the next step, exosomes were isolated and their quantity, size and proteomics were analyzed. The results did not show changes in the quantity or characteristics of the exosomes; however, the charge of the exosomes has been altered, which could contribute to placental nuclear damage and cellular injury.

### 8.4. Effect on cell signalling

As mentioned above (section: '*Endocrine disrupting potential*'), BPs by altering calcium ion signalling pathway may be capable of disrupting endocrine system activity. PBP inhibited transforming growth factor (TGF-β) signalling by increasing the rate of TGF-β receptor degradation in endocytosis with the participation of caveolae. PBP negatively regulated signalling of this cytokine by enhancing TGFβ receptor type-2 (TβRII) degradation. The authors of the study concluded that PBP acted by stimulating clearance of TβRII from the cell surface through caveolae-mediated endocytosis and subsequent proteasomal degradation (Chen et al., 2017).

TGF-β has been recognized as a factor participating in growth and tissue homeostasis. It is a protein involved in the processes of proliferation and apoptosis. Disruption of the TGF-β sequence may initiate the process of carcinogenesis (Chen et al., 2017). TGF-β suppresses function of various cells of the immune system, including CD8(+) and CD4(+) T cells, natural killer (NK) cells, dendritic cells, macrophages, and neutrophils. In addition, TGF-β induces action of regulatory T (Treg) cells and chemotaxis as well as regulates immune checkpoint proteins, such as programmed cell death protein (PD-1) (Kaboli et al., 2019). Hypersignalling *via* the TGF-β pathway is connected with increased tumour dissemination through various processes, including immune evasion, promotion of angiogenesis, and increased epithelial to mesenchymal transformation (Roane et al., 2019). Interestingly, TβRII occurs to be downregulated only in several human cancers, such as renal carcinomas and this reduction is supposed to be linked to increased proteasomal degradation (Willems et al., 2012; Meng et al., 2011). In various cell types, PBP has been shown to suppress TGF-β response by accelerating the turnover rate of TGF-β receptors. PBP was also capable of suppressing Smad2/3 and Smad4 phosphorylation and plasminogen activator inhibitor-1 (PAI-1) promoter activation, which dysregulated signalling pathway may be involved in cardiovascular disease development (Yildiz et al., 2014).

It has also been shown that PBP (but not 2,4,6-TBP) abolished TGF-β-mediated repression of E-cadherin expression, in addition to the induction of vimentin expression and N-cadherin and fibronectin upregulation, thus blocking TGF-β-induced epithelial-mesenchymal transition (EMT) signalling in lung adenocarcinoma cell line (A549) and nontransformed mouse mammary gland epithelial cell line (NMuMG) (Chen et al., 2017) (Fig. 2).

### 8.5. Disturbance of cellular redox balance

Jarosiewicz et al. (2019a; 2017) analyzed oxidative changes in human erythrocytes exposed to 2,4-DBP, 2,4,6-TBP and PBP. The results showed that 2,4-DBP and PBP from low concentration of 0.001 μg/mL and 2,4,6-TBP from 0.1 μg/mL caused an increase in total ROS level. In addition, all studied compounds at low concentrations caused oxidation of haemoglobin. The obtained results indicated high oxidative potential of BPs in human red blood cells. BPs also disturbed redox balance in human erythrocytes as described by Jarosiewicz et al. (2019a). Tested compounds decreased the activity of antioxidant enzymes, i.e. SOD and CAT (Fig. 3). Among BPs studied, 2,4-DBP most strongly changed redox balance. Similar results were achieved by Włuka et al. (2020) who assessed the effect of 2,4,6-TBP and PBP on oxidative stress parameters in human peripheral blood mononuclear cells (PBMCs). They observed that studied compounds at low concentrations range from 0.001 to 10 μg/mL induced total ROS level, and hydroxyl radical formation (only PBP), which led to oxidative damage to lipids and proteins in this cell type. In another study, Tang et al. (2018) noticed that 2,4-DBP caused ROS production, decreased the level of reduced glutathione (GSH), changed SOD activity and induced DNA damage in human hepatocyte carcinoma cell line (HepG2).

In *in vivo* study, Szymańska et al. (1995) showed that PBP caused a decrease of GSH level, increased the activity of γ-glutamyltransferase and caused lipid peroxidation in the liver of rats. They also observed a decrease in GSH level in kidneys, as well as observed an increase in protein content and the number of renal epithelial cells in the urine, which indicated on nephrotoxic effect of PBP in tested animals.

### 8.6. Necrotic and apoptotic potential

BPs exhibit proapoptotic activity, the most probably, due to their prooxidative properties. Ríos et al. (2003) treated retinoic acid differentiated and non-differentiated human neuroblastoma cell cultures (SH-SY5Y) with 2,4,6-TBP. The results showed that 2,4,6-TBP at significant concentrations (higher than 0.1 μmol/L) caused pyknosis and apoptosis of the tested cells. Recent study showed that PBP changed mitochondrial metabolism in BV-2

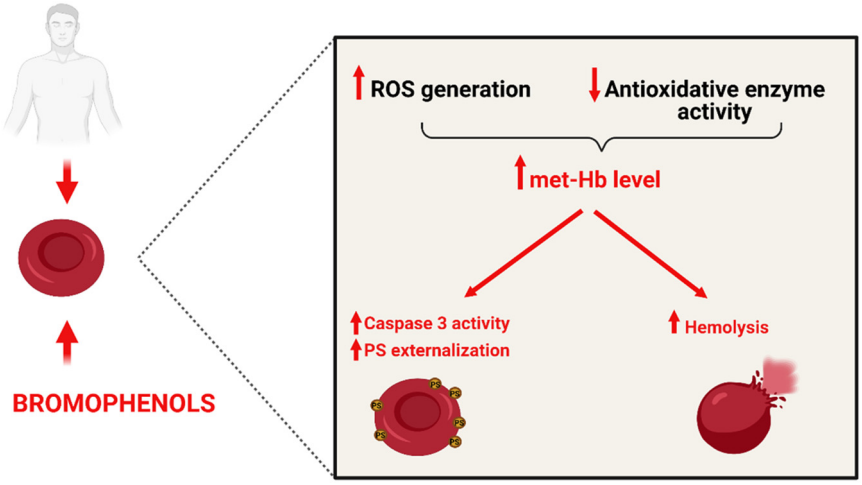



**Fig. 3.** Effects of BPs on oxidative stress, hemolysis and eryptosis in human erythrocytes (Jarosiewicz et al., 2017, 2019a, 2019b). ROS – reactive oxygen species; met-Hb – methemoglobin; PS – phosphatidylserine. Created with BioRender.com.

cells, which might have led to apoptosis induction *via* the intrinsic pathway. Although, limited alterations in pro-inflammatory responses were observed, changes in mitochondrial respiration (basal respiration, ATP-linked respiration and maximum respiration) were noted in studied cells (Bowen et al., 2020). Similarly, Włuka et al. (2020) observed significant depletion of intracellular ATP level in human PBMCs exposed to 2,4,6-TBP and PBP. Jarosiewicz et al. (2019a) analyzed eryptotic potential of 2,4-DBP, 2,4,6-TBP and PBP in unnucleated cells - human mature erythrocytes. All tested compounds caused apoptosis as phosphatidylserine externalization and caspase-3 activation were observed (Fig. 3); however tested BPs did not increase calpain activity nor raised cytosolic $Ca^{2+}$ level. It is considered that there are two pathways to induce apoptosis in erythrocytes. The first is associated with caspases, while the second with calpains. The authors suggested that BPs triggered of eryptosis through ROS-mediated caspase-3 activation.

Jarosiewicz et al. (2017) also studied hemolytic (necrotic) potential of 2,4-DBP, 2,4,6-TBP and PBP and showed that all examined compounds caused hemolytic changes and induced methemoglobin formation in human red blood cells. Hemolytic potential of BPs increased along with the increasing concentrations of these compounds and elongation of the incubation time. Similar results were achieved by Włuka et al. (2020) who observed that 2,4,6-TBP, and more strongly PBP induced a concentration- and time-dependent necrotic changes in human PBMCs (Fig. 3).

### 8.7. Effect on selected enzymes

The blood-brain barrier (BBB) is a selectively permeable barrier characterized by brain microvessels, which are composed of endothelial cells mortared by tight-junction proteins. ABC efflux transporters on the luminal membrane facilitate the removal of unwanted endobiotics and xenobiotics from the brain. Trexler et al. (2019) assessed the effect of 2,4,6-TBP *in vivo* and *ex vivo* on two blood-brain barrier transporters, i.e. P-glycoprotein (P-gp) and Multidrug Resistance-associated Protein 2 (MRP2) of female and male mice and rats. The study revealed that 2,4,6-TBP reduced P-gp activity; however it did not change MRP2 activity. The results have shown that 2,4,6-TBP may affect toxic compounds deposition in the brain and change elimination of these xenobiotics.

Some toxicants can inhibit the activity of UDP-glucuronosyltransferase isoforms (UGTs). Because UGTs are involved in the metabolism of phase II xenobiotics, the inhibition of these enzymes can facilitate the excretion of various substances, e.g. drugs into bile, urine and faeces. Wang et al. (2020) proved that 2,4-DBP and 2,4,6-TBP inhibited the activity of various UGTs isoforms. The most sensitive was the UGT1A7 isoform, which is implicated in the metabolism of endogenous substances like bile acids, androgens and estrogens.

