生態影響に関する化学物質審査規制／試験法セミナー（平成 25 年度）

＜東京＞　日時：平成 26 年 2 月 10 日（月）13:30～16:55
場所：津田ホール　3 階　ホール

＜大阪＞　日時：平成 26 年 2 月 14 日（金）13:30～16:55
場所：新梅田研修センター　本館 4 階　405 ホール

主催：環境省・（独）国立環境研究所

協力：日本環境毒性学会

【目次】

【プログラム】

（敬称略）

時　間	内　　容	講演者等
13:00～	受付	
13:30～13:35	開会挨拶	環境省
【第１部】　化学物質審査規制に関する動向		
13:35～14:05	化学物質審査規制法の 施行状況等について	環境省総合環境政策局環境保健部企画課 化学物質審査室 室長　木村　正伸（東京会場） 室長補佐　草川　祐介（大阪会場）
【第２部】　生態毒性試験及び生態毒性 QSAR に関する事項		
14:05～14:50	生態毒性に係る OECD テストガイドライン 210・211 改定について	鑪迫　典久 （独）国立環境研究所環境リスク研究センター
14:50～15:05	休憩	
15:05～15:35	生態毒性試験毒性値算出に当たっての統計的な留意点について	小田　重人 （独）国立環境研究所環境リスク研究センター
15:35～16:05	生態毒性試験実施に当たっての留意点について	菅谷　芳雄 （独）国立環境研究所環境リスク研究センター
16:05～16:35	生態毒性ＱＳＡＲモデル 「ＫＡＴＥ」について	蓮沼　和夫 （独）国立環境研究所環境リスク研究センター
16:35～16:50	総合質疑	
16:50～16:55	閉会挨拶	（独）国立環境研究所

＊各講演には質疑応答が含まれます。
＊プログラムの内容及び講演者は予告なく変更になることがあります。ご了承ください。

平成25年度生態影響に関する
化学物質審査規制／試験法セミナー

化学物質審査規制法の施行状況等について

平成26年2月10日(月)/14日(金)
環境省環境保健部企画課化学物質審査室

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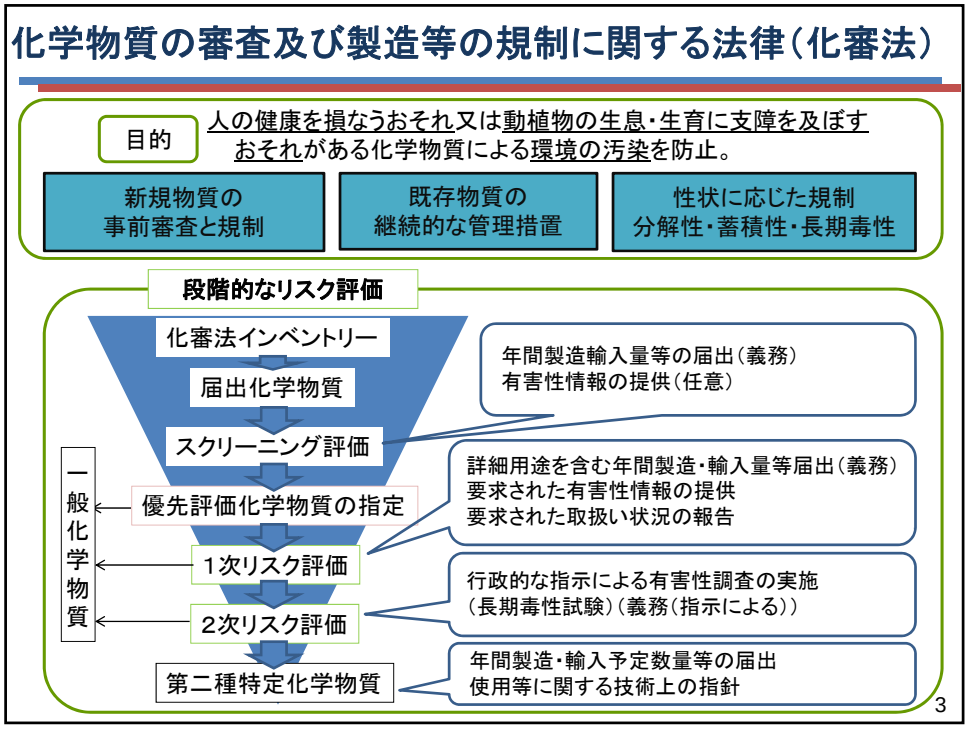
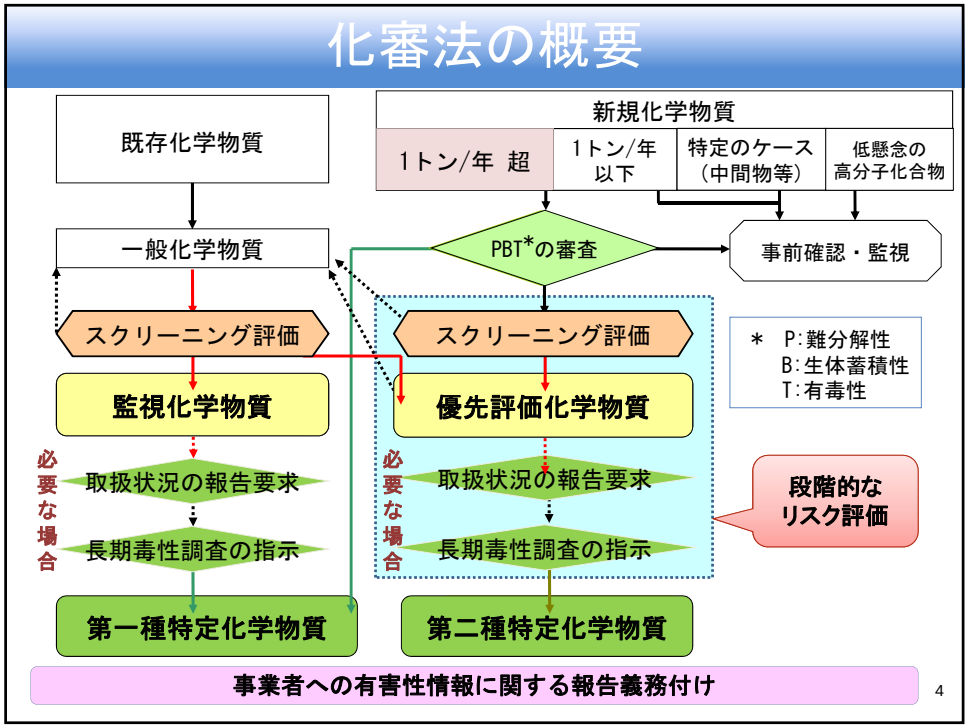

化学物質の審査及び製造等の規制に関する法律（化審法）
目的
人の健康を損なうおそれ又は動植物の生息・生育に支障を及ぼすおそれがある化学物質による環境の汚染を防止。
新規物質の事前審査と規制
既存物質の継続的な管理措置
性状に応じた規制 分解性・蓄積性・長期毒性
段階的なリスク評価
化審法インベントリー
届出化学物質
スクリーニング評価
優先評価化学物質の指定
1次リスク評価
2次リスク評価
第二種特定化学物質
一般化学物質
年間製造輸入量等の届出（義務）
有害性情報の提供（任意）
詳細用途を含む年間製造・輸入量等届出（義務）
要求された有害性情報の提供
要求された取扱い状況の報告
行政的な指示による有害性調査の実施
（長期毒性試験）（義務（指示による））
年間製造・輸入予定数量等の届出
使用等に関する技術上の指針
3


化審法の概要
既存化学物質
一般化学物質
スクリーニング評価
監視化学物質
必要な場合
取扱状況の報告要求
長期毒性調査の指示
第一種特定化学物質
新規化学物質
1トン/年 超
1トン/年 以下
特定のケース（中間物等）
低懸念の高分子化合物
PBT*の審査
事前確認・監視
スクリーニング評価
優先評価化学物質
必要な場合
取扱状況の報告要求
長期毒性調査の指示
第二種特定化学物質
＊ P:難分解性
B:生体蓄積性
T:有毒性
段階的なリスク評価
事業者への有害性情報に関する報告義務付け
4

規制対象物質の指定状況

H26年2月1日現在

規制対象物質の種類	定義	指定物質数
第一種特定化学物質	難分解性、高蓄積性、人又は高次捕食動物への長期毒性	28※
第二種特定化学物質	人又は生活環境動植物への長期毒性、相当広範な地域の環境中に相当程度残留	23
監視化学物質	難分解性、高蓄積性、人又は高次捕食動物への長期毒性は不明	38
優先評価化学物質	低蓄積性、第二種特定化学物質の有害性要件（人又は生活環境動植物への長期毒性）に該当しないことが明らかであるとは認められない、環境中に相当程度残留	160

※現在、第一種特定化学物質にエンドスルファン及びヘキサブロモシクロドデカンを追加すること等に関してパブリックコメントを実施中（平成26年2月15日（土）まで）。

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新規化学物質の審査

新規化学物質の届出又は審査の特例

	内容
少量新規化学物質	国内での年間の製造・輸入量の予定数量が一トン以下で既知見等から判断して環境の汚染が生じて人の健康又は生活環境動植物の生息等に関わる被害を生ずるおそれがない旨の確認を三大臣より受けた物質
中間物等	予定されている取り扱い方法からみて、その新規化学物質による環境の汚染が生じるおそれがないものとして、政令で定める場合（中間物、閉鎖系等用途、輸出専用品）に該当する旨の三大臣の確認を受けた物質
低懸念高分子化学物質	高分子化合物であって、これによる環境の汚染が生じて人の健康又は生活環境動植物の生息等に関わる被害を生ずるおそれがないものとして三大臣の確認を受けた物質
低生産新規化学物質	国内の一年間の製造・輸入予定数量が年間十トン以下の新規化学物質について、事前の審査の対象とした上で、難分解性であるものの高蓄積性ではないとの判定・通知を受けた場合には、十トン以下であること等について三大臣が事前の確認を行うとともに、事後の監視（報告徴収や立ち入り検査）がなされることを前提に、製造・輸入ができることとする物質

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化学物質審査小委員会の判定結果について

平成25年の化学物質審査小委員会では、346件（通常211件、低生産135件）の新規化学物質について判定を行った。

		130回（1月）	131回（3月）	132回（4月）	133回（5月）	134回（6月）	135回（7月）	136回（9月）	138回（10月）	139回（11月）	140回（12月）
審議件数		36	45	36	32	41	24	48	19	26	39
判定結果	第4条第一項第1号（難分解性かつ高蓄積性かつ人健康影響の疑い又は生態影響あり	0	0	0	0	0	0	0	0	0	0
	第2号（難分解性かつ人健康影響の疑いあり（高蓄積性でない）	1	2	1	0	0	1	4	2	0	0
	第3号（難分解性かつ生態影響あり（高蓄積性でない）	0	1	0	0	2	0	6	0	0	0
	第4号（難分解性かつ人健康影響の疑いあり・生態影響あり（高蓄積性でない）	1	3	0	2	6	5	1	2	1	3
	第5号（疑いなし又は良分解性	20	19	18	17	17	10	21	5	14	26
	第5条第二項第1号（難分解性（高蓄積性でない）毒性不明	14	20	17	13	16	8	16	10	11	10

