J. Vržina, L. Ulm, V. Schiesl, M. Šateva

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PRIPREMA U VODI TEŠKO TOPLJIVIH TVARI ZA EKOTOKSIKOLOŠKA ISPITIVANJA

Sažetak

Metode za određivanje toksičnosti kemijskih tvari na vodene organizme, propisane organizacijama kao što su OECD, EU ili ISO odnose se na kemijske supstancije s točnim generičkim opisom, tj. propisuju ispitivanje vodotopljivih, kemijski stabilnih i nehlapljivih tvari. Međutim, ako se te metode primjenjuju za tvari koje ne podliježu navedenim svojstvima (tvari koje su samo djelomično ili slabo topljive u vodi, nestabilne, hlapljive ili kompleksnog sastava), mogu se očekivati teškoće u procjeni toksičnosti.

Prema sadašnjim saznanjima najbolja raspoloživa metoda za ispitivanje toksičnosti u vodi slabo topljivih kemijskih tvari je WAF metodologija (Water Accommodated Fraction). WAF predstavlja vodeni medij koji sadrži frakciju supstancije zaostale u vodenom mediju nakon miješanja i odvajanja faza.

Predmet naših ispitivanja su maziva teško topljiva u vodi od kojih neka imaju svojstvo intenzivnog prijanjanja uz podlogu i maziva ulja koja s vodom stvaraju emulziju. Prema svom sastavu to su maziva na osnovi sintetičkih estera i biljnih ulja različito ugušćeni sa sapunima ili organofilnim bentonitima. Ispitivana maziva miješana su s deioniziranom vodom i mineralnim medijem pomoću elektromagnetske mješalice i u vremenskom razdoblju od 24-96 sati miješanja praćeni su određeni parametri (koncentracija lipofilnih tvari, totalni organski ugljik i prisutnost organskih kiselina eventualno nastalih hidrolizom estera).

UVOD

Metode za testiranje u vodi teško topljivih kompleksnih supstancija predmet su ekotoksikoloških istraživanja posljednjih dvadeset godina. Primarno su ta istraživanja potaknuta potrebom procjene toksičnosti sirove nafte prilikom izlijevanja iz tankera. Kasnije se pokazala potreba za testiranjem širokog spektra proizvoda i razvoj međunarodnih zakonskih regulativa i to prema proizvodima kao što su: kemijski priozvodi koji se koriste u naftnoj i plinskoj industriji, priozvodi koji se prevoze brodskim prijevozom na moru i proizvodi rafinerija nafte. Suočeni s takvim razvojem važno je prihvatiti dosljedan pristup testiranju i interpretaciji rezultata.

Zakonska regulativa temelji se na direktivama EU za opasne supstancije 67/548/EEC. Daljnje direktive EU (Europska zajednica) iz 1992. i 1993. godine donose propise o određivanju akvatičkog toksiciteta na [1]:

- aktivni mulj – respiratorna inhibicija
- alge (72 h) test inhibicije rasta
- Daphnia (48 h) akutni test
- Daphnia (21 dan) reprodukcijski test
- ribe (96 h) akutni test
- ribe – kronični test

Ovi se propisi odnose na čiste supstancije i proizvode dobro topljive u vodi, dok je za kompleksne supstancije (objavljene prema EINECS ili ELINCS) [2] u koje spadaju ulja i maziva, bilo potrebno doraditi postojeće metode. Ti su postupci obrađeni i objavljeni preko CONCAWE i GESAMP te ECETOC [3] u suglasnosti s publikacijama EU.

Prema sadašnjim saznanjima najbolja raspoloživa metoda za ispitivanje toksičnosti u vodi slabo topljivih kemijskih tvari je WAF metodologija (Water Accommodated Fraction). WAF predstavlja vodeni medij koji sadrži onu frakciju proizvoda koja ostaje u vodenoj fazi nakon miješanja i vremenskog razdoblja dovoljnog za odvajanje faza.