AChE is found in the brain and in the erythrocytes and is a crucial enzyme for peripheral and central nervous system (CNS). Retinoic acid differentiated and non-differentiated human neuroblastoma cell cultures (SH-SY5Y) were exposed to 2,4,6-TBP. The concentrations of 0.1 μmol/L and higher caused a reduction in cell growth and an increase in AChE activity, which was stronger in differentiated cell culture (Ríos et al., 2003).

### 8.8. Impact on aquatic organisms

Accumulation of BPs in the water environment raises great concerns because these compounds are suspected to exhibit multiple adverse effects on aquatic organisms. Geiger et al. (1988) and Broderius et al. (1995) showed that $LC_{50}$ values (96 h) for 2,4,6-TBP and PBP for Fathead minnow (*Pimephales promelas*) were 6.3 mg and 0.1 mg/L, respectively, which indicated on substantial differences in toxicity of tested substances. In another study, 4-BP at 3.4 mg/L was shown to exhibit teratogenic potential in embryos of zebrafish (*Danio rerio*) (Reineke et al., 2006). Casillas and Myers (1989) observed that 2-BP was hepatotoxic in English sole (*Parophrys vetulus*). The scientists observed the presence of hepatocellular coagulation necrosis and fatty change in the liver, altered glutathione and ascorbic acid levels, elevated serum aspartate aminotransferase and alkaline phosphatase activity as well as increased serum glucose and triglyceride content in tested fish (Casillas and Myers, 1989). BPs have been shown to exhibit endocrine disrupting potential both in *in vitro* models and in rats. Similarly, Deng et al. (2010) observed that 2,4,6-TBP caused an increase in testosterone and estradiol levels in males and a decrease in the levels of these hormones in females of zebrafish; however, Kudo et al. (2006) did not observe thyroid system-disrupting activity of BPs in amphibian *Xenopus laevis*. In other studies, Lu et al. (2018) exposed goldfish (*C. auratus*) to 2,4-DBP (0.2–100 mg/kg in food) for 7 days and noticed strong increase in GST activity, while Folle et al. (2020) reported that 2,4,6-TBP at very low concentrations of 0.3–3 μg/L reduced survival of embryos of Silver catfish (*Rhamdia quelen*) causing malformations, neurotoxicity symptoms and induction of oxidative stress. Recently, De Oliveira Riberio et al. (2021) showed that 2,4,6-TBP (0.5–50 ng/g) changed GST activity and caused damage to biomolecules of Nile tilapia (*Oreochromis niloticus*). They also observed changes in liver histopathology and damage to ultrastructure of hepatocytes of studied fish.

## 9. Conclusions

BPs are aromatic pollutants widely spread in the environment and human surrounding. Up to now, several BPs have been synthesized; however, most of them have minor industrial and environmental importance. 2,4-DBP, PBP, and particularly 2,4,6-TBP are the most commonly used BPs in the industry and are also most often detected in the air, aquatic and terrestrial ecosystems and the human body. These substances are used in the synthesis of BFRs, pigments and pesticides and are released as a result of transformation of TBBPA, PBDEs and other FRs through UV photolysis, thermal treatment and biological processes.

BPs occur in low concentration in the unpolluted and urban atmosphere with similar level reported for 2,4-DBP and 2,4,6-TBP and much lower for PBP. 2,4,6-TBP is the most commonly detected BP in aquatic ecosystems; although its content in surface water seems to be lower in comparison to 2,4-DBP. In sediments, 2,4,6-TBP is determined in much higher concentration than in water, while 2,4-DBP and PBP are seldom detected. The highest concentrations of 2,4,6-TBP and PBP have been detected in water and sediments adjacent to e-waste recycling sites. The compounds discussed are efficiently accumulated in living organisms, while marine fish and some marine invertebrates, such as clams and polychaetes accumulate the highest concentrations of BPs, and particularly 2,4,6-TBP.

BPs are transformed in the environment both under abiotic and biotic factors. Under UV radiation, metal oxides and temperature, usually more toxic and more persistent compounds, such as PBDEs, PBDFs and PBDDs are formed. Plants have limited ability to transform BPs, while bacteria efficiently degrade these substances by debromination and often by ring cleavage to form inorganic compounds.

Man is exposed to BPs mainly by food, and to a lower extent by indoor dust and drinking water. Among BPs discussed, 2,4,6-TBP is the most commonly detected in fish, sea food, foodstuffs and alcoholic beverages. Similarly, 2,4,6-TBP is the most commonly determined in indoor dust and drinking water. The occurrence of BPs, and particularly 2,4,6-TBP in humans was noted in blood, adipose and placental tissue, breast milk and urine. Data have shown that in humans and other mammals 2,4-DBP and 2,4,6-TBP are conjugated with sulphates and glucuronides and are readily excreted mainly in the urine; however the formation of highly toxic bromobenzenes and PBDEs is also possible.

2,4-DBP, 2,4,6-TBP and PBP by altering calcium ion and TGF-β signalling pathways, can interrupt thyroid hormone homeostasis, while 2,4-DBP and 2,4,6-TBP can also bind to estrogen receptors. Endocrine disrupting activity of these substances may lead to adverse reproductive and developmental outcomes and may be involved in cancer development in animals and human. 2,4-DBP, 2,4,6-TBP and particularly PBP alter cellular redox balance and cause damage to biomacromolecules, thereby they are capable of triggering of necrosis or/and apoptosis in various cell

types. Moreover, 2,4-DBP, and particularly 2,4,6-TBP by altering some ABC brain transporters and liver UGT isoforms may potentially handicap removal of xenobiotics from the brain and facilitate removal of endogenous compounds from the body. The studies have also shown that 2,4-DBP, and particularly 2,4,6-TBP alter sex hormones levels, change antioxidant response and exhibit hepatotoxic and neurotoxic potential in various fish species.

## Funding

This work was supported by statutory research (B2011000000191.01) admitted for Department of Biophysics of Environmental Pollution, Faculty of Biology and Environmental Protection, University of Lodz.

## CRediT authorship contribution statement

**Jaromir Michałowicz**: Conceptualization, Writing – original draft, Writing – review & editing. **Anna Włuka**: Visualization, Writing – original draft. **Bożena Bukowska**: Writing - original draft.

## 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.

## Acknowledgements

The authors would like to thank Katarzyna Mokra for help in preparing the schemes in BioRender.com.