※第5条第二項第1号は低生産量新規化学物質（製造輸入数量10トン以下）の確認対象物質
新規化学物質として届け出られた物質のうち、同一物質の届出については除いている。

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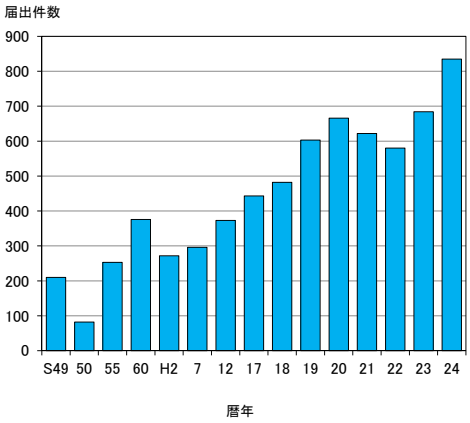
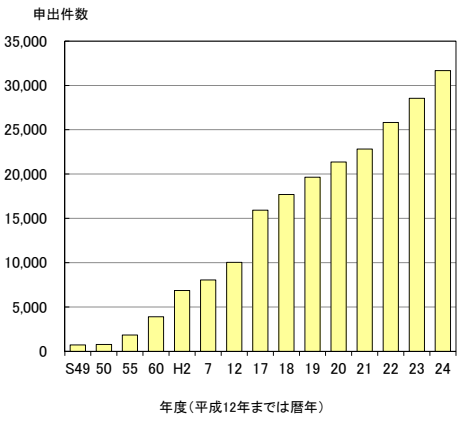
化審法の施行状況　新規化学物質の事前審査①

○新規化学物質の届出件数は増加傾向にあり、平成24年度の届出件数は835件。
○平成24年度の少量新規化学物質の申出件数は31,673件。

新規化学物質の届出件数



少量新規化学物質の申出件数



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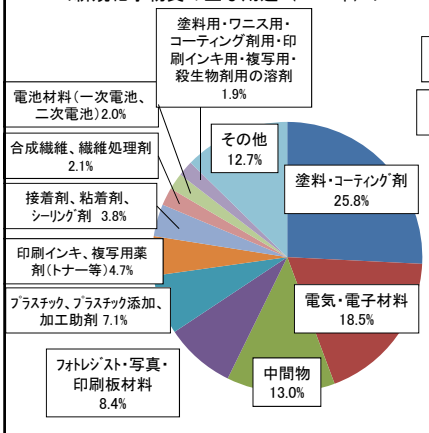
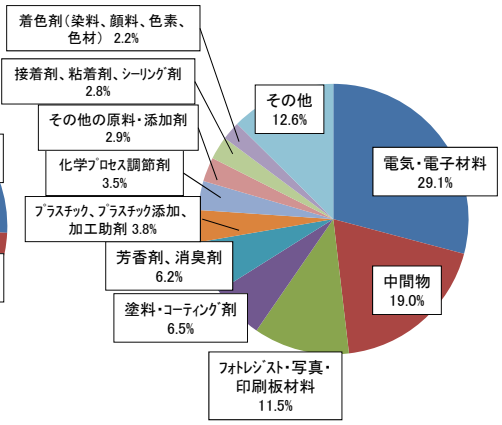
化審法の施行状況　新規化学物質の事前審査②

○新規化学物質、少量新規化学物質の用途分類は以下のとおり。

＜新規化学物質の主な用途（２４年）＞



＜少量新規化学物質の主な用途（２４年度）＞



出典：経済産業省「化審法の施行状況（平成24年）」

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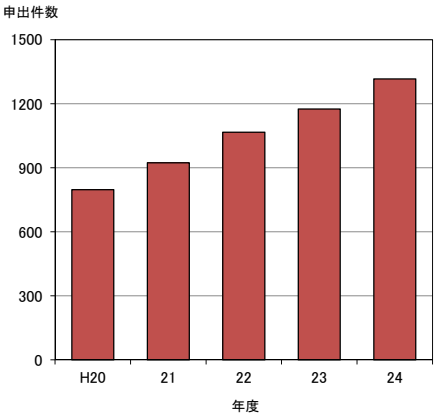
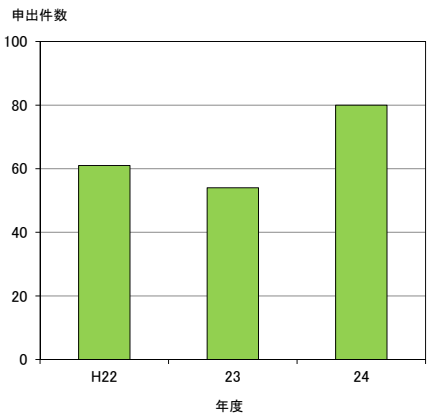
化審法の施行状況　新規化学物質の事前審査③

○低生産量新規化学物質の申出件数も増加傾向にあり、平成24年度の申出件数は1,316件。
○平成22年4月より運用が開始された低懸念高分子化合物の平成24年度の確認件数は80件。

低生産量新規化学物質の申出件数



低懸念高分子化合物の確認件数



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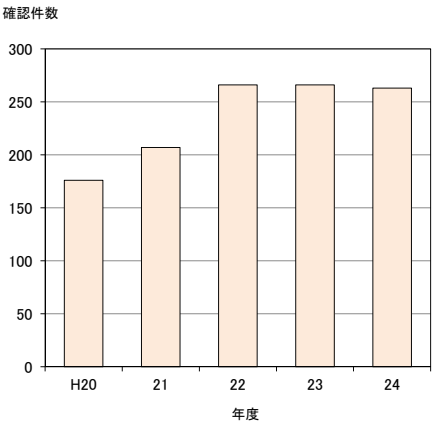
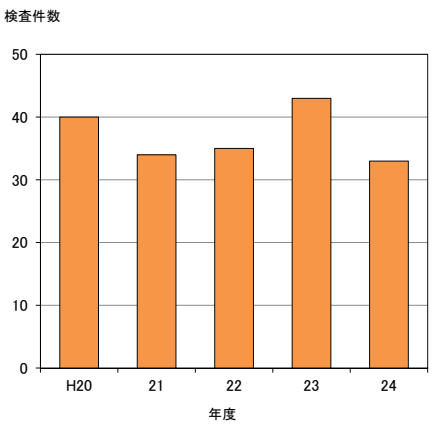
化審法の施行状況　新規化学物質の事前審査④

○中間物等の確認件数について、平成24年度の確認件数は263件。
○平成24年度の中間物等の事業所への立入検査件数は33件。

中間物等の確認件数



中間物等の立入件数



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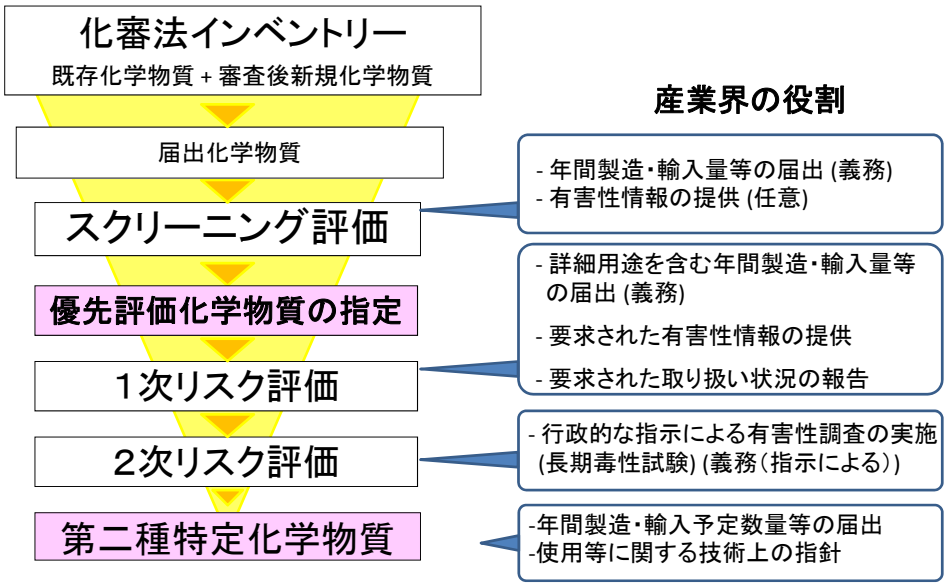
スクリーニング評価及びリスク評価の進捗状況

化審法に基づく段階的なリスク評価



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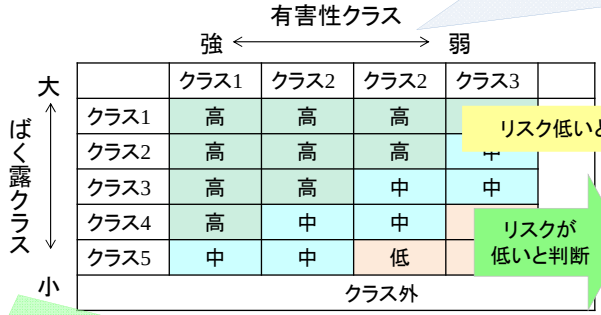
（1）スクリーニング評価手法

【人・健康】
一般毒性、生殖発生毒性、変異原性、発がん性から有害性クラスを設定
【生態】
水生生物の生態毒性試験データ（藻類・甲殻類・魚類）から有害性クラスを設定



リスク低いと判断できない
リスクが低いと判断
一般化学物質
（暴露クラスを毎年更新してスクリーニングを毎年再評価）
優先評価化学物質
さらなるリスク評価へ

【物化性状】
・製造・輸入数量等の届出情報
・分解性の判定結果
・全国総排出量を概算（毎年更新）

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①スクリーニング評価結果　暴露クラス

評価対象物質
（届出された11,979物質のうち、製造輸入数量が10t超の物質）　7,819物質

有害性評価の観点		人健康	生態
暴露クラス（平成24年度届出実績の確定値）	1	14物質	11物質
	2	67物質	48物質
	3	322物質	220物質
	4	744物質	551物質
	5	1,336物質	988物質
	外	5,336物質	6,001物質

○化審法に基づき事業者等より届出のあった製造/輸入数量及び用途分類並びにスクリーニング評価用の排出係数から推計される全国合計排出量に、分解性を加味した量により暴露クラスを付与している。

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②国による有害性情報の収集と有害性クラスの付与

○製造輸入数量10t超の物質7,819物質のうち、基本的にはCAS番号に基づいて一般化学物質の有害性情報の収集を実施している。

○下記の資料に基づいて信頼性の確認を行い、「化審法におけるスクリーニング評価手法について」に基づき、有害性クラスを付与している。

・「化審法における人健康影響に関する有害性データの信頼性評価等について」

・「化審法における生態影響に関する有害性データの信頼性評価等について」

○これまで、スクリーニング評価にあたっては国による一般化学物質の情報収集を行ってきたが、今後は事業者からの有害性情報等の提供を呼びかけることとしている（平成26年2月上旬に一般化学物質、優先評価化学物質の一部について、製造・輸入事業者に有害性情報の提供を依頼した。）。