WAF se priprema miješanjem poznate količine test supstancije u poznatom volumenu test medija. Količina test supstancije po jedinici volumena označava se kao “loading rate”. Miješanje treba biti snažno i kontinuirano kako bi se osigurala ravnoteža između vodene faze i supstancije koju ispitujemo, ali bez induciranja nastajanja emulzije. Praktična su iskustva pokazala da je vremensko razdoblje od 24 sata dovoljno za postizanje ravnoteže faza. Strategiju ispitivanja i interpretaciju rezultata razradili su autori Whitehouse i Mallet (1993.) a objavio ih je Chemical Notification Unit of the UK

Department of the Enviroment, a specijalne upute za testiranje kompleksnih ugljikovodičnih smjesa objavljene su u izvješću CONCAWE 1993. [1]. Također je važno poznavanje fizikalno-kemijskih svojstava, odnosno da li je ispitivana tvar ili proizvod stabilan u tijeku toksikološkog testiranja. Navedena strategija ispitivanja pomaže pri određivanju nepoznatih fizikalno kemijskih svojstava.

Rezultati ispitivanja moraju sadržavati sljedeće podatke:

- opis ispitivanog materijala
- test organizam
- tip medija za pripremu WAF
- tip sustava miješanja
- količina test materijala i medija
- volumen posude za miješanje
- otvorena ili zatvorena posuda
- tehnika miješanja uključujući brzinu vrtnje
- vrijeme trajanja miješanja / vrijeme separacije
- kvaliteta vode – pH, tvrdoća, salinitet, temperatura, na početku i kraju testa
- koncentracija kisika, na početku i kraju testa
- analitički rezultati

2. EKSPERIMENTALNI DIO

2.1 Uzorci maziva

Ispitivanja toksičnosti na vodene organizme izvršena su na sljedećim uzorcima maziva specifičnih svojstava:

Uzorak A: Mazivo na osnovi sintetičkog estera ugušćeno alumosilikatom i svojstvom jakog prianjanja na metalnu podlogu.
Uzorak B: Mazivo na osnovi biljnog ulja ugušćeno alumosilikatom i svojstvom jakog prianjanja na metalnu podlogu.
Uzorak C: Mazivo na osnovi sintetičkog estera ugušćeno litij-kalcij sapunom i jakim EP svojstvom.
Uzorak D: Mazivo na osnovi biljnog ulja ugušćeno litij sapunom.
Uzorak E: Mazivo ulje na osnovi sintetičkog estera.
Uzorak F: Mazivo ulje na osnovi biljnog ulja.

2.2.Instrumenti i pribor

Analizator ugljika SSM-5000 A Shimadzu
FTIR spektrometar, Paragon 1000, Perkin Elmer, infrasil kiveta 1cm

svjetlosnog puta
Precizna analitička vaga Mettler Toledo AB 204
Magnetske miješalice TMC 2072, Karl Hecht
Staklene čaše od 2l i ostalo laboratorijsko posuđe

2.3. Analitičke metode

Prilikom pripreme uzoraka vodootpornih maziva za ekotoksikološka ispitivanja korištene su metode:
Water Accommodated Fraction (WAF) [1] [3] [4] [5] [6].
DIN 38409 Teil 18-metoda za određivanje lipofilnih tvari u vodenoj frakciji [7].
EN 1484-metoda za određivanje ukupnog organskog ugljika (TOC) i otopljenog organskog ugljika (DOC) [8].