## References

Adams, S.R., Marvel, C.S., 1921. P-bromophenol. Org. Synth. 1, 39–43.
Ahn, Y.-B., Rhee, S.-K., Fennell, D.F., Kerkhof, L.J., Hentschel, U., Haggblom, M.M., 2003. Reductive dehalogenation of brominated phenolic compounds by microorganisms associated with the marine sponge aplysina aerophoba. Appl. Environ. Microbiol. 69, 4159–4166.
Albaladejo, G.J., Ros, J.A., Romero, A., Navarro, S., 2012. Effect of bromophenols on thetaste and odour of drinking water obtained by seawater desalination in south-easternSpain. Desalination 307, 1–8.
Anon, 1992. Initial Submission: Letter to US EPA Regarding Information on Pentabromophenol Enclosed Acute Oral Toxicity Study (LD50) in Albino Rats With Attachments (Report No.EPA/OTS 88-920003856S; NTIS/OTS 0536705) March 1990.
Aranda, A., Alonso-Merino, E., Zambrano, A., 2013. Receptors of thyroid hormones. Pediatr. Endocrinol. Rev. 11, 2–13.
Aznar-Alemany, Ó., Trabalón, L., Jacobs, S., Barbosa, V.L., Tejedor, F.M., Granby, K., Kwadijk, C., Cunha, S.C., Ferrari, F., Vandermeersch, G., Sioen, I., Verbeke, W., Vilavert, L., Domingo, J.L., Eljarrat, E., Barcelo, D., 2017. Occurrence of halogenated flame retardants in commercial seafood species available in european markets. Food Chem. Toxicol. 104, 35–47.
Bendig, P., Vetter, W., 2013. UV-induced formation of bromophenols from polybrominated diphenyl ethers. Environ. Sci. Technol. 47, 3665–3670.
Bendig, P., Lehnert, K., Vetter, W., 2014. Quantification of bromophenols in islay whiskies. J. Agric. Food Chem. 62, 2767–2771.
Bernstein, A., Ronen, Z., Levin, E., Halicz, L., Gelman, F., 2013. Kinetic bromine isotope effect: example from the microbial debromination of brominated phenols. Anal. Bioanal. Chem. 405, 2923–2929.
Bidleman, T.F., Andersson, A., Jantunen, L.M., Kucklick, J.R., Kylin, H., Letcher, R.J., Tysklind, M., Wong, F., 2019. A review of halogenated natural products in Arctic, subarctic and nordic ecosystems. Emerg. Contam. 5, 89–115.
Bjurlid, F., 2018. Polybrominateddibenzo-p-dioxins and Furans: From Source of Emission to Human Exposure. Örebro University ISSN: 0071-7886.
Bjurlid, F., Dam, M., Hoydal, K., Hagberg, J., 2018. Occurrence of polybrominated dibenzo-p-dioxins, dibenzofurans (PBDD/Fs) and polybrominated diphenyl ethers (PBDEs) in pilot whales (Globicephala melas) caught around the Faroe Islands. Chemosphere 195, 11–20.
Bowen, Ch., Childers, G., Perry, C., Martin, N., Mc Pherson, Ch., Lauten, T., Santos, J., Harry, G.J., 2020. Mitochondrial-related effects of pentabromophenol, tetrabromobisphenol A, and triphenyl phosphate on murine BV-2 microglia cells. Chemosphere 255 (126919).
Boyle, J.L., Lindsay, R.C., Stuiber, D.A., 1992. Bromophenol distribution in salmon and selected seafoods of fresh water and saltwater origin. J. Food Sci. 57, 918–922.
Bracha, P., Bonard, J.P., 1966. Determination of bromophos residues. J. Agric. Food Chem. 14, 609–612.
Broderius, S.J., Kahl, M.D., Hoglund, M.D., 1995. Use of joint toxic response to define the primary mode of toxic action for diverse industrial organic chemicals. Environ. Toxicol. Chem. 14, 1591–1605.
Cantón, R.F., Sanderson, J.T., Letcher, R.J., Bergman, Å., van den Berg, M., 2005. Inhibition and induction of aromatase (CYP19) activity by brominated flame retardants in H295R human adrenocortical carcinoma cells. Toxicol. Sci. 88, 447–455.
Casillas, E., Myers, M.S., 1989. Effect of bromobenzene and O-bromophenol on kidney and liver of English sole (Parophrys vetulus). Comp. Biochem. Physiol. Comp. Physiol. 93, 42–48.
Chatonnet, P., Bonnet, S., Boutou, S., Labadie, M.D., 2004. Identification and responsibility of 2,4,6-tribromoanisole in musty, corked odours in wine. J. Agric. Food Chem. 52, 1255–1262.
Chen, Ch., Yang, P., Kao, Y., Chen, P., Chung, Ch., Wang, S., 2017. Pentabromophenol suppresses TGF-β signaling by accelerating degradation of type II TGF-β receptors via caveolae-mediated endocytosis. Sci. Rep. 7, 43206.
Chen, D., Shen, J., Jiang, X., Su, G., Han, W., Sun, X., Li, J., Mu, Y., Wang, L., 2019. Simultaneous debromination and mineralization of bromophenol in an up-flow electricity-stimulated anaerobic system. Water Res. 157, 8–18.
Chi, X., Liu, J., Yu, M., Xie, Z., Jiang, G., 2017. Analysis of bromophenols in various aqueous samples using solid phase extraction followed by HPLC-MS. Talanta 164, 57–63.
Christiansson, A., Eriksson, J., Teclechiel, D., Bergman, A., 2009. Identification and quantification of products formed via photolysis of decabromodiphenyl ether. Environ. Sci. Pollut. Res. 16, 312–321.
Chung, H.Y., Ma, W.C.J., Ang, P.O., Kim, J.S., Chen, F., 2003b. Seasonal variations of bromophenols in brown algae (Padinaarborescens, sargassumsiliquastrum, and Lobophoravariegata) collected in Hong Kong. J. Agric. Food Chem. 51, 2619–2624.
Clayton, G.D., Clayton, F.E., 1993. Patty's industrial hygiene and toxicology. Toxicology. John Wiley and Sons, New York. NY.
Collet, B., Simon, E., Der Linden, S., Abdellaoui, N., Naderman, M., Man, H.-Y., Middelhof, I., Der Burg, B., Besselink, H., Brouwer, A., 2020. Evaluation of a panel of in vitro methods for assessing thyroid receptor β and transthyretin transporter disrupting activities. Reprod. Toxicol. 96, 432–444.
Covaci, A., Voorspoelsb, S., Abdallahc, M.A.E., Geensa, T., Harradc, S., Lawd, E.J., 2009. Analytical and environmental aspects of the flame retardant tetrabromobisphenol-a and its derivatives. J. Chromatogr. A 1216, 346–363.
Covaci, A., Harrad, S., Abdallah, M.A.-E., Ali, N., Law, R.J., Herzke, D., de Wit, C.A., 2011. Novel brominated flame retardants: a review of their analysis, environmental fate and behaviour. Environment 37, 532–556.
De Oliveira Riberio, C.A., Bozza, D.A., Esquivel, L., De Oliveira, E.C., Neto, F., 2021. Comparative effects of oral exposure to 2,4,6-tribromophenol and decabromodiphenyl ether in Nile tilapia. Environ. Sci. Pollut. Res. Int. 16, 34655032.
Deng, J., Liu, C., Yu, L., Zhou, B., 2010. Chronic exposure to environmental levels of tribromophenol impairs zebrafish reproduction. Toxicol. Appl. Pharmacol. 243, 87–95.
Dong, H., Dong, S., Hansen, P.E., Stagos, D., Liu, X., Liu, M., 2020. Progress of bromophenols in marine algae from 2011 to 2020: structure, bioactivities, and applications. Mar. Drugs 18, 411.
Dopico, M., Gomez, A., 2015. Review of the current state and main sources of dioxins around the world. J. Air Waste Manag. Assoc. 65, 1033–1049.
Dufour, P., Pirard, C., Charlier, C., 2017. Determination of phenolic organohalogens in human serum from a Belgian population and assessment of parameters affecting the human contamination. Sci. Total Environ. 599–600, 1856–1866.
Eguchi, A., Nomiyama, K., Devanathan, G., Subramanian, A., Bulbule, A.K., Parthasarathy, P., Takahashi, S., Tanabe, S., 2012. Different profiles of anthropogenic and naturally produced organohalogen compounds in serum from residents living near a coastal area and e-waste recycling workers in India. Environ. Int. 47, 8–16.
Erratico, C.A., Szeitz, A., Bandiera, S.M., 2012. Oxidative metabolism of BDE-99 by human liver microsomes: predominant role of CYP2B6. Toxicol. Sci. 129, 280–292.
European Food Safety Authority, 2012. Scientific opinion on brominated flame retardants (BFRs) in food: brominated phenols and their derivatives. EFSA J. 10, 2634–2676.
Evangelopoulos, P., Arato, S., Persson, H., Kantarelis, E., Yang, W., 2019. Reduction of brominated flame retardants (BFRs) in plastics from waste electrical and electronic equipment (WEEE) by solvent extraction and the influence on their thermal decomposition. Waste Manag. 94, 165–171.
Ezechiáš, M., Svobodová, K., Cajthaml, T., 2012. Hormonal activities of new brominated flame retardants. Chemosphere 87, 820–824.
Ezechiáš, M., Covino, S., Cajthaml, T., 2014. Ecotoxicity and biodegradability of new brominated flame retardants: a review. Ecotoxicol. Environ. Saf. 110, 153–167.
Feng, C., Xu, Q., Jin, Y., Lin, Y., Qui, X., Lu, D., Wang, G., 2016. Determination of urinary bromophenols (BrPs) as potential biomarkers for human exposure to polybrominated diphenyl ethers (PBDEs) using gas chromatography-tandem mass spectrometry (GC–MS/MS). J. Chromatogr. B 1022, 70–74.
Folle, N.M.T., Azevedo-Linhares, M., Garcia, J.R.E., da Costa Souza, A.T., Grötzner, S.R., de Oliveira, E.C., Paulin, A.F., Leite, N.F., Neto, F.F., de Oliveira Ribeiro, C.A., 2020. Low concentration of 2,4,6-tribromophenol (TBP) represents a risk to south american silver catfish ramdiaquelen (Quoy a nd gaimard, 1824) population. Ecotoxicol. Environ. Saf. 187, 109815.
Fujii, Y., Harada, K.H., Hitomi, T., Kobayashi, H., Koizumi, A., Haraguchi, K., 2014. Temporal trend and age-dependent serum concentration of phenolic organohalogen contaminants in japanese men during 1989–2010. Environ. Pollut. 185, 228–233.
Fujishima, A., Fujiwara, M., 1999. Single dose oral toxicity test of 2,4,6-tribromophenol in rats. Shizuoka, Japan Ministry of Health & Welfare. Biosafety Research Centre. Toxicity Testing Reports of Environmental Chemicals No. 7.
Fuller, S.C., Frank, D.C., Fitzhenry, M.J., Smyth, H.E., Poole, S.E., 2008. Improved approach for analyzing bromophenols in seafood using stable isotope dilution analysis in combination with SPME. J. Agric. Food Chem. 56, 8248–8254.