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③ スクリーニング評価実施結果

	平成22年度（平成23年1月審議）		平成23年度（平成24年1月審議）		平成24年度（平成24年7月審議）		平成25年度（平成25年7月審議）	
	人健康	生態	人健康	生態	人健康	生態	人健康	生態
評価対象の物質区分	旧二監	旧三監	一般化学物質の一部		届出のあった全ての一般化学物質			
曝露情報	平成21年度実績		平成22年度実績		平成22年度実績		平成23年度実績	
有害性情報	二監・三監の判定根拠		OECD/HPV判定根拠など		国が保有している・収集した情報で信頼性等が確認できたもの			
評価単位物質	682物質	212物質	109物質	275物質	10,792物質		11,979物質	
製造輸入数量10t超	447物質	166物質	101物質	188物質	7,054物質		7,819物質	
優先評価化学物質相当	88物質		8物質		46物質		40物質	
	75物質	20物質	6物質	4物質	31物質	21物質	17物質	23物質

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④ 指定された優先評価化学物質

- 優先評価化学物質　160物質（平成26年2月1日現在）
  平成25年7月審議において優先判定相当とされた物質の一部について、12月20日付けで優先評価化学物質として指定。残りの物質についても公示準備中。

リスト公開サイト
（English）
J-CHECK（Japan Chemicals Collaborative Knowledge Database）
http://www.safe.nite.go.jp/jcheck/list7.action?category=230&request locale=en
NITE　CHRIP
http://www.safe.nite.go.jp/english/sougou/view/IntrmSrchYusenList en.faces
（日本語）
環境省化審室サイト
http://www.env.go.jp/chemi/kagaku/kisei/yuusen.html

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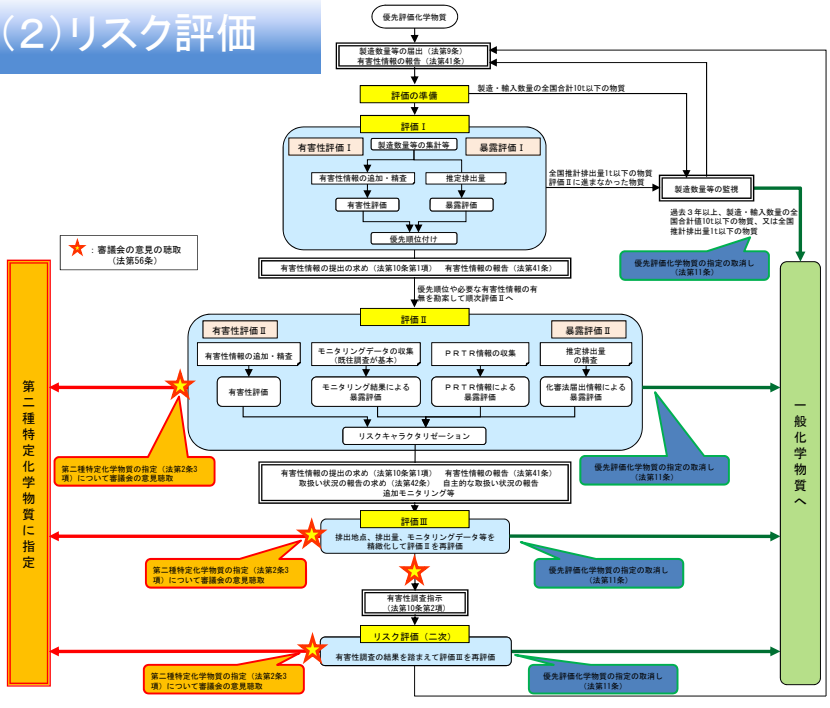
（2）リスク評価



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① リスク評価（1次）について

リスク評価（1次）は、評価Ⅰ、Ⅱ、Ⅲの3段階構成

＜評価 Ⅰ＞

有害性評価は、スクリーニング評価時と同じ情報を用いて行い、暴露評価は、製造・輸入数量等の届出情報のみを用いて行う。これにより、評価Ⅱを進める優先順位づけを行う。

＜評価 Ⅱ＞

有害性評価は、有害性情報を追加的に収集して行い、暴露評価は対象範囲を増やしてリスク評価を行う。既往のPRTRデータやモニタリングデータも活用して行う。これらにより、リスク評価を行い、直ちに第二種特定化学物質への指定又は有害性調査の指示の可否を判断する。それらの判断に至らないときは評価Ⅲに進む。

＜評価 Ⅲ＞

取扱い情報や追加モニタリングデータ等も用いてリスク評価を精緻化し、有害性調査指示の必要性について判断する。

21

② リスク評価（1次）評価Ⅰについて

評価対象となった全ての優先評価化学物質（年間製造数量等合計10t超）

↓

○化審法第9条第1項に基づく優先評価化学物質の届出情報（製造数量、輸入数量、用途等）
○スクリーニング評価で用いた有害性情報

↓

リスク評価（1次）評価Ⅰ

有害性評価

スクリーニング評価で対象としているエンドポイントについて、スクリーニング評価とおなじ不確実係数を用いて有害性評価値を導出

暴露評価

事業者から届出のあった製造・出荷数量をもとに、排出に係る一連の仮定に沿って都道府県・ライフサイクルステージ・用途別に仮想的排出源を仮定

⇒ 詳細用途分類別の排出係数を乗じて排出量を推計

⇒ ばく露に係る一連の仮定に沿って環境中濃度や人の摂取量を推計

＜指標＞
人：リスクが懸念される排出源の全国の箇所数及びリスクが懸念される影響地域の全国の合計面積
生態：リスク懸念の箇所数

22

③ リスク評価（1次）Ｉ結果

＜平成25年度　評価Ｉの結果を踏まえた対応＞

優先評価化学物質　（平成23年度までに指定）		95 物質
リスク評価（一次） 評価Ｉの対象		79 物質
	平成25年度より 評価Ⅱに着手する物質	**8 物質 (人健康：1 物質) (生　態：7 物質)**
	上記に該当せず、次年度、 引き続き評価Ｉを行う物質	62 物質
	当面の間、数量監視を行い、 次年度、評価Ｉを行う物質 （全国推計排出量1t以下）	6 物質
当面の間、数量監視を行い、次年度、評価Ｉを行う物質 （製造・輸入数量の全国合計値10t以下）		2 物質

（参考）＜既に評価Ⅱを実施している物質数＞

平成24年度から評価Ⅱを実施しているもの	**18物質（人健康:11物質、生態:7物質）**

23

④ リスク評価Ⅱ着手物質

平成24年度　18物質

＜人健康影響（11物質）＞
○ヒドラジン　　○１，３－ブタジエン
○ジクロロメタン
○１，２－ジクロロプロパン
○クロロエチレン　○エチレンオキシド
○１，２－エポキシプロパン
○ホルムアルデヒド○アクリロニトリル
○ベンゼン　　○ｏ－トルイジン

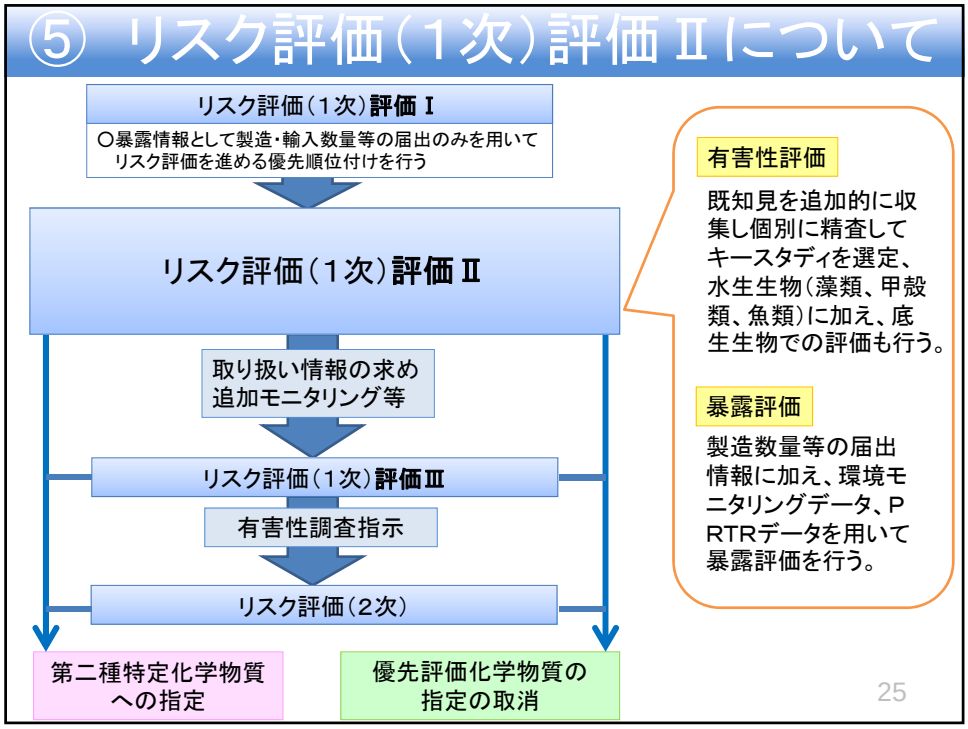
＜生態影響（７物質）＞
○１，３－ジクロロプロペン
○アクリル酸ｎ－ブチル
○イソプロペニルベンゼン
○ｐ－ジクロロベンゼン
○２，６－ジ－ｔｅｒｔ－ブチル－４－メチルフェノール
○［３－（２－エチルヘキシルオキシ）プロピルアミン］トリフェニルホウ素（ＩＩＩ）
○４，４’－（プロパン－２，２－ジイル）ジフェノール（ビスフェノールA）

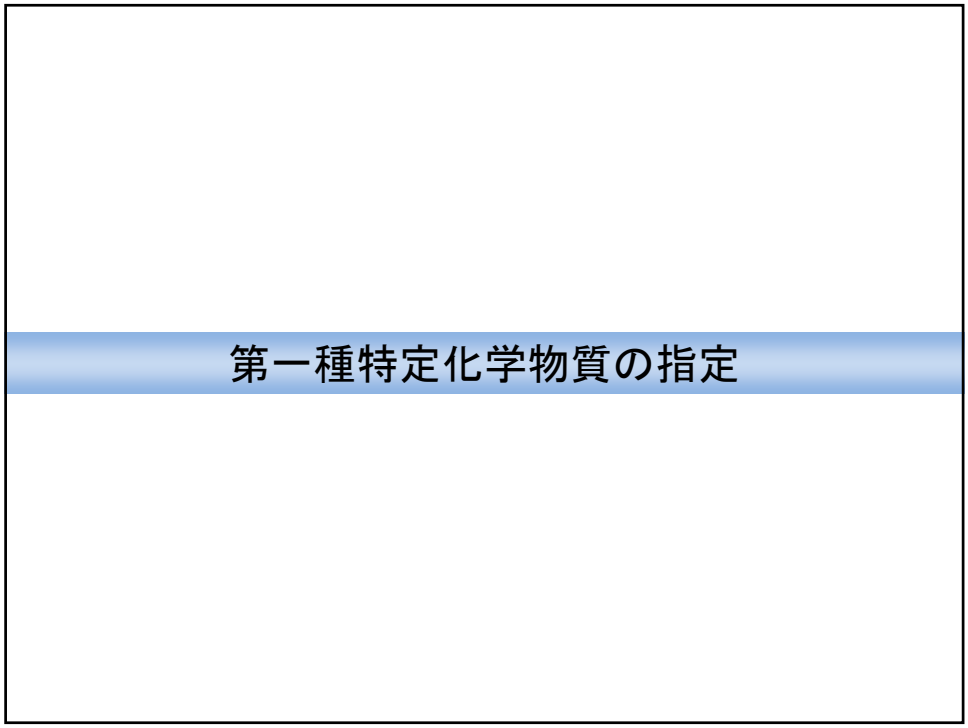
平成25年度　8物質

＜人健康影響(1物質)＞
○N，N－ジメチルホルムアミド

＜生態影響(7物質)＞
○ヒドラジン
○ブロモメタン
（別名臭化メチル）
○1，2，4－トリメチルベンゼン
○ナフタレン
○α－（ノニルフェニル）－ω－ヒドロキシポリ（オキシエチレン）（別名ポリ（オキシエチレン）＝ノニルフェニルエーテル）
○過酸化水素
○アクリル酸