2.4. Priprema uzoraka za ekotoksikološka ispitivanja

Za pripremu WAF načinjena su najprije preliminarna ispitivanja topljivosti maziva u vodi. U tu svrhu upotrijebljene su veće odvage maziva, tj. odvagano je po 0,5 g maziva i miješano s 500 ml deionizirane vode u staklenoj čaši pomoću magnetske miješalice brzine miješanja 600 okretaja na minutu. Bilo je potrebno ustanoviti koje je vrijeme miješanja potrebno da se postigne ravnoteža faza između dijela maziva koje je otopljeno u vodi i dijela neotopljenog maziva. Premda je prema literaturnim podacima preporučeno vrijeme miješanja do postizavanja ravnoteže 24 h, ispitivana maziva miješana su do 96 sati, a ispitivanja vodene faze vršena su nakon 24, 48, 72 i 96 sati. Za svako vremensko razdoblje korištena je posebna odvaga maziva (0,5 g/500 ml deionizirane vode) te je nakon prekida miješanja i odvajanja faza od jednog sata za analizu uzimana bistra vodena otopina. S obzirom da je bilo potrebno ustanoviti koja se količina maziva otopila u vodi, određivana je količina ukupnih lipofilnih tvari metodom infracrvene spektrometrije [4]. Lipofilne tvari se iz vode ekstrahiraju s pogodnim otapalom (tetraklormetan) i ekstrakt se snimi prema čistom otapalu na infracrvenom spektrometru u valnom području od 3200–2800 cm^{-1}. Za kvantitativno određivanje lipofilnih tvari u dobivenom IR spektru korištene su veličine absorpcijskih vrpci kod 2958 cm^{-1}, 2924 cm^{-1} i 3030 cm^{-1}. Iz istog CCl_4 ekstrakta, otparavanjem otapala (CCl_4) određene su karboksilne kiseline eventualno nastale hidrolizom maziva esterskog tipa. Iz dobivenih IR spektara moglo se ustanoviti da se pored esterske apsorpcijske vrpce koja se javlja ovisno o mazivu u području od 1750–1735 cm^{-1}, javljaju se i apsorpcijske vrpce kod 1693,3 cm^{-1}, 1697,5 cm^{-1} i 1696 cm^{-1} koje se mogu pripisati prisutnosti karboksilnih kiselina.

Ove su se apsorpcijske vrpce javile samo u uzorcima C i D nakon 48, 72 i 96 sati. U istoj vodenoj frakciji uzoraka C i D, ali samo nakon 48 sati miješanja određene su titracijom lužinom slobodne masne kiseline. Nakon završenog miješanja maziva i vode u preliminarnom pokusu tijekom 96 sati i dobivenih rezultata svih analiza vodene frakcije ustanovljeno je da je za daljnja ispitivanja dovoljno razdoblje miješanja od 24 sata.

Daljnja ispitivanja za WAF metodologiju vršena su s manjom odvagom maziva tj. sa 100 mg maziva koje se miješalo s jednom litrom mineralnog medija za uzgoj Daphnia magna. Pokusi su izvedeni u staklenoj čaši od 2 litre uz miješanje pomoću magnetske mješalice brzinom miješanja od 600 okretaja na minutu. Mineralni medij za uzgoj Daphnia magna je vodena otopina soli: kalcij i kalij klorida, magnezij sulfata i natrij hidrogen karbonata [9]. Ova se priprema uzoraka svodi na 3 stupnja:

1. STUPANJ: Odvagano mazivo (100 mg/l mineralnog medija) miješa se 24 sata, a uzorak za ispitivanje toksičnosti na vodene organizme uzima se uz miješanje i **bez odvajanja** faza. Ako je rezultat ispitivanja EC_{50}>100 mg/l [10] nisu potrebna daljnja ispitivanja čime se procjenjuje da u akutnom testu na vodene organizme proizvod nije toksičan. U protivnom slučaju ispitivanje se nastavlja primjenom WAF – metodologije:

2. STUPANJ: Odvaga maziva od 100 mg/l mineralnog medija miješa se 24 sata i nakon jednog sata **odvajanja faza** uzima se **samo vodena faza** za ispitivanje toksičnosti na vodene organizme. Ako je ponovno EC_{50} >100 mg/l, nisu potrebna daljnja ispitivanja. U protivnom se ispitivanja nastavljaju.

3. STUPANJ: Postupkom WAF metodologije načini se **niz odvaga** maziva manjih od 100 mg i svaka se odvaga miješa 24 sata u mineralnom mediju. Nakon jednog sata odvajanja faza, uzima se vodena faza za ispitivanje toksičnosti na vodene organizme. Na taj način testiranjem niza koncentracija istog uzorka primjenjujući WAF postupak dobiva se točniji rezultat za EC_{50} nego da se test otopina priprema razrjeđivanjem one koja ima najviši "loading rate".

U našem smo eksperimentalnom dijelu primijenili sva tri stupnja ispitivanja. Svaka je vodena frakcija ispitana na sadržaj ukupnih lipofilnih tvari i sadržaj totalnog organskog ugljika, a rezultati su prikazani u tablicama 2 i 3.

REZULTATI

Rezultati ispitivanja uzoraka za ekotoksikološka ispitivanja prikazani su tablicama i IR spektrima.