Gao, S., Wan, Y., Zheng, G., Luo, K., Kannan, K., Giesy, J.P., Lam, M.H.W., Hu, J., 2015. Organobromine compound profiling in human adipose: assessment of sources of bromophenol. Environ. Pollut. 204, 81–89.
Geiger, D.L., Call, D.J., Brooke, L.T., 1988. Acute Toxicities of Organic Chemicals to Fathead Minnows (Pimephales promelas). Vol. IV. University of Wisconsin–Superior, Superior, WI.
Goerke, H., Weber, K., 1991. Bromophenols in Laniceconchilega (Polychaeta, Terebellidae) - the influence of sex, weight and season. Bull. Mar. Sci. 48, 517–523.
Golan, R., Gelman, F., Kuder, T., Taylor, A.A., Ronen, Z., Bernstein, A., 2019. Degradation of 4-bromophenol by Ochrobactrum sp. HI1 isolated from desert soil: pathway and isotope effects. Biodegradation 30, 37–46.
Goto, A., Tue, N., Someya, M., Isobe, T., Takahashi, S., Tanabe, S., Kunisue, T., 2017. Occurrence of natural mixed halogenated dibenzo-p-dioxins: specific distribution and profiles in mussels from Seto Inland Sea, Japan. Environ. Sci. Technol. 51, 11771–11779.
Gribble, G.W., 2010. Naturally occurring organohalogen compounds: a comprehensive update. Progress in the Chemistry of Organic Natural Products. 91. Springer Verlag ISSN: 0071-7886.
Gutierrez, M., Becerra, J., Godoy, J., Barra, R., 2005. Occupational and environmental exposure to tribromophenol used for wood surface protection in sawmills. Int. J. Environ. Health Res. 15, 171–179.
Halawani, A., Latif, N., Taka, A., 1951. On pentabromophenol and pentachlorophenol as molluscacides in the prevention of bilharziasis. J. Egypt. Med. Assoc. 34, 163–170.
Haldén, A., Nyholm, J., Andersson, P., Holbech, H., Norrgren, L., 2010. Oral exposure of adult zebrafish (Danio rerio) to 2,4,6-tribromophenol affects reproduction. Aquat. Toxicol. 100, 30–37.
Han, W., Wang, S., Huang, H., Luo, L., Zhang, S., 2013. Simultaneous determination of brominated phenols in soils. J. Environ. Sci. 25, 2306–2312.
Haraguchi, K., Kotaki, Y., Relox, J.R., Romero, M.L.J., Terada, R., 2010. Monitoring of naturally produced brominated phenoxyphenols and phenoxyanisoles in aquatic plants from the Philippines. J. Agric. Food Chem. 58, 12385–12391.
Harrad, S., de Wit, C.A., Abdallah, M.A., Bergh, C., Björklund, J.A., Covaci, A., Darnerud, P.O., de Boer, J., Diamond, M., Huber, S., Leonards, P., Mandalakis, M., Östman, C., Haug, L.S., Thomsen, C., Webster, T.F., 2010. Indoor contamination with hexabromocyclododecanes, polybrominated diphenyl ethers, and perfluoroalkyl compounds: an important exposure pathway for people? Environ. Sci. Technol. 44, 3221–3231.
Hassenklöver, T., Predehl, S., Pilli, J., Ledwolorz, J., Assmann, M., Bickmeyer, U., 2006. Bromophenols, both present in marine organisms and in industrial flame retardants, disturb cellular Ca2+ signalling in neuroendocrine cells (PC12). Aquat. Toxicol. 76, 37–45.
Ho, K.L., Yau, M.S., Murphy, M.B., Wan, Y., Fong, B.M.W., Tam, S., Giesy, J.P., Leung, K.S.Y., Lam, M.H.W., 2015. Urinary bromophenol glucuronide and sulfate conjugates: potential human exposure molecular markers for polybrominated diphenyl ethers. Chemosphere 133, 6–12.
Howe, P.D., Dobson, S., Malcolm, H.M., 2005. 2,4,6-Tribromophenol and other simple brominated phenols. IPCS Concise International Chemical Assessment Documents. 66. WHO, Geneva, pp. 1–35.
Huber, S., Warner, N.A., Nygard, T., Remberger, M., Harju, M., Uggerud, H.T., Kaj, L., Hanssen, L., 2015. A broad cocktail of environmental pollutants found in eggs of three seabird species from remote colonies in Norway. Environ. Toxicol. Chem. 34, 1296–1308.
International Research Development Corporation, 1974. Acute Inhalation Toxicity in the Albino Rat. International Research Development Corporation (Report No. EPA/OTS 86-900000305; NTIS/OTS 0523297; submitted to US EPA with test data and cover letter by Great Lakes Chemical Corporation, West Lafayette, IN, Mattawan, MI March 1990.
Jarosiewicz, M., Duchnowicz, P., Włuka, A., Bukowska, B., 2017. Evaluation of the effect of brominated flame retardants on haemoglobin oxidation and hemolysis in human erythrocytes. Food Chem. Toxicol. 109, 264–271.
Jarosiewicz, M., Krokosz, A., Marczak, A., Bukowska, B., 2019a. Changes in the activities of antioxidant enzymes and reduced glutathione level in human erythrocytes exposed to selected brominated flame retardants. Chemosphere 227, 93–99.
Jarosiewicz, M., Michałowicz, J., Bukowska, B., 2019b. In vitro assessment of eryptotic potential of tetrabromobisphenol A and other bromophenolic flame retardants. Chemosphere 215, 404–412.
Jiang, J., Zhao, H., Sun, S., Wang, Y., Liu, S., Xie, Q., Li, X., 2018. Occurrence and profiles of halogenated phenols, polybrominated diphenyl ethers and hydroxylated polybrominated diphenyl ethers in the effluents of waste water treatment plants around Huang-Bo Sea, North China. Sci. Total Environ. 622–623, 1–7.
Kaboli, P.J., Zhang, L., Xiang, S., Shen, J., Li, M., Zhao, Y., Wu, X., Zhao, Q., Zhang, H., Lin, L., Yin, J., Wu, Y., Wan, L., Yi, T., Li, X., Cho, Ch.H., Li, J., Xiao, Z., Wen, Q., 2019. Molecular markers of regulatory T cells in cancer immunotherapy with special focus on Acute Myeloid Leukemia (AML) - a systematic review. Curr. Med. Chem. 27, 4673–4698.
Kapustka, K., Ziegmann, G., Klimecka-Tatar, D., 2018. Problems in waste management in the aspect of the secondary use of plastics from WEEE. MATEC Web Conf. 183.
Kawashiro, Y., Fukata, H., Omori-Inoue, M., Kubonoya, K., Jotaki, T., Takigami, H., Sakai, S., Mori, C., 2008. Perinatal exposure to brominated flame retardants and polychlorinated biphenyls in Japan. Endocr. J. 55, 1071–1084.
Kicklighter, C.E., Kubanek, J., Hay, M.E., 2004. Do brominated natural products defend marine worms from consumers? Some do, most don't. Limnol. Oceanogr. 49, 430–441.
Kitamura, S., Shinohara, S., Iwase, E., Sugihara, K., Uramaru, N., Shigematsu, H., Fujimoto, N., Ohta, S., 2008. Affinity for thyroid hormone and estrogen receptors of hydroxylated polybrominated diphenyl ethers. J. Health Sci. 54, 607–614.
Knudsen, G.A., Trexler, A.W., Richards, A.C., Hall, S.M., Hughes, M.F., Birnbaum, L.S., 2019. 2,4,6-Tribromophenol disposition and kinetics in rodents: Effects of dose, route, sex, and species. Toxicol. Sci. 169, 167–179.