24


⑤ リスク評価（1次）評価Ⅱについて
リスク評価（1次）評価Ⅰ
○暴露情報として製造・輸入数量等の届出のみを用いてリスク評価を進める優先順位付けを行う
リスク評価（1次）評価Ⅱ
取り扱い情報の求め
追加モニタリング等
リスク評価（1次）評価Ⅲ
有害性調査指示
リスク評価（2次）
第二種特定化学物質への指定
優先評価化学物質の指定の取消
有害性評価
既知見を追加的に収集し個別に精査してキースタディを選定、水生生物（藻類、甲殻類、魚類）に加え、底生生物での評価も行う。
暴露評価
製造数量等の届出情報に加え、環境モニタリングデータ、PRTRデータを用いて暴露評価を行う。
25


第一種特定化学物質の指定

POPs条約（残留性有機汚染物質に関するストックホルム条約）

POPs（Persistent Organic Pollutants、残留性有機汚染物質）
＝ ①毒性があり、
②分解しにくく、
③生物中に蓄積され、
④長距離を移動する物質。

➡ 1国に止まらない国際的な汚染防止の取組が必要。

POPsによる汚染防止のため、**国際的に協調してPOPsの廃絶、削減等**を行う。
〇2001年5月採択。我が国は2002年8月に締結。2004年5月に発効。）
〇締約国会議は2年に1回、これまで6回開催。
〇専門・技術的事項は、残留性有機汚染物質検討委員会（POPRC）で審議。

対象物質（当初12物質）

農薬・殺虫剤
アルドリン、ディルドリン、エンドリン、クロルデン、ヘプタクロル、DDT、マイレックス、トキサフェン、ヘキサクロロベンゼン、

意図せず生成される副産物等
ダイオキシン、ジベンゾフラン

工業化学品
PCB

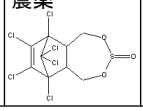
（注）2009年5月に9物質群の追加に合意

条約を履行するための国内実施計画を策定して実施。

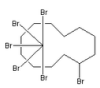
27

COP5及びCOP6：附属書A（廃絶）へ追加された物質

COP5において決定された事項

物質	主な用途	除外
エンドスルファン及びその異性体	農薬 	・製造・使用等の禁止 （以下の用途を除外する規定あり） -特定作物-害虫への農薬用の製造と使用

COP6において決定された事項

物質	主な用途	除外
ヘキサブロモシクロドデカン 1,2,5,6,9,10-ヘキサブロモシクロドデカン及びその主な異性体： α-ヘキサブロモシクロドデカン β-ヘキサブロモシクロドデカン γ-ヘキサブロモシクロドデカン	難燃剤 	・製造・使用等の禁止 （以下の用途を除外する規定あり） -建築用のビーズ法発泡ポリスチレン及び押出発泡ポリスチレン用の製造と使用

- 上記の2物質を、中央環境審議会の第一次答申に基づき、化審法の第一種特定化学物質に指定し、製造・輸入・使用の原則禁止等の措置を講ずる予定。※
- また、中央環境審議会の第二次答申に基づき、HBCDを含む製品（繊維用難燃処理薬剤、難燃性EPS用ビーズ及び防炎生地・防炎カーテン）について、化審法に基づく輸入禁止措置を講ずる予定。

※ エンドスルファンについては農薬取締法に基づき、既に農薬としての製造、販売等は禁止されている。

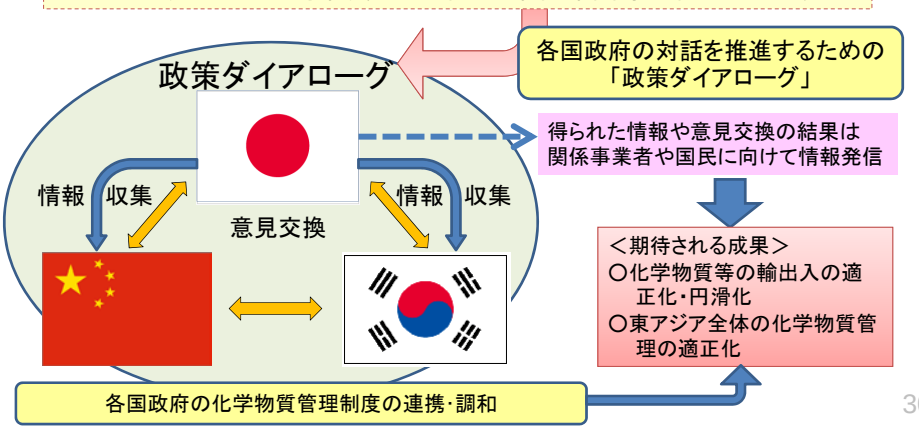
28

日中韓化学物質政策ダイアローグの開催

日中韓化学物質政策ダイアローグ

〇平成18年12月　第8回日中韓三カ国環境大臣会合
- 「化学物質管理に関する政策や規制に関する情報交換の推進」について合意
- 平成19年から、毎年、日中韓化学物質政策ダイアローグを開催（計7回開催）

東アジア域内（特に日中韓三カ国間）の化学物質等の輸出入等は頻繁に行われているため、地域内の化学物質の適正管理は各国・関係事業者にとって重要



30

第7回日中韓化学物質政策ダイアローグの概要

平成25年11月13日～15日　＠日本・京都府京都市

（1）　13日（水）：日中韓の化学物質管理に関する専門家会合（非公開）
- ①　化学物質に係る生態毒性試験に関する共同研究の進捗について
- ②　中国のGLP施設への現地調査の結果について
- ③　化学物質のリスク評価手法等について

（2）　14日（木）：第7回日中韓政府事務レベル会合（非公開）
- ①　化学物質管理政策に関する意見交換
- ②　化学物質管理に関する国際動向への対応に関する意見交換
- ③　今後の取組

（3）　15日（金）：日中韓の化学物質管理政策に関するセミナー（公開）
- ①　韓国の化学物質管理政策及び産業行動計画の変更
  講演者：韓国化学物質管理協会副会長　Jeeyoon LEE
- ②　中国における化学物質管理政策の最新動向
  講演者：中国環境部准教授　Jing Ye
- ③　日本における化学物質管理政策の最新動向
  講演者：環境省化学物質審査室　室長　木村　正伸

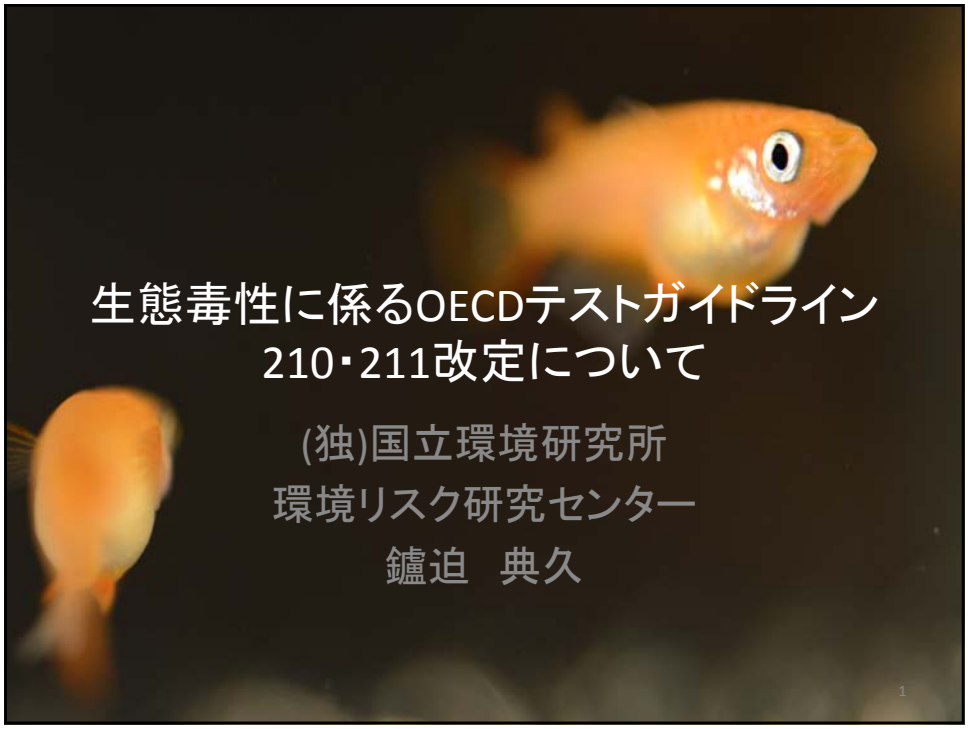
31

化学物質情報検索支援システム（ケミココ）

ケミココとは、化学物質の性質や有害性などの情報が調べやすい検索サイトです
信頼性の高いデータベースにリンク！　約22000物質の情報にアクセス可能

URL: http://www.chemicoco.go.jp/

32



OECD 試験ガイドラインの改定

ｵｵﾐｼﾞﾝｺ繁殖試験　*Daphnia magna* Reproduction Test

TG 202 Part 2　　1984年採択

TG 211　　1998年採択、 2008年改定

改定：2012年10月2日

発効：2014年4月2日

魚類初期生活段階毒性試験(TG 210)

1998年採択

改定：2013年7月26日

発効：2015年1月26日

藻類生長阻害試験(TG 201)

1981年採択、1984年改定、2006年改定、

改定：2011年7月28日

発効：2013年1月28日

2

RECOMMENDATIONS EMANATING FROM THE OECD WORKSHOP ON A FISH TOXICITY TESTING FRAMEWORK, SEPTEMBER 2010

24th Meeting of the Working Group of National Co-ordinators of the Test Guidelines Programme (WNT) 24th-27th April 2012, OECD Headquarters, Paris, France

2010年の魚類専門者会議で話し合われたこと

- TG204の廃止、TG210の改定、繁殖を含む魚類生活史試験の開発
- ガイダンスドキュメント23の改定、魚種の検討
- 魚類AOP（Adverse Outcome Pathways）の開発の基礎固め