Tablica 1: Sadržaj otopljenih lipofilnih tvari u vodenoj fazi **tijekom 96 sati** određenih metodom infracrvene spektroskopije (IR)
Table 1: The content of dissolved lipophilic substances in aqueous phase **during 96 hours**, determined by the Infrared Spectroscopy (IR) method

Otopljene lipofilne tvari u vodenoj fazi/Dissolved lipophilic substances in aqueous phase (%)						
Vrijeme miješanja Stirring time (h)	Uzorak Sample A	Uzorak Sample B	Uzorak Sample C	Uzorak Sample D	Uzorak Sample E	Uzorak Sample F
24	13,04	0,68	1,80	0,40	58,10	31,56
48	12,40	0,76	2,12	0,48	59,31	29,80
72	13,00	0,68	2,08	0,46	57,65	29,80
96	12,26	0,84	2,20	0,48	58,10	24,10

Određivanjem slobodnih masnih kiselina u vodi nakon 24 sata miješanja ustanovljeno je da hidrolizom nisu nastale slobodne masne kiseline. U vodenom mediju nastalom miješanjem uzorka C nakon 48, 72 i 96 sati u IR spektru pored esterske absorpcijske vrpce javljaju se absorpcijske vrpce koje upućuju na prisutnost kiselina. U tim vodenim uzorcima titracijom je također potvrđena prisutnost slobodnih masnih kiselina, ali u vrlo maloj količini.

Tablica 2: Sadržaj otopljenih lipofilnih tvari u vodenoj fazi **nakon 24 sata** miješanja određenih metodom infracrvene spektroskopije (IR)
Table 2: The content of dissolved lipophilic substances in aqueous phase after **24 hours** of stirring, determined by the Infrared Spectroscopy (IR) method

Otopljene lipofilne tvari u vodenoj fazi/Dissolved lipophilic substances in aqueous phase (%)						
Odvaga maziva lubricant content (mg/l)	Uzorak Sample A	Uzorak Sample B	Uzorak Sample C	Uzorak Sample D	Uzorak Sample E	Uzorak Sample F
100 (stupanj/degree 1)	9,4	1,0	1,4	1,3	77,9	27,2
100 (stupanj/degree 2)	10,0	1,3	1,5	2,1	12,6	3,7

Tablica 3: Sadržaj totalnog organskog ugljika (TOC) u vodenoj fazi nakon 24 sata miješanja
Table 3: Total Organic Carbon (TOC) content in aqueous phase after 24 hours of stirring

Totalni organski ugljik/Total Organic Carbon (TOC) (%)						
Odvaga maziva lubricant content (mg/l)	Uzorak Sample A	Uzorak Sample B	Uzorak Sample C	Uzorak Sample D	Uzorak Sample E	Uzorak Sample F
100 (stupanj/degree 1)	62,6	28,0	23,1	22,3	58,1	34,8
100 (stupanj/degree 2)	29,8	5,19	4,63	4,64	18,6	1,73

DISKUSIJA

Za WAF-metodologiju u preliminarnom ispitivanju određen je sadržaj lipofilnih tvari metodom infracrvene spektrometrije (IR) u vodenoj frakciji nakon 24, 48, 72 i 96 sati miješanja maziva u vodi (tablica 1). S obzirom da je njihov sadržaj bio gotovo konstantan u svim izvršenim mjerenjima, zaključeno je da je vremensko razdoblje od 24 sata dovoljno za postizanje ravnoteže faza. Iz istih ekstrakata lipofilnih tvari pristupilo se određivanju slobodnih masnih kiselina kako bi se ustanovila eventualna hidroliza maziva esterskog tipa u tijeku pokusa. Rezultati su pokazali da nakon 24 sata miješanja niti jedno mazivo na osnovi estera nije hidroliziralo, dok su nakon 48, 72 i 96 sati miješanja nastale slobodne masne kiseline samo u uzorcima C i D. U IR spektrima (slika 1-4) uzorka C vidljive su apsorpcijske vrpce karboksilnih kiselina.