Koch, Ch., Sures, B., 2018. Environmental concentrations and toxicology of 2,4,6-tribromophenol (TBP). Environ. Pollut. 233, 706–713.
Kollitz, E.M., De Carbonnel, L., Stapleton, H.M., Ferguson, P.L., 2018. The Affinity of brominated phenolic compounds for human and zebrafish thyroid receptor β: Influence of chemical structure. Toxicol. Sci. 163, 226–239.
Kudo, Y., Yamauchi, K., Fukazawa, H., Terao, Y., 2006. In vitro and in vivo analysis of the thyroid system-disrupting activities of brominated phenolic and phenol compounds in Xenopus laevis. Toxicol. Sci. 92, 87–95.
Kumar, P., 2018. Electronic waste- hazards, management and available green technologies for remediation – a review. Int. Res. J. Environ. Sci. 7, 57–68.
Kunze, C., Bommer, M., Hagen, W.R., Uksa, M., Dobbek, H., Schubert, T., Diekert, G., 2017. Cobamide-mediated enzymatic reductive dehalogenation via long-range electron transfer. Nat. Commun. 8 (15858).
Lang, Z., Li, G., Mai, B., An, T., 2019. Biodegradation of typical BFRs 2,4,6-tribromophenol by an indigenous strain Bacillus sp. GZT isolated from e-waste dismantling area through functional heterologous expression. Sci. Total Environ. 697, 134159.
Lee, D., Ahn, C., Hong, E., An, B., Hyun, S., Choi, K., Jeung, E., 2016. 2,4,6-Tribromophenol interferes with the thyroid hormone system by regulating thyroid hormones and the responsible genes in mice. Int. J. Environ. Res. Public Health 13, 697.
Leonetti, C., Butt, C.M., Hoffman, K., Hammel, S.C., Miranda, M.L., Stapleton, H.M., 2016. Brominated flame retardants in placental tissues: associations with infant sex and thyroid hormone endpoints. Environ. Health 15, 113.
Leonetti, C.P., Butt, C.M., Stapleton, H.M., 2018. Disruption of thyroid hormone sulfotransferase activity by brominated flame retardant chemicals in the human choriocarcinoma placenta cell line, BeWo. Chemosphere 197, 81–88.
Li, Z., Yoshida, N., Wang, A., Nan, J., Liang, B., Zhang, C., Zhang, D., Suzuki, D., Zhou, X., Xiao, Z., Katayama, A., 2015. Anaerobic mineralization of 2,4,6-tribromophenol to CO2 by a synthetic microbial community comprising Clostridium, Dehalobacter, and Desulfatiglans. Bioresour. Technol. 176, 225–232.
Lin, K.D., Yan, C., Gan, J., 2014a. Production of hydroxylated polybrominated diphenyl ethers (OH-PBDEs) from bromophenols by manganese dioxide. Environ. Sci. Technol. 48, 263–271.
Lin, K.D., Gan, J., Liu, W.P., 2014b. Production of hydroxylated polybrominated diphenyl ethers from bromophenols by bromoperoxidase-catalyzed dimerization. Environ. Sci. Technol. 48, 11977–11983.
Lin, K., Zhou, S., Chen, X., Ding, Y., Kong, X., Gan, J., 2015. Formation of hydroxylated polybrominated diphenyl ethers from laccase-catalyzed oxidation of bromophenols. Chemosphere 138, 806–813.
Lin, K., Song, L., Zhou, S., Chen, D., Gan, J., 2016. Formation of brominated phenolic contaminants from natural manganese oxides-catalyzed oxidation of phenol in the presence of Br. Chemosphere 155, 266–273.
Liu, A., Shi, J., Qu, G., Hu, L., Ma, Q., Song, M., Jing, C., Jiang, G., 2017. Identification of emerging brominated chemicals as the transformation products of tetrabromobisphenol A (TBBPA) derivatives in soil. Environ. Sci. Technol. 51, 5434–5444.
Lopez, M.G., Driffield, M., Fernandes, A.R., Smith, F., Tarbin, J., Lloyd, A.S., Christy, J., Holland, M., Steel, Z., Tlustos, C., 2018. Occurrence of polybrominated diphenyl ethers, hexabromocyclododecanes, bromophenols and tetrabromobisphenols A and S in Irish foods. Chemosphere 197, 709–715.
Lu, G., Liu, J., Zhang, F., Nkoom, M., Wu, D., 2018. Interactive effects of BDE-153 and its metabolite 2,4-DBP on biochemical status in Crucian Carp (Carassius auratus). Pol. J. Environ. Stud. 27, 1–9.
Lyubimov, A.V., Babin, V.V., Kartashov, A.I., 1998. Developmental neurotoxicity of 2,4,6-tribromophenol in Wistar rats. Neurotoxicology 19, 303–312.
Malysheva, S.V., Goscinny, S., Malarvannan, G., Poma, G., Andjelkovic, M., Jacobs, G., Voorspoels, S., Covaci, A., Van Loco, J., 2017. Occurrence of HBCDs, TBBPA, brominated phenols and their derivative in foodstuffs in Belgium. BFR 2017, 1–3.
Manasfi, T., Lebaron, K., Verlande, M., Dron, J., Demelas, C., Vassalo, L., Revenko, G., Quivet, E., Boudenne, J.-L., 2019. Occurrence and speciation of chlorination byproducts in marine waters and sediments of a semi-enclosed bay exposed to industrial chlorinated effluents. Int. J. Hyg. Environ. Health 222, 1–8.
Meerts, I.A.T.M., van Zanden, J.J., Luijks, E.A.C., van Leeuwen-Bol, I., Marsh, G., Jakobsson, E., Bergman, A., Brouwer, A., 2000. Potent competitive interactions of some brominated flame retardants and related compounds with human transthyretin in vitro. Toxicol. Sci. 56, 95–104.
Meng, W., Xia, Q., Wu, L., Chen, S., He, X., Zhang, L., Gao, Q., Zhou, H., 2011. Downregulation of TGF-beta receptor types II and III in oral squamous cell carcinoma and oral carcinoma-associated fibroblasts. BMC Cancer 11, 88.
Mizukawa, H., Nomiyama, K., Nakatsu, S., Yamamoto, M., Ishizuka, M., Ikenaka, Y., Nakayama, S.M.M., Tanabe, S., 2017. Anthropogenic and naturally produced brominated phenols in pet blood and pet food in Japan. Environ. Sci. Technol. 51, 11354–11362.
Mizukawa, H., Nomiyama, K., Nakatsu, S., Yamamoto, M., Ischizuka, M., Ikenaka, Y., Nakayama, S., Tanabe, S., 2017. Anthropogenic and naturally produced brominated phenols in pet blood and pet food in Japan. Environ. Sci. Technol. 51, 11354–11362.
Mota da Silva, V., da Cunha Veloso, M.C., de Oliveira, A.S., Vieira Santos, G., de Pereira, P.A., de Andrade, J.B., 2005. Determination of simple bromophenols in marine fishes by reverse-phase high performance liquid chromatography (RP-HPLC). Talanta 68, 323–328.
Na, Y.C., Hong, J.K., Kim, K.J., 2007. Formation of polybrominated dibenzo-p-dioxins/furans (PBDDs/Fs) by the pyrolysis of 2,4-dibromophenol, 2,6-dibromophenol and 2,4,6-tribromophenol. Bull. Kor. Chem. Soc. 28, 547–552.
Nichkova, M., Germani, M., Marco, M.P., 2008. Immunochemical analysis of 2,4,6-tribromophenol for assessment of wood contamination. J. Agric. Food Chem. 56, 29–34.
Nomani, A.A., Ajmal, M., Ahmad, S., 1996. Gas chromatography - mass spectrometric analysis of four polluted rivers for phenolic and organic compounds. Environ. Monit. Assess. 40, 1–9.