3

OECD試験法の種類

慢性

マルチジェネレーション試験
(7ヶ月間，多世代への影響)

TG230
TG229
(3週間，繁殖、VTG)

TG234
(10週間，二次性徴)

TG210
(約40日，発達・生存・成長)

TG212
(8～9日間，発達・生存)

短期

長期

TG204

TG215
(28日間，発達・生存)

TG203
(4日間，生存)

TG236
(3日間，胚発達)

急性

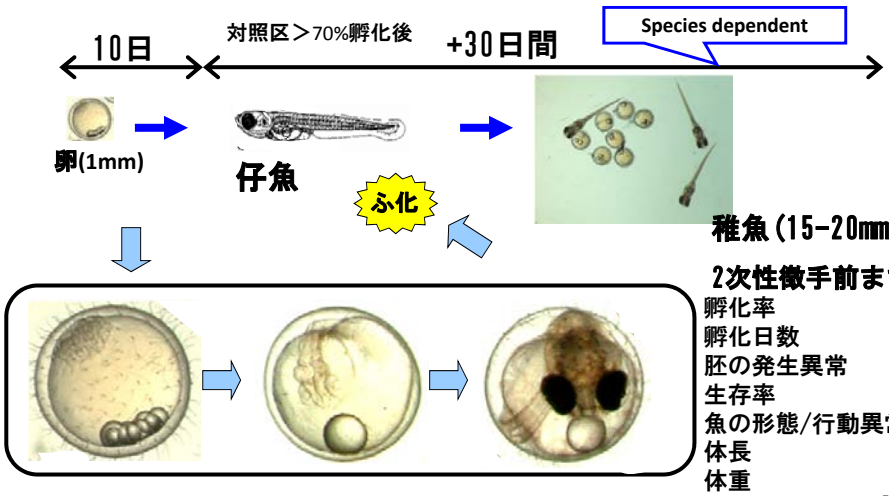
図．魚類を用いた生態影響試験の分類（概念）

4

OECD TG210

魚類初期生活段階毒性（ELST）試験

目的：連続曝露による化学物質の胚から稚魚期への影響を評価



孵化率
孵化日数
胚の発生異常
生存率
魚の形態/行動異常
体長
体重

5

TG210　2013年改定箇所について

パラグラフ3、32

- 分かりにくく書いてあるが、従来の統計値であるNOECだけではなくECx使用についても記載されている。ただし、強制的ではない。

- Lethal and sub-lethal effects are assessed and compared with control values to determine the lowest observed effect concentration (LOEC) in order to determine the (i) no observed effect concentration (NOEC) and/or (ii) ECx (e.g. EC10, EC20) by using a regression model to estimate the concentration that would cause a x % change in the effect measured. Reporting of relevant effect concentrations and parameters may depend upon the regulatory framework. The test concentrations should bracket the ECx so that the ECx comes from interpolation rather than extrapolation (see Annex 1 for definitions).

6

TG210　2013年改定箇所について

パラグラフ4,6

- 旧法では暗にTG203を行ってTG210を行う流れが記載されていたが、新法では急性毒性は必須ではないと明言された。ただし物性情報としてのTG104、105の利用、急性毒性試験としてTG203に加えてTG236の利用が書き加えられた。
- パラグラフ6には生分解性情報としてTG301にTG310が追加された。

- Test chemical refers to what is being tested. The water solubility (see Guideline 105) and the vapour pressure (see Guideline 104) of the test chemical should be known and a reliable analytical method for the quantification of the chemical in the test solutions with known and reported accuracy and limit of quantification should be available. Although not necessary to conduct the test, results from an acute toxicity test (see Guideline 203 or Guideline 236), preferably performed with the species chosen for this test, may provide useful information.

7

TG210　2013年改定箇所について

パラグラフ5

- 混合物の試験について記載されている。旧法にはない。

被験物質の化学的同一性を明らかにする必要がある。結果を規制に用いるかどうかは慎重を要す。

- If the Test Guideline is used for the testing of a mixture, its composition should as far as possible be characterised, e.g., by the chemical identity of its constituents, their quantitative occurrence and their substance-specific properties (like those mentioned above). Before use of the Test Guideline for regulatory testing of a mixture, it should be considered whether it will provide acceptable results for the intended regulatory purpose.

8

TG210　2013年改定箇所について

VALIDITY OF THE TEST　パラグラフ7、Annex2

- 溶存酸素量が60～100%から、60%以上に変更。
- 温度変動（1.5℃）がmustからshouldに変更。
- 化学物質の分析定量は義務付けられた。
- 物質濃度が20%以内という記述が無くなった。
- コントロールの孵化率と孵化後生存率が決められた。

For a test to be valid the following conditions apply:

- the dissolved oxygen concentration should be >60% of the air saturation value throughout the test;
- the water temperature should not differ by more than + 1.5°C between test chambers or between successive days at any time during the test, and should be within the temperature ranges specified for the test species (Annex 2);
- the analytical measure of the test concentrations is compulsory.
- overall survival of fertilised eggs and post-hatch success in the controls and, where relevant, in the solvent controls should be greater than or equal to the limits defined in Annex 2.

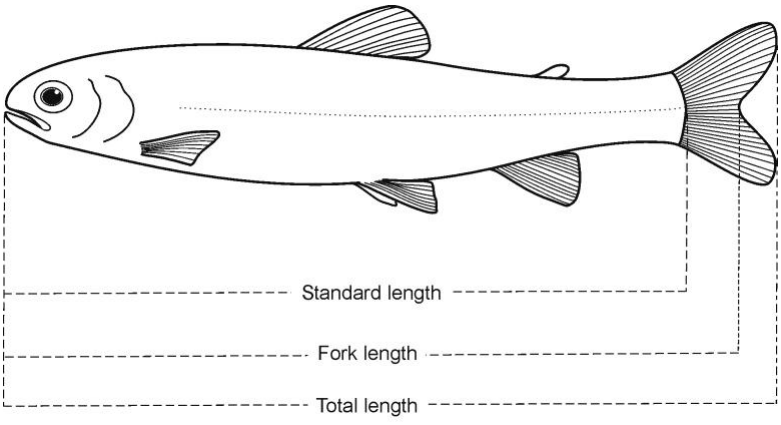
9

Annex2

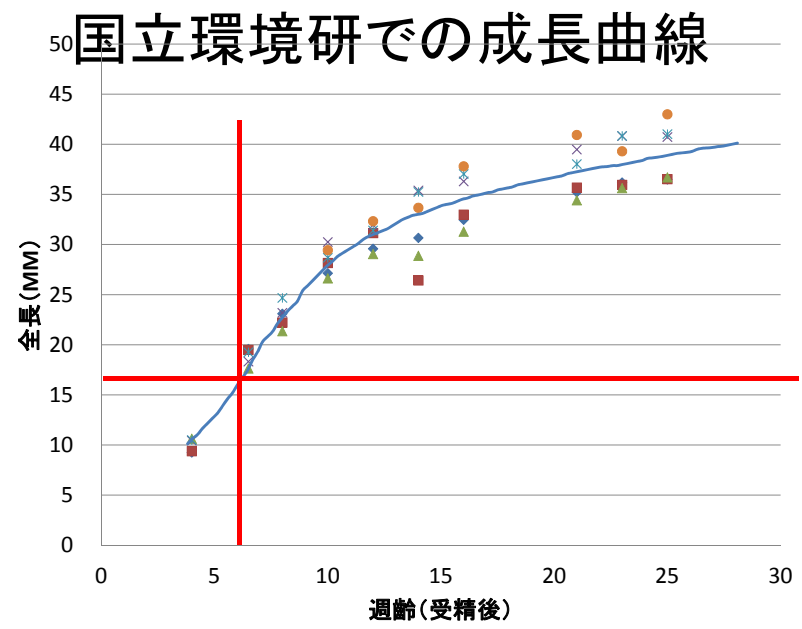
Species	Temperature(℃)	Photo period (hrs)	RECOMMENDED DURATION OF TEST	Typical minimum mean total length of control fish at the end of the study (mm) *	Hatching success	Post-hatch success
Oryzias latipes Japanese Ricefish or Medaka	25 ± 2	12 - 16	30 days post-hatch	17	80%	80%
Danio rerio Zebrafish	26 ± 1.5	12 –16	30 days post-hatch	11	70%	75 %

* Typical minimum mean total length is not a validity criterion but deviations below the figure indicated should be carefully examined in relation to the sensitivity of the test. The minimum mean total length is derived from a selection of data available at the current time.