Daljnja ispitivanja odnosila su se na određivanje otopljenih lipofilnih tvari i totalnog organskog ugljika u vodenoj fazi nakon 24 sata miješanja uzoraka maziva i mineralnog medija (za uzgoj *Daphnia magna*) **bez vremenskog razdoblja za odvajanje faza (stupanj 1)**. Za određivanje potrebnih parametara u drugom stupnju primijenjena je WAF metodologija tj. 24-satno miješanje i **vremensko razdoblje od 1 sata potrebnog za odvajanje faza**. Temeljem dobivenih rezultata može se zaključiti da za uzorke A-D ne postoji značajna razlika u sadržaju lipofilnih tvari u vodenoj fazi nakon 1. i 2. stupnja ispitivanja (tablica 2). Nasuprot tome određivanjem totalnog organskog ugljika (tablica 3) postoji znatna razlika između prvog i drugog stupnja ispitivanja što ukazuje da je za takvu vrstu maziva potrebno primijeniti WAF metodologiju. Nadalje, kod uzoraka E i F koja imaju bitno različita fizikalno-kemijska svojstva (s vodom stvaraju blagu emulziju) postoji razlika između prvog i drugog stupnja ispitivanja izraženih i preko sadržaja lipofilnih tvari i preko sadržaja totalnog organskog ugljika u vodenom mediju.

ZAKLJUČAK

Na temelju dobivenih eksperimentalnih podataka za pripremu uzoraka maziva za ekotoksikološka ispitivanja proizlaze sljedeći zaključci:

1. WAF (Water Accommodated Fraction) predstavlja najbolju raspoloživu metodu za ispitivanje toksičnosti maziva teško topljivih u vodi.
2. Primjenom WAF metodologije za pripremu uzoraka za ekotoksikološka ispitivanja ustanovljeno je vremensko razdoblje od 24 sata kao optimalno vrijeme miješanja maziva i mineralnog medija do postizanja ravnoteže faza.

3. Rezultati određivanja sadržaja lipofilnih tvari i totalnog organskog ugljika u vodenom mediju uzoraka maziva ukazuju da je određivanje totalnog organskog ugljika bolji pokazatelj sadržaja otopljene organske tvari u vodi.

Slika 1: Uzorak C: IR spektar originalnog uzorka
Figure **1**: Sample C: Original sample IR spectrum

Slika 2: Uzorak C: IR spektar ekstrakta nakon 24 sata miješanja
Figure 2: Sample C: Extract IR spectrum after 24 hours of stirring

Slika 3: Uzorak C: IR spektar ekstrakta nakon 48 sata miješanja
Figure 3: Sample C: Extract IR spectrum after 48 hours of stirring

Slika 4: Uzorak C: IR spektar ekstrakta nakon 72 sata miješanja
Figure 4: Sample C: Extract IR spectrum after 72 hours of stirring

PREPARATION OF WATER-RESISTANT SUBSTANCES FOR ECOTOXICOLOGICAL TESTING

Abstract

Test methods for assessment toxicity chemical substances to aquatic organisms have been described by various organizations including the OECD, EU or ISO. Those test

methods are typically designed for substances with precise generic description and substances, which are readily water soluble, chemically stable and non-volatile. When test methods are applied to substances with different chemical properties (sparingly soluble, unstable or volatile substances or those of a complex composition) it is possible to expect some difficulties in toxicity evaluation.

According to present knowledge the best test method for toxicity examination of water-resistant substances is WAF methodology (Water Accommodated Fraction). WAF is an aqueous medium containing only that fraction of a substance which remains in the aqueous phase after mixing and phase separation.

The subjects for our examination were water-resistant greases (some of them have very strong adhesive characteristics) and lubricant oils which form emulsion with water. According to their chemical composition there are synthetic based esters and plant oils thickned with soaps and organophylic bentonits. The greases were mixed with deionised water and mineral media on magnetic stirrer and during the time period of 24-96 hours some parameters were defined (concentration of lipophylic substances, total organic carbon and organic acids eventually made by ester hydrolyzis).

INTRODUCTION

Test methods for complex water-resistant substances have been the subject of ecotoxicological testing for the past twenty years. The research was initially caused by the need to evaluate crude oil toxicity in cases of oil spills from tankers. Later on, the need arose to test a wide variety of products, given the development of international legal regulations, including products such as: Chemical products used in oil and gas industry, products transported across the sea by ships, and oil refinery products. Viewing such development, it is important to adopt a proper approach to tests and results interpretation.