Öberg, K., Warman, K., Öberg, T., 2002. Distribution and levels of brominated flame retardants in sewage sludge. Chemosphere 48, 805–809.
Ohta, S., Okumura, T., Nishimura, H., Nakao, T., Shimizu, Y., Ochiai, F., Aozasa, O., Miyata, H., 2004. Levels of PBDEs, TBBPA, TBPs, PCDDs/DFs, PXDDs/DFs AND PBDDs/DFs in human milk of nursing women and dairy milk products in Japan. Organohalogen Compd. 66, 2857–2862.
Okon, D.E., Choo, G., Barcelo, D., Oh, J.-E., 2020. Chapter one - introduction of emerging halogenated flame retardants in the environment. Compr. Anal. Chem. 88, 1–39.
Oliveira, A.S., Silva, V.M., Veloso, M.C.C., Santos, G.V., de Andrade, J.B., 2009. Bromophenol concentrations in fish from Salvador, BA, Brasil. Ann. Br. Acad. Sci. 81, 165–172.
Olsen, C.M., Meussen-Elholm, E.T.M., Holme, J.A., Hongslo, J.K., 2002. Brominated phenols: characterization of estrogen-like activity in the human breast cancer cell-line MCF-7. Toxicol. Lett. 129, 55–63.
Orisakwe, O.E., Frazzoli, C., Elochukwullo, C., Oritsemuelebi, B., 2019. Public health burden of e-waste in Africa. J. Health Pollut. 9, 190610.
Ortuno, N., Molto, N., Conesa, J.A., Font, R., 2014. Formation of brominated pollutants during the pyrolysis and combustion of tetrabromobisphenol A at different temperatures. Environ. Pollut. 191, 31–37.
Pezzi, V., Mathis, J.M., Rainey, W.E., Carr, B.R., 2003. Profiling transcript levels for steroidogenic enzymes in fetal tissues. J. Steroid Biochem. Mol. Biol. 87, 181–189.
Pivnenko, K., Granby, K., Eriksson, E., Astrup, T.F., 2017. Recycling of plastic waste: Screening for brominated flame retardants (BFRs). Waste Manag. 69, 101–109.
Poma, G., Malysheva, S.V., Goscinny, S., Malarvannan, G., Voorspoels, S., Covaci, A., Van Loco, J., 2018. Occurrence of selected halogenated flame retardants in Belgian foodstuff. Chemosphere 194, 256–265.
Pratt, I., Anderson, W., Crowley, D., Daly, S., Evans, R., Fernandes, A., Fitzgerald, M., Geary, M., Keane, D., Morrison, J., 2013. Brominated and fluorinated organic pollutants in the breast milk of first-time Irish mothers: is there a relationship to levels in food? Food Addit. Contam. Part A: Chem. Anal. Control Expo. Risk Assess. 30, 1788–1798.
Reineke, N., Biselli, S., Franke, S., Francke, W., Heinzel, N., Huhnerfuss, H., Iznaguen, H., Kammann, U., Theobald, N., 2006. Brominated indoles and phenols in marine sediment and water extracts from the North and Baltic Seas–concentrations and effects. Arch. Environ. Contam. Toxicol. 51, 186–196.
Remberger, M., Sternbeck, J., Strömberg, K., 2002. Screening av triclosan och visa bromerade fenoliska ämnen I Sverige. IVL Report B1477-2 Stockholm, November.
Ríos, J.C., Repetto, G., Jos, A., del Peso, A., Salguero, M., Cameán, A., Repetto, M., 2003. Tribromophenol induces the differentiation of SH-SY5Y human neuroblastoma cells in vitro. Toxicol. in Vitro 17, 635–641.
Roane, B.M., Arend, R.C., Birrer, M.J., 2019. Review: targeting the transforming growth factor-beta pathway in ovarian cancer. Cancers 11, 668.
Sahoo, N.K., 2020. Kinetics of simultaneous degradation of 4-bromophenol and 4-chlorophenol by Arthrobacter chlorophenolicus A6. Biointerface Res. Appl. Chem. 10, 4939–4943.
Saito, I., Onuki, A., Seto, H., 2007. Indoor organophosphate and polybrominated flame retardants in Tokyo. Indoor Air 17, 28–36.
Sanchez, M.A., Gonzalez, B., 2007. Genetic characterization of 2,4,6-trichlorophenol degradation in *Cupriavidus necator* JMP134. Appl. Environ. Microbiol. 73, 2769–2776.
Saponaro, F., Sestito, S., Runfola, M., Rapposelli, S., Chiellini, G., 2020. Selective thyroid hormone receptor-beta (TRβ) agonists: new perspectives for the treatment of metabolic and neurodegenerative disorders. Front. Med. 9 (7), 331.
Schlabach, M., Remberger, M., Brorström-Lundén, E., Norström, K., Kaj, L., Andersson, H., Herzke, D., Borgen, A., Harju, M., 2011. Brominated Flame Retardants (BFR) in the Nordic Environment. TemaNord. 528. Nordic Council of Ministers, Copenhagen, p. 86.
Sha, B., Dahlberg, A.-K., Wiberg, K., Ahrens, A., 2018. Fluorotelomer alcohols (FTOHs), brominated flame retardants (BFRs), organophosphorus flame retardants (OPFRs) and cyclic volatile methylsiloxanes (cVMSs) in indoor air from occupational and home environments. Environ. Pollut. 241, 319–330.
Sharma, V.K., Zboril, R., McDonald, T., 2014. Formation and toxicity of brominated disinfection by-products during chlorination and chloramination of water: a review. J. Environ. Sci. Health A 49, 212–228.
Sheller-Miller, S., Radnaa, E., Arita, Y., Getahun, D., Jones, R.J., Peltier, M.R., Menon, R., 2020. Environmental pollutant induced cellular injury is reflected in exosomes from placental explants. Placenta 89, 42–49.
Sim, W.J., Lee, S.H., Lee, I.S., Choi, S.D., Oh, J.E., 2009. Distribution and formation of chlorophenols and bromophenols in marine and riverine environments. Chemosphere 77, 552–558.
Simpson, E.R., Clyne, C., Rubin, G., Boon, W.C., Robertson, K., Britt, K., Speed, C., Jones, M., 2002. Aromatase - a brief overview. Annu. Rev. Physiol. 64, 93–127.
Sindiku, O., Babayemi, J.O., Tysklind, M., Osibanjo, O., Weber, R., Watson, A., Schlummer, M., Lundstedt, S., 2015. Polybrominated-dibenzo-p-dioxins and dibenzofurans (PBDD/Fs) in e-waste plastic in Nigeria. Environ. Sci. Pollut. Res. 22, 14515–14529.
Sithole, B.B., Williams, D.T., Lastoria, C., Robertson, J.L., 1986. Determination of halogenated phenols in raw and potable water by selected ion gas chromatography–mass spectrometry. J. Assoc. Off. Anal. Chem. 69, 466–473.
Smeds, A., Saukko, P., 2003. Brominated flame retardants and phenolic endocrine disrupters in Finnish human adipose tissue. Chemosphere 53, 1123–1130.
Stekol, J.A., Dash, H.H., 1939. P-bromophenol as the intermediate in synthesis of p-bromophenylmercapturic acid from bromobenzene in the rat. Proc. Soc. Exp. Biol. Med. 40, 261–263.
Sun, J., Chen, Q., Qian, Z., Zheng, Y., Yu, S., Zhang, A., 2018. Plant uptake and metabolism of 2,4-dibromophenol in carrot: in vitro enzymatic direct conjugation. J. Agric. Food Chem. 66, 4328–4335.
Szymańska, J.A., Bruchajzer, E., Piotrowski, J.K., 1995. Investigations on acute hepato- and nephrotoxicity of pentabromophenol. Int. J. Occup. Environ. Health 8, 245–254.
Takigami, H., Suzuki, G., Hirai, Y., Sakai, S., 2009. Brominated flame retardants and other polyhalogenated compounds in indoor air and dust from two houses in Japan. Chemosphere 76, 270–277.
Tanaka, R., Yamada, R., Oba, K., Iga, T., Mikami, S., 1999. Combined repeat dose and reproductive/developmental toxicity screening test of 2,4,6-tribromophenol by oral administration in rats. Japan Ministry of Health & Welfare, Biosafety Research Centre (Toxicity Testing Reports of Environmental Chemicals No. 7, Shizuoka (in Japanese) http://wwwdb.mhlw.go.jp/ginc/dbfile1/paper/paper118-79-6d.html).
Tang, S., Liu, H., Yin, H., Liu, X., Peng, H., Lu, G., Dang, Z., He, C., 2018. Effect of 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) and its metabolites on cell viability, oxidative stress, and apoptosis of HepG2. Chemosphere 193, 978–988.
Thomsen, C., Lundanes, E., Becher, G., 2001. Brominated flame retardants in plasma samples from three different occupational groups in Norway. J. Environ. Monit. 3, 366–370.
Thomsen, C., Lundanes, E., Becher, G., 2002a. Brominated flame retardants in archived serum samples from Norway: A study on temporal trends and the role of age. Environ. Sci. Technol. 36, 1414–1418.
Thomsen, C., Leknes, H., Lundanes, E., Becher, G., 2002b. A new method for determination of halogenated flame retardants in human milk using solid-phase extraction. J. Anal. Toxicol. 26, 129–137.
Tolosa, I., Bayona, J.M., Albaiges, J., 1991. Identification and occurrence of brominated and nitrated phenols in estuarine sediments. Mar. Pollut. Bull. 22, 603–607.
Trexler, A.W., Knudsen, G.A., Nicklisch, S.C.T., Birnbaum, L.S., Cannon, R.E., 2019. 2,4,6-tribromophenol exposure decreases P-glycoprotein transport at the blood-brain barrier. Toxicol. Sci. 171, 463–472.
Tue, N.M., Takahashi, S., Suzuki, G., Isobe, T., Viet, P.H., Kobara, Y., Seike, N., Zhang, G., Sudaryanto, A., Tanabe, S., 2013. Contamination of indoor dust and air by polychlorinated biphenyls and brominated flame retardants and relevance of non-dietary exposure in Vietnamese informal e-waste recyckling sites. Environ. Int. 51, 160–167.
Uhnakova, B., Petrikova, A., Biedermann, D., 2009. Biodegradation of brominated aromatics by cultures and Laccase of *Trametes versicolor*. Chemosphere 76, 826–832.
Vetter, W., Gallistl, C., Schlienz, A., Preston, T., Müller, J., von der Trenck, K.T., 2017. Brominated flame retardants (BFRs) in eggs from birds of prey from Southern Germany. Environ. Pollut. 231, 569–577.
Wang, X., Hu, X., Zhang, H., Chang, F., Luo, Y., 2015. Photolysis kinetics, mechanisms, and pathways of tetrabromobisphenol A in water under simulated solar light irradiation. Environ. Sci. Technol. 49, 6683–6690.
Wang, J.-J., Rhew, R.C., Chow, A.T., 2016. Haloform formation in coastal wetlands along salinity gradient at South Carolina, United States. Environ. Chem. 13, 745–756.
Wang, J., Hao, Z., Shi, F., Yin, Y., Cao, D., Yao, Z., Liu, J., 2018. Characterization of brominated disinfection by-products formed during the chlorination of aquaculture seawater. Environ. Sci. Technol. 52, 5662–5670.
Wang, F., Wang, S., Yang, K., Liu, Y.-Z., Yang, K., Chen, Y., Fang, Z.-Z., 2020. Inhibition of UDP-glucuronosyltransferases (UGTs) by bromophenols (BPs). Chemosphere 238, 124645.
Wanga, K., Huang, X., Lina, K., 2019. Multiple catalytic roles of chloroperoxidase in the transformation of phenol: products and pathways. Ecotoxicol. Environ. Saf. 179 96-10.
Watanabe, I., Sakai, S., 2003. Environmental release and behavior of brominated flame retardants. Environ. Int. 29, 665–682.
Watanabe, I., Kashimoto, T., Tatsukawa, R., 1984. Brominated phenol production from the chlorination of wastewater containing bromide ions. Bull. Environ. Contam. Toxicol. 33, 395–399.
Watanabe, I., Takahashi, K., Tatsukawa, R., 1985. Brominated phenols and anisoles in river and marine sediments in Japan. Bull. Environ. Contam. Toxicol. 35, 272–278.
Whitfield, F.B., Helidoniotis, F., Shaw, K.J., Svoronos, D., 1999. Distribution of bromophenols in species of marine algae from Eaestern Australia. J. Agric. Food Chem. 47, 2367–2373.
Whitfield, F.B., Shaw, K.J., Walker, D.I., 1992. The source of 2,6-dibromophenol: cause of an iodoform taint in Australian prawns. Water Sci. Technol. 25, 131–138.
Willems, E., Cabral-Teixeira, J., Schade, D., Cai, W., Reeves, P., Bushway, P.J., Lanier, M., Walsh, Ch., Kirchhausen, T., Belmonte, J.C.I., Cashman, J., Mercola, M., 2012. Small molecule-mediated TGF-beta type II receptor degradation promotes cardiomyogenesis in embryonic stem cells. Cell Stem Cell 11, 242–252.
Włuka, A., Woźniak, A., Woźniak, E., Michałowicz, J., 2020. Tetrabromobisphenol A, terabromobisphenol S and other bromophenolic flame retardants cause cytotoxic effects and induce oxidative stress in human peripheral blood mononuclear cells (in vitro study). Chemosphere 261, 127705.
Wong, F., Suzuki, G., Michinaka, C., Yuan, B., Takigami, H., 2017. Dioxin-like activities, halogenated flame retardants, organophosphate esters and chlorinated paraffins in dust from Australia, the United Kingdom, Canada, Sweden and China. Chemosphere 168, 1248–1256.
World Health Organisation, 2005. 2,4,6-Tribromophenol and Other Simple Brominated Phenols. Concise International Chemical Assessment Document 66. 2005 Geneva.
Xiong, J.K., An, T.C., Zhang, C.S., Li, G.Y., 2015. Pollution profiles and risk assessment of PBDEs and phenolic brominated flame retardants in water environments within a typical electronic waste dismantling region. Environ. Geochem. Health 37, 457–473.
Xiong, J., Li, G., An, T., 2016a. The microbial degradation of 2,4,6-tribromophenol (TBP) in water/sediments interface: investigating bioaugmentation using Bacillus sp. GZT. Sci. Total Environ. 575, 573–580.
Xiong, J., Li, G., An, T., Zhang, Ch., Wei, Ch., 2016b. Emission patterns and risk assessment of polybrominated diphenyl ethers and bromophenols in water and sediments from the Beijiang River, South China. Environ. Pollut. 219, 596–603.
Yadu, A., Sahariah, B.P., Anandkumar, J., 2016. Studies on biological degradation of 4-bromophenol using anaerobic, anoxic and aerobic bioreactors. J. Modern Chemistry Chem. Technol. 7, 37–41.
Yanxiao, Z., Jinsong, L., 2018. Emissions, environmental levels, sources, formation pathways, and analysis of polybrominated dibenzo-p-dioxins and dibenzofurans: a review. Environ. Sci. Pollut. Res. 25, 33082–33102.