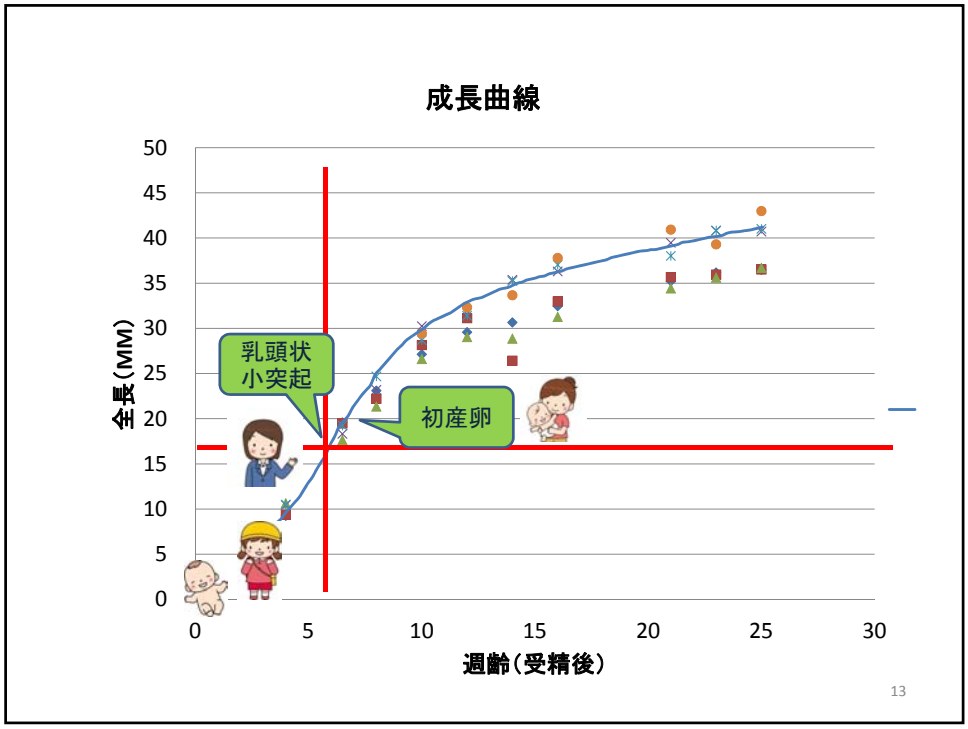
10

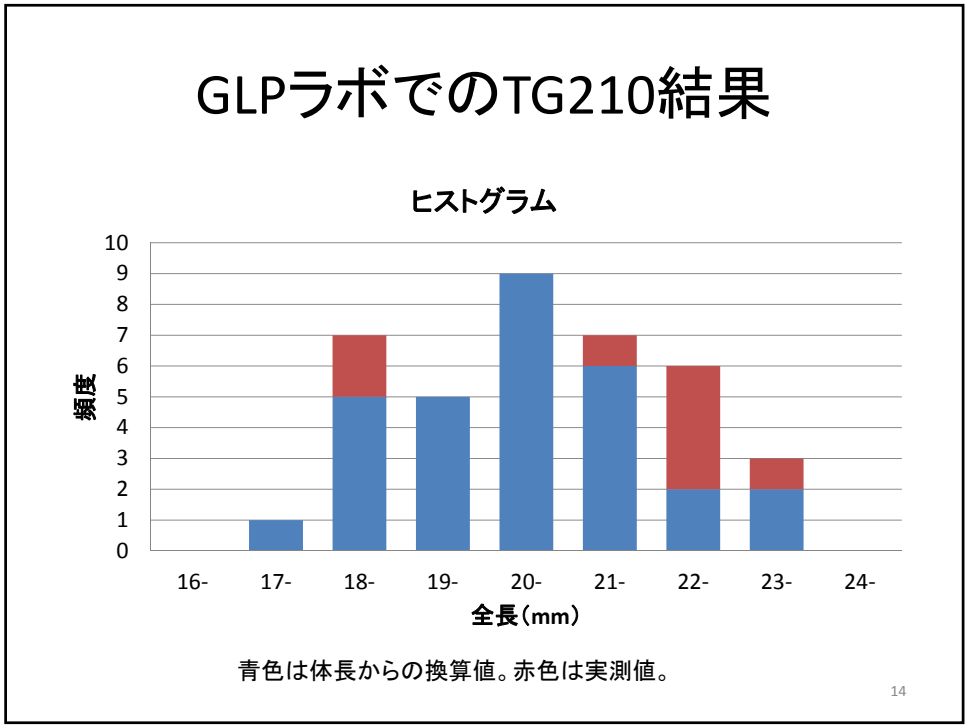

Standard length
Fork length
Total length

11


国立環境研での成長曲線
全長(MM)
週齢(受精後)
0
5
10
15
20
25
30
35
40
45
50

12


成長曲線
全長(MM)
週齢(受精後)
乳頭状
小突起
初産卵
13


GLPラボでのTG210結果
ヒストグラム
頻度
全長(mm)
青色は体長からの換算値。赤色は実測値。
14

TG210　2013年改定箇所について

Test chambersパラグラフ9

- 試験容器に関する記載。シリコンを避けるためオールガラス容器の使用を推奨している。魚の成長や溶存酸素確保のために、小型魚で7Lを推奨している。
- Any glass, stainless steel or other chemically inert vessels can be used. As silicone is known to have a strong capacity to absorb lipophilic substances, the use of silicone tubing in flow-through studies and use of silicone seals in contact with water should be minimised by the use of e.g. monoblock glass aquaria. The dimensions of the vessels should be large enough to allow proper growth in the control, maintenance of dissolved oxygen concentration (e.g. for small fish species, a 7 L tank volume will achieve this) and compliance with the loading rate criteria given in paragraph 19. It is desirable that test chambers be randomly positioned in the test area. A randomised block design with each treatment being present in each block is preferable to a completely randomised design. The test chambers should be shielded from unwanted disturbance. The test system should preferably be conditioned with concentrations of the test chemical for a sufficient duration to demonstrate stable exposure concentrations prior to the introduction of test organisms.

15

TG210　2013年改定箇所について

パラグラフ16その1

- ストックソリューションは溶剤を極力用いずに作成することが好ましいと記載された。

- Test solutions of the chosen concentrations are prepared by dilution of a stock solution. The stock solution should preferably be prepared by simply mixing or agitating the test chemical in dilution water by using mechanical means (e.g. stirring and/or ultrasonication). Saturation columns (solubility columns) or passive dosing methods (6) can be used for achieving a suitable concentrated stock solution.

16

TG210　2013年改定箇所について

パラグラフ16その2

- どうしても溶剤を使用する場合には、濃度を一定にする、ガイダンスドキュメント23に従うなどの記載が加わった。
- The use of a solvent carrier is not recommended. However, in case a solvent is necessary, a solvent control should be run in parallel, at the same solvent concentration as the chemical treatments; *i.e.* the solvent level should preferably be equal across all concentrations as well as the solvent control. For some diluter systems this might be technically difficult; here the solvent concentration in the solvent control should be equal to the highest solvent concentration in the treatment group. For difficult to test substances, the OECD Guidance Document No. 23 on aquatic toxicity testing of difficult substances and mixtures should be consulted (2). If a solvent is used, the choice of solvent will be determined by the chemical properties of the substance. The OECD Guidance Document No. 23 recommends a maximum concentration of 100 µl/L. To avoid potential effect of the solvent on endpoints measured (7), it is recommended to keep solvent concentration as low as possible.

17

TG210　2013年改定箇所について

Conditions of Exposure　パラグラフ18、19、21、22

- ***Duration*** においてThe test should continue at least until all the control fish have been free-feeding. が削除された。Annex2に日数で記載されている。
- ***Loading***において、少なくとも60卵を2水槽に分ける、から少なくとも80卵を4水槽に分ける、に変更された。また卵・仔魚期のエアレーションはしないことが明記されている。
- ***Feeding***において、水槽間で差がないように死亡率を考慮して与える、が加えられたまた、生き餌投与についての注意が付加された。
- ***Test concentrations***において、通常5濃度区、最低4連が明記された。急性毒性試験、胚急性毒性試験や予備試験の結果を利用して、設定濃度範囲を決めることが記載されている。
- ***Controls***において、溶剤コントロールの設置が記載されている（関連パラ16）
- ***Frequency of Analytical Determinations and Measurements*** において、繰り返し間は同一条件であることが記載されている。実測されることが義務付けられたので、定量下限の記載が求められる。週1回の測定、設定値との乖離が20%いないなどの記載がある。

18

TG210　2013年改定箇所について

旧パラグラフ32

These observations will result in some or all of the following data being available for statistical analysis:

- cumulative mortality;
- numbers of healthy fish at end of test;
- time to start of hatching and end of hatching;
- numbers of larvae hatching each day;
- length and weight of surviving animals;
- numbers of deformed larvae;
- numbers of fish exhibiting abnormal behaviour.

が削除された

19

TG210　2013年改定箇所について

Test report パラグラフ34

- 化学物質の物化性状を記載する。
- 一般水質等の記載箇所
- テストコンディション等を記載する
- 統計処理の記載。特にECxの取り扱い。

- Test chemical:

Mono-constituent substance

- physical appearance, water solubility, and additional relevant physicochemical properties;

- chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc. (including the organic carbon content, if appropriate.

20

TG210改定の留意点まとめ

・ 溶剤の使用を極力なくし、実測値を重視することになった。

・ 試験終了日が日にちで切られる。そのため、目標となる成長量がクライテリアに導入された。

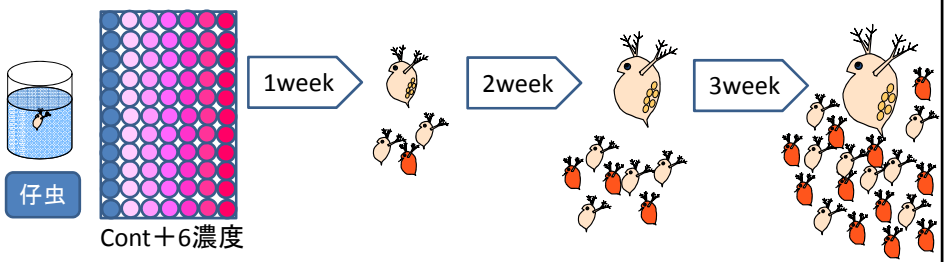
・ メダカの場合、全長17mmが目標値となるが、日本のGLPラボで行われた過去の試験結果はすべて目標値を超えている。

・ 統計処理の結果として、NOEC/LOEC表記だけではなく、ECxを使用しても良くなった。しかしその扱いについてはまだ詳細が明らかになっていない。

21

TG211

Daphnia magna Reproduction Test



試験期間：21d
試験生物：オオミジンコを推奨、ふ化後1日以内、1個体／容器：10繰り返し／濃度
試 験 区： 対照区（助剤対照区）と最低5暴露濃度区
エンドポイント： 繁殖阻害 （試験期間中の総産仔数、初産日までの期間・・・）
妥当性クライテリア： 対照区の1親当たり総産仔数60個体以上
算出する毒性値： ECx NOEC（LOEC）

22

TG211、2012年改定版の概要

パラグラフ 2.(抄訳)

TG 202 Part 2 (1984)からTG 211(1998)への主な変更点

(a) 推奨種をオオミジンコとする
(b) 試験期間は21日とする
(c) 半止水式試験においては、使用する生物個体数を最低40から、最低10個体に減らし、反復数を4であったものを10(1濃度区当たり、1容器1個体)とする。
(流水式試験においては、反復数最低4(1濃度区当たり4容器40個体とする)
(d) 試験培地および給餌について試験特有の規定を設けた、

TG211(1998)から本改定版への主な変更点

(e) 2008年に付録7を追加した。これは産仔個体の性比を調べる手順を規定した。
(f) 2012年に、反応変数として、
「試験終了時まで生存した親個体当たりの産仔数」に事故や予期しない死亡を除き、「試験開始時の親個体当たりの産仔数」を追加。
(g) 統計処理のための指針をさらに追加した
(h) 限度試験を導入した.

23

TG211 2012年改定箇所について

変更点 1 (パラグラフ4 、パラグラフ51)

曝露濃度と死亡率に有意な相関がみられる場合には、被験物質の影響であるので繁殖阻害率を算出する際の反応変数は、「試験開始時の親個体当たりの試験期間に正常に産出された仔の総数」を使う(従前の手法と比較して厳しい毒性値となる場合)

そのため
試験の結果曝露区で親の死亡が見られた場合には、(1)曝露に起因するものかどうか、(2)曝露濃度依存的に増加しているかどうかを確認する。後者の場合は、統計手法として、Cochran-Armitage trend test が有効であろう。

親個体は、ハンドリング上の間違いで死亡(事故死)や、曝露とは無関係の意図しない死亡が見られるので、親の死亡については記録にその理由を記載すること。

24

TG211　2012年改定箇所について

変更点2 （パラグラフ11、21、60）

試験生物種は、オオミジンコとするものの、その他のDaphnids（枝角類）でもよい。ただし妥当性基準を満たさなければならないし、満たしたことを示さなければならない。

留意点
一方、パラグラフ21では、ニセネコゼミジンコの使用に言及しているが、オオミジンコの妥当性基準や試験期間をニセネコゼミジンコを使っては達成できないので、もし、ニセネコゼミジンコを使う場合には別途科学的妥当性を示す必要がある（パラグラフ60）。

試験手順の変更
オオミジンコ以外の種を用いる場合には、妥当性基準を満たすこと、または、科学的妥当性を有することを示す（パラグラフ　60）。

25

TG211　2012年改定箇所について

変更点3 （パラグラフ24）

流水式試験の場合には、1つの容器に複数（例えば5，10）個体入っており、もし一部の親が死亡し、死亡した親個体を含めどの親の産仔であるかわからない場合には、反応変数は、「試験開始時の親当たりの総産仔数」を用いることとする。

試験手順の変更
例えば事故で死亡したことが明確で、かつ、その時点では産仔が見られていない場合の場合には、反応変数を「試験開始時の親当たりの総産仔数」とすることはない。
どの反応変数を利用したかについては、十分な考察が必要となる。

26

TG211　2012年改定箇所について

変更点4 （パラグラフ33、34）

もし必要がある場合には、曝露濃度設定予備試験を実施する。その場合は、1濃度区当たり2回の反復とする。追加的に、文献から得られた似た化学物質の情報、Daphnia属の急性毒性値その他は、この予備試験の濃度設定に有用である。

曝露濃度設定予備試験の試験期間は21日間、もしくは影響レベルを予測するに十分な期間とする。試験結果を記録すること。

試験手順の変更
曝露濃度設定予備試験の手順の追加
曝露濃度設定予備試験を行う必要としない場合の判断と記載方法

27

TG211　2012年改定箇所について

パラグラフ35

試験設計の段階で、統計処理方法を検討すること、その場合には検出力についても検討する..