Legal regulations are based on EU Directives for Harmful Substances 67/548/EEC. Further EU (European Union) Directives, from 1992 and 1993 respectively, bring regulations on determining aquatic toxicity on /1/:

- active sludge - respiratory inhibition
- algae (72 h) growth inhibition test
- Daphnia (48 h) acute test
- Daphnia (21 days) reproductive test
- fish (96 h) acute test
- fish - cronical test

These regulations refer to pure substances and readily water- soluble products. For complex substances, however, (listed according to EINECS or ELINCS /2/, including oils and lubricants, it was necessary to advance the existing methods. These procedures have been processed and released according to CONCAWE and GESAMP, as well as ECETOC /3/, in compliance with EU publications.

According to our present knowledge, the best method for testing the toxicity of water-resistant chemical substances is the WAF (Water Accommodated Fraction) methodology. WAF constitutes an aqueous medium containing the fraction of the product remaining in aqueous phase after mixing and phase separation.

WAF is prepared by mixing the known quantity of test substance with the known test medium volume. Test substance quantity per volume unit is conceived as the loading rate. The mixing has to be aggressive and continuous, in order to maintain the balance between the aqueous phase and the test substance, but without any emulsion generation induction. Experience has shown that 24 hours are enough to achieve phase balance. Whitehouse and Mallet (1993) have elaborated test strategy and results interpretation, which have been published in the Chemical Notification Unit of the UK Department of the Environment, while the special guidelines for testing complex hydrocarbon blends have been published in the CONCAWE report in 1993. /1/. It is also important to know the physico-chemical properties, i.e. whether the tested substance or product remains stable in the course of toxicological tests. The said test strategy helps to determine the unknown physico-chemical properties.

Test results must include the following information:

- tested material description
- test organism
- medium type for WAF preparation
- mixing system type
- test material and medium quantity

- stirring container volume
- whether container is opened or closed
- stirring technique, including rotation velocity
- mixing duration/separation time
- water quality - pH, hardness, salinity, temperature at the beginning and at the end of the test
- oxygen concentration at the beginning and at the end of the test
- analytical results

2. THE EXPERIMENTAL PART

2.1. Lubricant Samples

The testing of toxicity for aquatic organisms has been performed on the following lubricant samples, with specific properties:

Sample A: Lubricant based on synthetic ester, thickened with alumosilicate, highly adhesive to metal surfaces.
Sample B: Lubricant based on vegetable oil, thickened with alumosilicate, highly adhesive to metal surfaces.
Sample C: Lubricant based on synthetic ester, thickened with lithium-calcium soap, with pronounced EP properties.
Sample D: Lubricant based on vegetable oil, thickened with lithium soap.
Sample E: Lubricant oil based on synthetic ester.
Sample F: Lubricant oil based on vegetable oil.

2.2. Instruments & Tools

Carbon analyzer SSM-5000 A Shimadzu
FTIR spectrometer, Paragon 1000, Perkin Elmer, infrasyl cuvette, 1 cm of light path
Precision analytical balance Mettler Toledo AB 204
Magnetic stirrers TMC 2072, Karl Hecht
2 l glasses (made of glass), and other laboratory vessels

2.3. Analytical Methods

While preparing samples of water-resistant lubricants for ecotoxicological testing, we have used the following methods:
Water Accommodated Fraction (WAF) /1/ /3/ /4/ /5/ /6/
DIN 38409 Teil 18 - method for identifying lipophylic substances in water fraction /7/

EN 1848 - method for determining Total Organic Carbon (TOC) and Dissolved Organic Carbon (DOC) /8/