Yildiz, S.Y., Kuru, P., Oner, E.T., Agirbasli, M., 2014. Functional stability of plasminogen activator inhibitor-1. Sci. World J. 2014, 858293.

Yu, G., Bu, Q., Cao, Z., Du, X., Xia, J., Wu, M., Huang, J., 2016. Brominated flame retardants (BFRs): A review on environmental contamination in China. Chemosphere 150, 479–490.

Zhang, Q., Liu, Y., Lin, Y., Kong, W., Zhao, X., Ruan, T., Liu, J., Schnoor, J., Jiang, G., 2019. Multiple metabolic pathways of 2,4,6-tribromophenol in rice plants. Environ. Sci. Technol. 53, 7473–7482.

Zhao, H., Jiang, J., Wang, Y., Xie, Q., Qu, B., 2017. Phototransformation of 2,4,6-tribromophenol in aqueous solution: kinetic sand photolysis products. J. Environ. Sci. Health A 52, 45–54.

Zhu, Q., Maeno, S., Nishimoto, R., Miyamoto, T., Fukushima, M., 2014. Oxidative degradation of pentabromophenol in the presence of humic substances catalyzed by a SBA-15 supported iron-porphyrin catalyst. J. Mol. Catal. A Chem. 385, 31–37.

# OŚWIADCZENIA WSPÓŁAUTORÓW PRAC

Łódź, dn. 24.11.2022

Mgr Anna Barańska
Katedra Biofizyki Skażeń Środowiska
Uniwersytet Łódzki

# Oświadczenie współautora

Dotyczy publikacji:

**Włuka Anna**, Woźniak Agnieszka, Woźniak Ewelina, Michałowicz Jaromir (2020) *Tetrabromobisphenol A, terabromobisphenol S and other bromophenolic flame retardants cause cytotoxic effects and induce oxidative stress in human peripheral blood mononuclear cells (in vitro study).* Chemosphere, 261(103):127705.

Oświadczam, że mój wkład w powstanie publikacji polegał na pomocy w opracowaniu koncepcji pracy oraz zaplanowaniu części eksperymentów. Jestem wykonawcą większości analiz dotyczących cytotoksycznego i oksydacyjnego wpływu badanych związków na jednojądrzaste komórki krwi obwodowej człowieka poza analizą uszkodzeń oksydacyjnych białek oraz części analiz dotyczących peroksydacji lipidów. Dokonałam również interpretacji i analizy statystycznej uzyskanych wyników oraz napisałam publikację. Mój udział oceniam na **60%**.

Anna Barańska (Włuka)

Łódź, dn. 24.11.2022

Mgr Agnieszka Woźniak
Klinika chorób wewnętrznych i farmakologii klinicznej
Uniwersytet Medyczny w Łodzi

# Oświadczenie współautora

Dotyczy publikacji:
Włuka Anna, **Woźniak Agnieszka**, Woźniak Ewelina, Michałowicz Jaromir (2020) *Tetrabromobisphenol A, terabromobisphenol S and other bromophenolic flame retardants cause cytotoxic effects and induce oxidative stress in human peripheral blood mononuclear cells (in vitro study).* Chemosphere, 261(103):127705.

Oświadczam, że mój wkład w powstanie publikacji polegał na wykonaniu części analiz dotyczących określenia potencjału oksydacyjnego badanych związków w jednojądrzastych komórkach krwi obwodowej człowieka. Mój udział oceniam na **5%.**

Agnieszka Woźniak

Łódź, dn. 24.11.2022

Dr Ewelina Woźniak
Klinika chorób wewnętrznych i farmakologii klinicznej
Uniwersytet Medyczny w Łodzi

# Oświadczenie współautora

Dotyczy publikacji:
Włuka Anna, Woźniak Agnieszka, **Woźniak Ewelina**, Michałowicz Jaromir (2020) *Tetrabromobisphenol A, terabromobisphenol S and other bromophenolic flame retardants cause cytotoxic effects and induce oxidative stress in human peripheral blood mononuclear cells (in vitro study).* Chemosphere, 261(103):127705.

Oświadczam, że mój wkład w powstanie publikacji polegał na pomocy merytorycznej w napisaniu publikacji. Mój udział oceniam na **5%.**

Ewelina Woźniak

Łódź, dn. 24.11.2022

Prof. dr hab. Jaromir Michałowicz
Katedra Biofizyki Skażeń Środowiska
Uniwersytet Łódzki

## Oświadczenie współautora

Dotyczy publikacji:
Włuka Anna, Woźniak Agnieszka, Woźniak Ewelina, **Michałowicz Jaromir** (2020) *Tetrabromobisphenol A, terabromobisphenol S and other bromophenolic flame retardants cause cytotoxic effects and induce oxidative stress in human peripheral blood mononuclear cells (in vitro study).* Chemosphere, 261(103):127705.

Oświadczam, że mój wkład w powstanie publikacji polegał na współudziale w opracowaniu koncepcji pracy oraz zaplanowaniu części eksperymentów. Ponadto dotyczył on pomocy w interpretacji uzyskanych wyników, pomocy merytorycznej oraz pomocy w napisaniu publikacji. Mój udział oceniam na **30%**.

J. Michałowicz

Łódź, dn. 24.11.2022

Mgr Anna Barańska
Katedra Biofizyki Skażeń Środowiska
Uniwersytet Łódzki

# Oświadczenie współautora

Dotyczy publikacji:

**Barańska Anna**, Woźniak Agnieszka, Mokra Katarzyna, Michałowicz Jaromir (2022) *Genotoxic mechanism of action of TBBPA, TBBPS and selected bromophenols in human peripheral blood mononuclear cells*. Frontiers in Immunology, 13:869741.

Oświadczam, że mój wkład w powstanie publikacji polegał na pomocy w opracowaniu koncepcji pracy oraz zaplanowaniu części eksperymentów. Jestem wykonawcą większości analiz dotyczących genotoksycznego wpływu badanych związków na jednojądrzaste komórki krwi obwodowej człowieka poza analizą uszkodzeń dwuniciowych DNA oraz naprawy DNA. Dokonałam również interpretacji i analizy statystycznej uzyskanych wyników oraz napisałam publikację. Mój udział oceniam na **65%**.

...Anna Barańska.....

Łódź, dn. 24.11.2022

Mgr Agnieszka Woźniak
Klinika chorób wewnętrznych i farmakologii klinicznej
Uniwersytet Medyczny w Łodzi

## Oświadczenie współautora

Dotyczy publikacji:
Barańska Anna, **Woźniak Agnieszka**, Mokra Katarzyna, Michałowicz Jaromir (2022) *Genotoxic mechanism of action of TBBPA, TBBPS and selected bromophenols in human peripheral blood mononuclear cells*. Frontiers in Immunology, 13:869741.