Normally there should be at least five test concentrations, bracketing effective concentration (e.g. ECx), and arranged in a geometric series with a separation factor preferably not exceeding 3.2 An appropriate number of replicates for each test concentration should be used (see paragraphs 24–25). Justification should be provided if fewer than five concentrations are used. Substances should not be tested above their solubility limit in test medium. Before conducting the experiment it is advisable to consider the statistical power of the tests design and using appropriate statistical methods (4). In setting the range of concentrations, the following should be borne in mind:
1）……
2）……
3）…….

28

TG211　2012年改定箇所について

パラグラフ36、58
繁殖試験の限度試験を行うことができる。コントロールと最高濃度区をそれぞれ繰り返し10で行うことができる。流水式の時は少なくても良い。
試験最高濃度は、例示では10mg/Lとなっているがこの数値は規定ではなく、試験結果の利用目的や化学物質規制当局からのデータ要求に従って試験を実施すべきであると解釈される。

試験手順の変更
- 限度試験に移行する手順を明確に規定すること。その際、統計処理方法についても、指針を参照して適切に対処すること
-

36. If no effects are observed at the highest concentration in the range-finding test (e.g. at 10 mg/l), or when the test substance is highly likely to be of low/ no toxicity based on lack of toxicity to other organisms and/or low/no uptake, the reproduction test may be performed as a limit test, using a test concentration of e.g.10 mg/l and the control. Ten replicates should be used for both the treatment and the control groups. When a limit test might need to be done in a flow-through system less replicates would be adequate. A limit test will provide the opportunity to demonstrate that there is no statistically significant effect at the limit concentration, but if effects are recorded a full test will normally be required.

29

TG211　2012年改定箇所について

パラグラフ38
対照区における平均産仔数の変動係数は、25%未満であること。（パラグラフ38）
今回の改定で変更になったものではないが・・・・検出力を高める規定で特に重要

38. Generally in a well-run test, the coefficient of variation around the mean number of living offspring produced per parent animal in the control(s) should be ≤ 25%, and this should be reported for test designs using individually held animals.

30

TG211　2012年改定箇所について

パラグラフ　44、51、52
親ミジンコの死亡率が、明らかに濃度依存的に増加している場合には･･･
　「試験終了時まで生存した親個体当たりの産仔数」ではなく、
　事故や予期しない死亡を除き、「試験開始時の親個体当たりの産仔数」とする

1）　親の死亡の原因が、事故や予期しない死亡であるかどうかを判断し、適切に記録する手順の追加

2）　死亡率と暴露濃度との関係の統計解析する手順の追加（Cochran-Armitage trend test ）

3）　反応変数「試験開始時の親個体当たりの産仔数」による毒性値算出の手順の追加

4）　2つの反応変数による毒性値の比較と、毒性値の決定の手順の追加

31

TG211　2012年改定箇所について

パラグラフ　56-57

OECD ガイダンス文書　No. 54　の引用
対照区との比較においては、悪影響をみるものであり、片側検定が基本である。

試験手順の変更
統計的な手法を紹介しているので、適した手法をここから引用して、必要に応じて毒性値を算出する

有意差検定は片側でp0．05　で検定を実施する（必要に応じて、手順の見直しを行う）。

32

TG211　2012年改定箇所について

変更点10 （パラグラフ　59）

対照区の取り扱いについての新たな記述

手順の変更
1） 無処理対照区と助剤対照区の間に有意差があるかどうかを判断するには、限度試験の場合と同じ手法が利用できる（パラグラフ　58）
※ 助剤の利用により、有意に毒性が緩和もしくは増悪することが観察された場合には、当該助剤の妥当性に問題がないか検討が必要であろう。

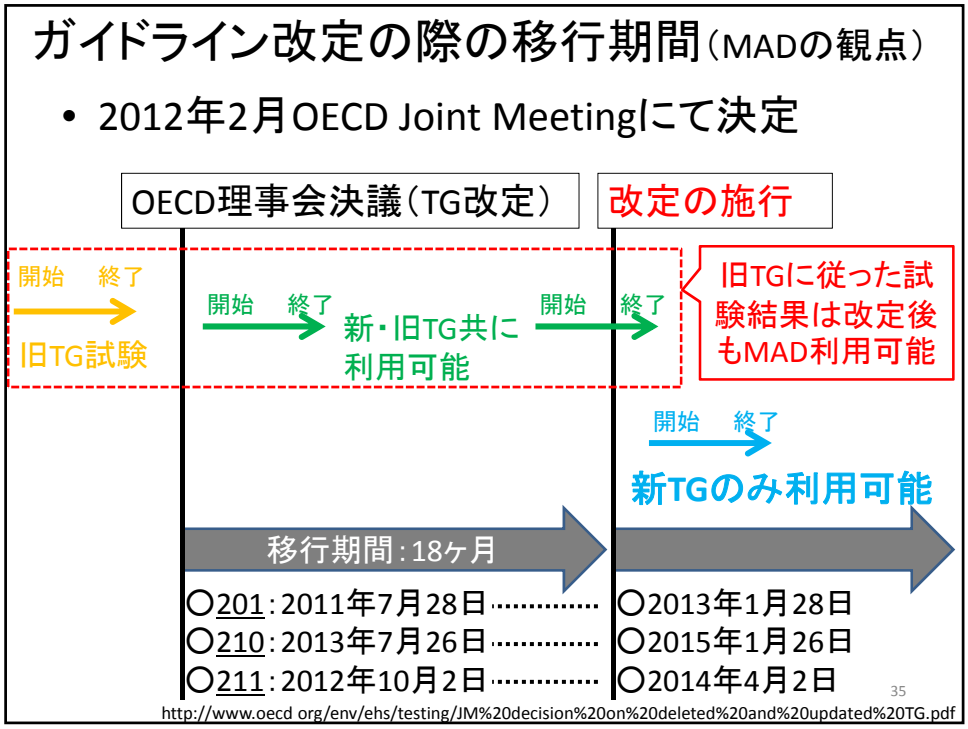
2） 無処理対照区と助剤対照区間に有意差がないと判定された場合には、pooled control を用いてよい。そうでない場合には、助剤対照区を用いる。
※ pooled controlに関しては、OECD GD 54 とは結論が異なっている。そのため、ここでの規定は、一般化することは危険であり、ある限られた場合にpooled controlを利用できると解釈すべきであろう。

33

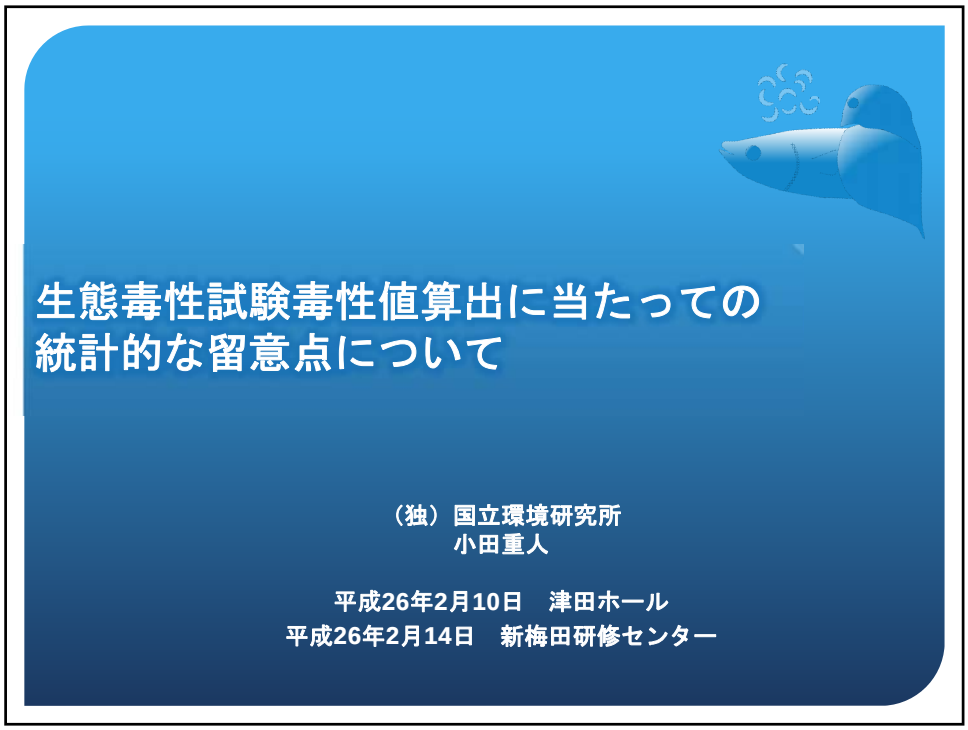
TG211改定の留意点

- 化学物質はミジンコにさまざまな影響を与えるが、致死的影響が現れる前に、必ず産仔数の低下が起こる、とは限らない
- 致死的影響は2値（生と死）データ、産仔数は連続数データであり、両者を一緒に解析することはこれまで提案された統計手法にはなじまない。
  改定案はこのようなデータを解析する手法を提示していない。
- 改定案は親世代の死亡原因が被験物質曝露によらず偶発的または操作ミスなどの事故による場合は毒性値算出データから除外するとしている。この判断は試験担当者により異なることが予想されため、除外のルールを明確にする必要がある。
- 被験物質影響が産仔数低下よりも致死影響が顕著でより低い濃度で起こる場合は、死亡率からNOECが最低値となるため、最低繰り返し数10（10個体／濃度区）では統計的検出力が十分ではない。死亡率がNOECの根拠とする場合は他の繁殖試験同様のNested designが適当であろう。

34


ガイドライン改定の際の移行期間（MADの観点）
• 2012年2月OECD Joint Meetingにて決定
OECD理事会決議（TG改定）
改定の施行
開始
終了
旧TG試験
開始
終了
新・旧TG共に利用可能
開始
終了
旧TGに従った試験結果は改定後もMAD利用可能
開始
終了
新TGのみ利用可能
移行期間：18ヶ月
○201：2011年7月28日
○210：2013年7月26日
○211：2012年10月2日
○2013年1月28日
○2015年1月26日
○2014年4月2日
35
http://www.oecd org/env/ehs/testing/JM%20decision%20on%20deleted%20and%20updated%20TG.pdf