2.4. Preparation of Samples for Ecotoxicological Testing

In the scope of preparations for WAF, preliminary tests of lubricant solubility in water were conducted first. Somewhat larger lubricant volumes were used for this purpose, i.e. 0,5 g of the lubricant was mixed with deionized water in a glass, using magnetic stirrer with 600 rpm. It was necessary to determine how long does it take to stir in order to achieve phase balance between the part of the lubricant dissolved in water and the undissolved part of the lubricant. Although references recommend stirring in duration of 24 h in order to achieve the said balance, the tested lubricants were stirred for up to 96 hours, while the aqueous phase tests were performed after 24, 48, 72, and 96 h. A new lubricant amount was used for each period (0.5 g/500 ml od deionized water). After the stirring was completed and the phases separated for an hour, the clear water solution was taken for analysis. Since it was necessary to establish which amount of the lubricant was dissolved in water, we have used Infrared Spectrometry /4/ in order to establish the quantity of total lipophilic substances. Lipophilic substances were extracted from water using a suitable solvent (tetrachlormethane), and the extract shot - in comparison with the clean solvent - on an infrared spectrometer in the wavelength area of 3,200-2,800 cm^{-1}. For a quantitative determination of lipophilic substances in the obtained IR spectrum, we have used absorption bands of the following sizes: 2,958 cm^{-1}, 2,924 cm^{-1}, and 3,030 cm^{-1}. From the same CCl_4 extract, through solvent (CCl_4) evaporation, we have determined carboxylic acids possibly generated through hydrolysis of the ester-based lubricant. From the IR spectra obtained, we were able to establish that, apart from the absorption bands that appear, depending on the lubricant, in the 1,750-1,735 cm^{-1} area, there were also absorption bands appearing at 1,693.3 cm^{-1}, 1,697.5 cm^{-1}, and 1,696 cm^{-1}, pointing to the presence of carboxylic acids. These absorption bands appeared only in C and D samples, after 48, 72, and 96 hours respectively. In the same water fraction of C and D samples, but only after 48 hours of stirring, free fatty acids were determined through titration with alkali. After the stirring of lubricant and water was completed in a preliminary test during 96 hours, and after results were obtained for all water fraction analyses, it was established that the stirring period of 24 hours is sufficient for further tests.

Further tests for WAF methodology were performed using a smaller lubricant amount i.e. 100 g, mixed with a litre of mineral medium for the cultivation of Daphnia magna. The tests were performed in a 2 l glass, using a magnetic stirrer of 600 rpm. The mineral medium for the cultivation of Daphnia magna is a water saline solution: Calcium and potassium chloride, magnesium sulphate and sodium hydrogen carbonate /9/. The sample preparation proceeds in the following 3 degrees:

1st DEGREE: The weighed lubricant (100 mg/l of mineral medium) is stirred for 24 hours, while the sample for testing the toxicity for aquatic organisms is taken with stirring and **without phase separation**. If the test result is EC_{50} > 100 mg/l /10/, no further tests are necessary. This means that the product has been evaluated as non-toxic for aquatic organisms in the acute test. If this is not the case, the test is continued by the application of WAF methodology:

2nd DEGREE: Lubricant in the amount of 100 mg/l of the mineral medium is stirred for 24 hours. After one hour of **phase separation**, **only the aqueous phase** is taken, in order to test its possible toxicity for aquatic organisms. If the result is EC_{50} > 100 mg/l, again, no further tests are necessary. If this is not the case, they are continued.

3rd DEGREE: Using WAF methodology, **several lubricant amounts** lower than 100 mg are taken, and each one of them is stirred for 24 hours in a mineral medium. After one hour of phase separation, the aqueous phase is taken in order to test its possible toxicity for aquatic organisms. That is how, by testing a series of concentrations of the same sample, using WAF procedure, more accurate results for EC_{50} are obtained than if the test solution were prepared by diluting the one with the highest loading rate.

In our experimental part, we have applied all three degrees of testing. Each water fraction was tested in terms of total lipophilic substances content and Total Organic Carbon content, while the results are shown in tables 2 and 3.

RESULTS

The results of testing samples for exotoxicological testing are shown in tables and through IR spectra.

By determining free fatty acids in the water, after 24 h of stirring, it has been established that no free fatty acids were generated through hydrolysis. In the water medium generated by stirring Sample C after 48, 72, and 96 h, the IR spectrum has revealed, apart from ester absorption bands, also the absorption bands pointing to the presence of acids. In these water samples,

titration has also confirmed the presence of free fatty acids, although in very low volumes.