Oświadczam, że mój wkład w powstanie publikacji polegał na wykonaniu części analiz dotyczących genotoksycznego wpływu badanych związków w jednojądrzastych komórkach krwi obwodowej człowieka. Mój udział oceniam na **5%.**

Agnieszka Woźniak

Łódź, dn. 24.11.2022

Dr Katarzyna Mokra
Katedra Biofizyki Skażeń Środowiska
Uniwersytet Łódzki

# Oświadczenie współautora

Dotyczy publikacji:
Barańska Anna, Woźniak Agnieszka, **Mokra Katarzyna**, Michałowicz Jaromir (2022) *Genotoxic mechanism of action of TBBPA, TBBPS and selected bromophenols in human peripheral blood mononuclear cells*. Frontiers in Immunology, 13:869741.

Oświadczam, że mój wkład w powstanie publikacji polegał na pomocy merytorycznej w napisaniu publikacji. Mój udział oceniam na **5%**.

Katarzyna Mokra

Łódź, dn. 24.11.2022

Prof. dr hab. Jaromir Michałowicz
Katedra Biofizyki Skażeń Środowiska
Uniwersytet Łódzki

## Oświadczenie współautora

Dotyczy publikacji:
Barańska Anna, Woźniak Agnieszka, Mokra Katarzyna, **Michałowicz Jaromir** (2022) *Genotoxic mechanism of action of TBBPA, TBBPS and selected bromophenols in human peripheral blood mononuclear cells*. Frontiers in Immunology, 13:869741.

Oświadczam, że mój wkład w powstanie publikacji polegał na współudziale w opracowaniu koncepcji pracy oraz zaplanowaniu części eksperymentów. Ponadto dotyczył on pomocy w interpretacji uzyskanych wyników, pomocy merytorycznej oraz pomocy w napisaniu publikacji. Mój udział oceniam na **25%**.

J. Michałowicz

Łódź, dn. 24.11.2022

Mgr Anna Barańska
Katedra Biofizyki Skażeń Środowiska
Uniwersytet Łódzki

## Oświadczenie współautora

Dotyczy publikacji:

**Barańska Anna**, Sicińska Paulina, Michałowicz Jaromir (2022) *Apoptosis-inducing potential of selected bromophenolic flame retardants 2,4,6-tribromophenol and pentabromophenol in human peripheral blood mononuclear cells*. Molecules, 27(18):5056.

Oświadczam, że mój wkład w powstanie publikacji polegał na pomocy w opracowaniu koncepcji pracy oraz zaplanowaniu części eksperymentów. Jestem wykonawcą wszystkich analiz dotyczących apoptotycznego wpływu badanych związków na jednojądrzaste komórki krwi obwodowej człowieka. Dokonałam również interpretacji i analizy statystycznej uzyskanych wyników oraz napisałam publikację. Mój udział oceniam na **65%**.

.....Anna Barańska.....

Łódź, dn. 24.11.2022

Dr Paulina Sicińska
Katedra Biofizyki Skażeń Środowiska
Uniwersytet Łódzki

## Oświadczenie współautora

Dotyczy publikacji:
Barańska Anna, **Sicińska Paulina**, Michałowicz Jaromir (2022) *Apoptosis-inducing potential of selected bromophenolic flame retardants 2,4,6-tribromophenol and pentabromophenol in human peripheral blood mononuclear cells*. Molecules, 27(18):5056.

Oświadczam, że mój wkład w powstanie publikacji polegał na pomocy merytorycznej w napisaniu publikacji. Mój udział oceniam na **5%.**

Paulina Sicińska

Łódź, dn. 24.11.2022

Prof. dr hab. Jaromir Michałowicz
Katedra Biofizyki Skażeń Środowiska
Uniwersytet Łódzki

## Oświadczenie współautora

Dotyczy publikacji:
Barańska Anna, Sicińska Paulina, **Michałowicz Jaromir** (2022) *Apoptosis-inducing potential of selected bromophenolic flame retardants 2,4,6-tribromophenol and pentabromophenol in human peripheral blood mononuclear cells.* Molecules, 27(18):5056.

Oświadczam, że mój wkład w powstanie publikacji polegał na współudziale w opracowaniu koncepcji pracy oraz zaplanowaniu części eksperymentów. Ponadto dotyczył on pomocy w interpretacji uzyskanych wyników, pomocy merytorycznej oraz pomocy w napisaniu publikacji. Mój udział oceniam na **30%**.

J. Michałowicz

Łódź, dn. 24.11.2022

mgr Anna Barańska
Katedra Biofizyki Skażeń Środowiska
Uniwersytet Łódzki

## Oświadczenie współautora

Dotyczy publikacji:

**Barańska Anna,** Bukowska Bożena, Michałowicz Jaromir (2022) *Determination of apoptotic mechanism of action of Tetrabromobisphenol A and Tetrabromobisphenol S in human peripheral blood mononuclear cells*: A comparative study. Molecules, 27(18):6052.

Oświadczam, że mój wkład w powstanie publikacji polegał na pomocy w opracowaniu koncepcji pracy oraz zaplanowaniu części eksperymentów. Jestem wykonawcą wszystkich analiz dotyczących apoptotycznego wpływu badanych związków na jednojądrzaste komórki krwi obwodowej człowieka. Dokonałam również interpretacji i analizy statystycznej uzyskanych wyników oraz napisałam publikację. Mój udział oceniam na **65%**.

.....Anna Barańska.....

Łódź, dn. 24.11.2022

Prof. dr hab. Bożena Bukowska
Katedra Biofizyki Skażeń Środowiska
Uniwersytet Łódzki

## Oświadczenie współautora

Dotyczy publikacji:
Barańska Anna, **Bukowska Bożena**, Michałowicz Jaromir (2022) *Determination of apoptotic mechanism of action of Tetrabromobisphenol A and Tetrabromobisphenol S in Human Peripheral Blood Mononuclear Cells*: A Comparative Study. Molecules, 27(18):6052.

Oświadczam, że mój wkład w powstanie publikacji polegał na pomocy merytorycznej w napisaniu publikacji. Mój udział oceniam na **5%**.

B. Bukowska

Łódź, dn. 24.11.2022

Prof. dr hab. Jaromir Michałowicz
Katedra Biofizyki Skażeń Środowiska
Uniwersytet Łódzki

## Oświadczenie współautora

Dotyczy publikacji:
Barańska Anna, Bukowska Bożena, **Michałowicz Jaromir** (2022) *Determination of apoptotic mechanism of action of tetrabromobisphenol A and tetrabromobisphenol S in human peripheral blood mononuclear cells*: A comparative study. Molecules, 27(18):6052.

Oświadczam, że mój wkład w powstanie publikacji polegał na współudziale w opracowaniu koncepcji pracy oraz zaplanowaniu części eksperymentów. Ponadto dotyczył on pomocy w interpretacji uzyskanych wyników, pomocy merytorycznej oraz pomocy w napisaniu publikacji. Mój udział oceniam na **30%**.

J. Michałowicz

Łódź, dn. 24.11.2022

Prof. dr hab. Jaromir Michałowicz
Katedra Biofizyki Skażeń Środowiska
Uniwersytet Łódzki

## Oświadczenie współautora

Dotyczy publikacji:
**Michałowicz Jaromir**, Włuka Anna, Bukowska Bożena (2021) *A review on environmental occurrence, toxic effects and transformation of man-made bromophenols*. Science of the Total Environment, 811(126919):152289.

Oświadczam, że mój wkład w powstanie publikacji polegał na współudziale w opracowaniu koncepcji pracy. Ponadto dokonałem oceny merytorycznej pracy oraz napisałem część publikacji. Mój udział oceniam na **45%**.

J. Michałowicz

Łódź, dn. 24.11.2022

Mgr Anna Barańska
Katedra Biofizyki Skażeń Środowiska
Uniwersytet Łódzki

# Oświadczenie współautora

Dotyczy publikacji:
Michałowicz Jaromir, **Wluka Anna**, Bukowska Bożena (2021) *A review on environmental occurrence, toxic effects and transformation of man-made bromophenols.* Science of the Total Environment, 811(126919):152289.

Oświadczam, że mój wkład w powstanie publikacji polegał na współudziale w opracowaniu koncepcji pracy oraz napisaniu rozdziałów tj.: wstęp; właściwości fizykochemiczne, produkcja i zastosowanie bromofenoli; występowanie w środowisku; narażenie ludzi na bromofenole oraz części rozdziału dotyczącego toksyczności bromofenoli. Mój udział oceniam na **35%**.

....Anna Barańska (Wluka)

Łódź, dn. 24.11.2022

Prof. dr hab. Bożena Bukowska
Katedra Biofizyki Skażeń Środowiska
Uniwersytet Łódzki

## Oświadczenie współautora

Dotyczy publikacji:
Michałowicz Jaromir, Włuka Anna, **Bukowska Bożena** (2021) *A review on environmental occurrence, toxic effects and transformation of man-made bromophenols.* Science of the Total Environment, 811(126919):152289.

Oświadczam, że mój wkład w powstanie publikacji polegał na pomocy merytorycznej w napisaniu publikacji. Mój udział oceniam na **20%**.

B Bukowska
.....