DISCUSSION

In preliminary tests for WAF methodology, the content of lipophilic substances was determined using the method of Infrared Spectrometry (IR) in water fraction, after 24, 48, 72, and 96 hours of lubricant stirring in water (Table 1). Since their content was nearly constant in all performed measurements, it has been concluded that 24 hours are enough to achieve phase balance. In the same extracts of lipophilic substances, the content of free fatty acids was determined, in order to identify the possible hydrolysis of ester-based lubricants during the test.

The results have shown that, after 24 hours, there was no hydrolysis of any of the ester-based lubricants, while, after 48, 72, and 96 hours of stirring, free fatty acids were generated only in samples C and D. IR spectra (Figures 1-4) of sample C show carboxylic acids absorption bands.

Further tests concerned the identification of dissolved lipophilic substances and Total Organic Carbon in aqueous phase after 24 hours of mixing lubricant samples and mineral medium (for the cultivation of *Daphnia magna*) **without the phase separation period (1st degree).** In order to determine the necessary parameters, the 2nd degree included the application of WAF methodology i.e. 24-hour stirring **and the period of 1 hour necessary for phase separation**.

Based on the results obtained, we may conclude that, for samples A-D, there is no major difference in the content of lipophilic substances in aqueous phase after the 1st and 2nd degree of testing (Table 2). On the other hand, when it comes to the determination of Total Organic Carbon (Table 3), there is a major difference between the 1st and the 2nd degree of testing, which means that, for such lubricants, it is necessary to apply WAF methodology. Furtherly, when it comes to samples E and F, which have considerably different physico-chemical properties (they create a mild emulsion with water), there is a difference between the 1st and the 2nd degree of testing expressed both through the content of lipophilic substances, and through Total Organic Carbon content in the aqueous medium.

CONCLUSION

Based on the experimental data obtained for the preparation of lubricant samples for ecotoxicological testing, we may conclude the following:

1. WAF (Water Accompanied Fraction) represents the best possible method available for testing the toxicity of water-resistant lubricants.
2. By applying WAF methodology for the preparation of samples for ecotoxicological testing, the period of 24 hours has been set as optimal for the mixing of lubricant and mineral medium, until phase balance is achieved.
3. The results of determining the content of lipophilic substances and Total Organic Carbon in the aqueous medium of lubricant samples have shown that the determination of Total Organic Carbon is a better indicator of the dissolved organic substance content in water.

Literatura/References:

1. ECETOC Monograph No.26 Aquatic Toxicity Testing of Sparingly Soluble, Volatile and Unstabile Substances, Brusseles, September 1996.
2. EINECS ili ELINCS, CONCAWE Report no. 95/59
3. GIRLING, A.E., WHALE, G.F., ADEMA, D.M.: *Chemosphere*, 29, 2645.-2649., 1994.
4. GIRLING, A.E.: *Chemosphere*, 19, 1635.-1641., 1989.
5. BENNETT D., GIRLING A.E., BOUNDS, A.: *Chemosphere,* 21, 659.-669., 1990.
6. CONCAWE: Ecotoxicological Testing of Petroleum Products: Test Methodology. R.No. 92/56 1993.
7. DIN 38409 Teil 18: Bestimmung von Kohlenwasserstoffen
8. EN 1484 Anleitungen zur Bestimmung des gesamten organischen Kohlenstoffs (TOC) und des gelösten organischen Kohlenstoffs (DOC)
9. ISO 6341:1996 Water quality-Determination of the inhibition of the mobility *of Daphnia magna Straus* (*Cladocera, Crustacea*)-Acute toxicity test
10. Official Journal of the European Communities No L 110A/68, 1993: Classification on the basis of environmental effects

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543.053 priprema uzorka vodom izlučene frakcije (WAF) teško topljive tvari	sampling by water accomodated fraction (WAF) of sparingly soluble substances
543.39 određivanje toksičnosti teško topljivih sastojaka vode	toxicity determination of sparingly soluble water constituents
665.765.035.7 mazive masti, toksična svojstva	toxicity properties of lubricating greases

Autori/Authors:
[1]J. Vržina, [1]L. Ulm, [1]V. Schiesl, [2]M. Šateva
[1] Zavod za javno zdravstvo grada Zagreba, Zagreb [2] Ivasim, Ivanić Grad

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