ご清聴ありがとうございました
Nori
36

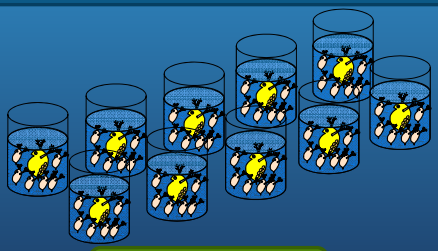

生態毒性試験毒性値算出に当たっての
統計的な留意点について
（独）国立環境研究所
小田重人
平成26年2月10日　津田ホール
平成26年2月14日　新梅田研修センター


2
はじめに
• OECD TGの改訂
• TG 211
• 試験個体の死亡を伴う産仔データの新たな扱い方
• TG 210
• Annex 5, “Statistical guidance for NOEC determination”
• Annex 6, “Statistical guidance for regression estimates”
• 生態毒性試験法
• 試験生物の配置とデータ構造の特徴
• 処理-容器-個体
• 解析の単位とプーリング
• 容器ごとの平均値の使用
• 容器を無視したデータのプーリング

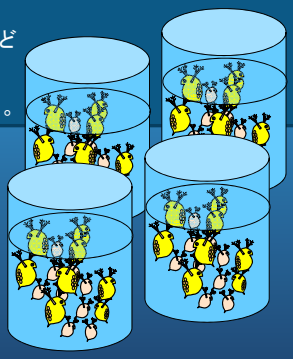
3

試験生物の配置とデータ構造

- 多くの生態毒性試験では、処理-容器-複数個体といったように試験生物を入れ子状に配置している。
- 得られるデータは、入れ子状になることもあれば、容器ごとの値や平均となってしまうものもある。
  - 試験終了時の体長、体重、ふ化、死亡、羽化など
  - 産卵数、産仔数など
- 容器あたり1個体で完全に個体識別できる配置もある。



処理（容器＝個体）



処理（容器（複数個体））

4

入れ子構造を持つデータの解析

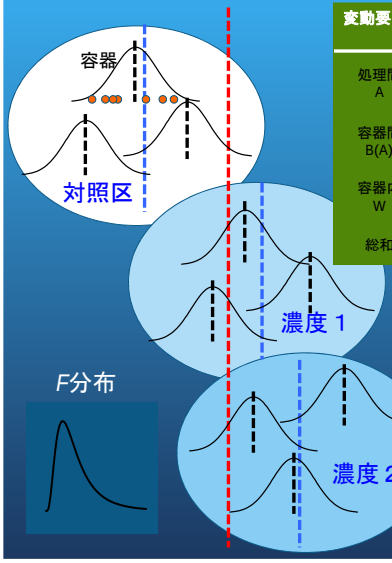
- 生態毒性試験法により得られるデータの多くは、処理-容器-個体といった入れ子構造を持っている
  - 解析方法としてはNested ANOVAと呼ばれる入れ子構造を考慮した分散分析を用いることが多い
- OECD TGでは、「解析の単位を容器とする」との記述がある（TG 210など）
  - 個体データが得られていても、容器ごとの平均を用いる
  - 複数個体の容器ごとのデータ（個体識別できない）
  - TG 211半止水式曝露では容器と個体の区別なし

- 容器ごとの平均を扱うこと
- 容器を無視してプールすること

5

分散分析による解析

入れ子構造を持つ分散分析（Nested ANOVA）



変動要因	df	SS	用いる平均	MS	F	Expected MS
処理間 A	$a-1$	SS_A		$\frac{SS_A}{(a-1)}$	$\frac{MS_A}{MS_{B(A)}}$	$\sigma_e^2+n\sigma_{B(A)}^2+\frac{bn\sum(A_i-\bar{A})^2}{a-1}$
容器間 B(A)	$a(b-1)$	$SS_{B(A)}$		$\frac{SS_{B(A)}}{a(b-1)}$	$\frac{MS_{B(A)}}{MS_W}$	$\sigma_e^2+n\sigma_{B(A)}^2$
容器内 W	$ab(n-1)$	SS_W		$\frac{SS_W}{ab(n-1)}$		σ_e^2
総和	$abn-1$	SS_{Total}				

$$SS_A = nb\sum_{i=1}^{a}\left(\bar{X}_i-\bar{X}\right)^2$$

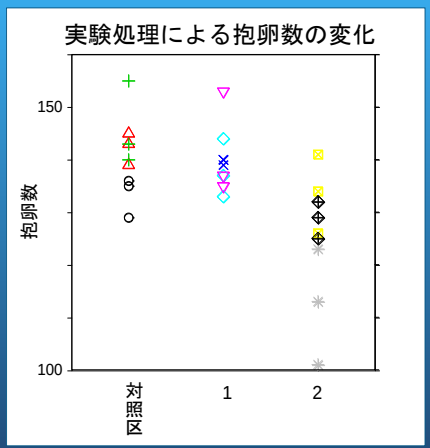
a: 処理区数
b: 処理区あたりの容器数
n: 容器あたりの個体数

分散の比をF分布と比較することにより、処理、容器の違いによって平均値の違いがもたらされているかを検定する。

- 処理効果の検定には容器ごとの平均が用いられている。
- 容器の効果が見られるときに容器を無視したプーリングを行わない。

6

容器ごとの平均の使用



Nested Anovaによる解析結果

変動要因	df	SS	MS	F	P
処理間 A	2	1403	701.6	4.120	0.0748
容器間 B(A)	6	1022	170.3	3.781	0.0130
容器内 W	18	810.7	45.04		

容器ごとの平均を用いた解析結果

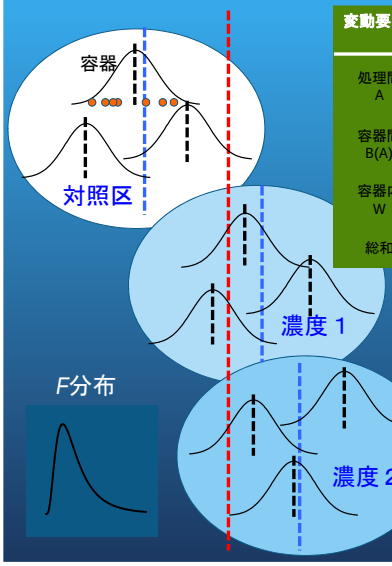
変動要因	df	SS	MS	F	P
処理間 A	2	467.7	233.86	4.119	0.0748
容器間 B(A)	6	340.7	56.78		

- 容器あたりの試験個体数が同じであれば、容器ごとの平均を用いて解析しても処理効果の検定は同じとなる。

7

分散分析による解析

入れ子構造を持つ分散分析（Nested ANOVA）



変動要因	df	SS	用いる平均	MS	F	Expected MS
処理間 A	$a-1$	SS_A		$\frac{SS_A}{(a-1)}$	$\frac{MS_A}{MS_{B(A)}}$	$\sigma_e^2+n\sigma_{B(A)}^2+\frac{bn\sum(A_i-\bar{A})^2}{a-1}$
容器間 B(A)	$a(b-1)$	$SS_{B(A)}$		$\frac{SS_{B(A)}}{a(b-1)}$	$\frac{MS_{B(A)}}{MS_W}$	$\sigma_e^2+n\sigma_{B(A)}^2$
容器内 W	$ab(n-1)$	SS_W		$\frac{SS_W}{ab(n-1)}$		σ_e^2
総和	$abn-1$	SS_{Total}				

$$SS_A = nb\sum_{i=1}^{a}\left(\bar{X}_i-\bar{X}\right)^2$$

a: 処理区数
b: 処理区あたりの容器数
n: 容器あたりの個体数

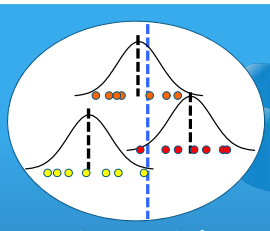
分散の比をF分布と比較することにより、処理、容器の違いによって平均値の違いがもたらされているかを検定する。

- 処理効果の検定には容器ごとの平均が用いられている。
- 容器の効果が見られるときに容器を無視したプーリングを行わない。

8

プーリング

- 効果の見られない要因を除いて解析しなおすことにより検出力を上げる



Nested Anovaによる解析結果

変動要因	df	SS	MS	F	P
処理間 A	2	1403	701.6	4.120	0.0748
容器間 B(A)	6	1022	170.3	3.781	0.0139
容器内 W	18	810.7	45.04		

容器間の効果が有意だがプールしてみると

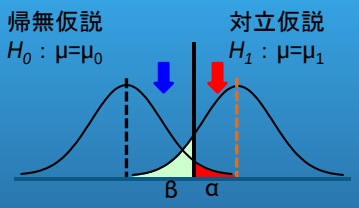
変動要因	df	SS	MS	F	P
処理間 A	2	1403	701.6	9.188	0.00109
容器内 W	24	1833	76.4		

正しい「繰り返し」でない
*F*分布からのずれ
自由度の水増し

- 容器間の違いを無視してプールすれば誤った結論を導くことも
- 効果の検出には棄却率をα=0.05に設定しているが、$p > \alpha$であればプーリングを行ってよいのか？
  - たとえば*p*が0.25以上であれば良いとする

9

プーリングの条件



- 第一種の過誤：α
  帰無仮説が正しいにもかかわらず棄却してしまう確率
- 第二種の過誤：β
  対立仮説が正しいにもかかわらず帰無仮説を誤って受け入れてしまう確率
- 検出力：1-β

- 対立仮説の母平均が帰無仮説の母平均に近づくと第二種の過誤が増大する（検出力が低下する）
- 棄却率を操作することによって第二種の過誤を減らすことが可能

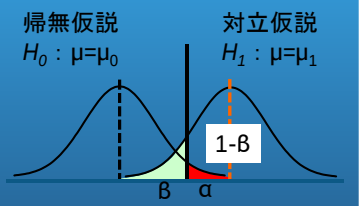
助剤対照区の扱いについても同様のことが言える

10

検出力

- 第二種の過誤：β
  対立仮説が正しいにもかかわらず帰無仮説を誤って受け入れてしまう確率
- 検出力：1-β

- TG 210 Annex 5
  - 75-80%の検出力となる差を示すこと
- TG 211
  - 段落35：試験を行う前に、解析に用いる統計手法と試験系が持つ検出力について検討しておくこと
  - 段落38：対照区における生存仔虫数の変動係数（CV）が25%以下であること

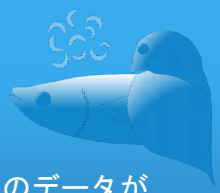


- どの程度の母平均の差が、どの程度の確率で検出できるのかを知っておきたい
- LOEC, NOECを求める試験系では検出力を把握しておくことが大事

- サンプルサイズ
- 棄却率：α
- 平均の差：$|\mu_0-\mu_1|$
- 母分散

11

入れ子構造からの変形

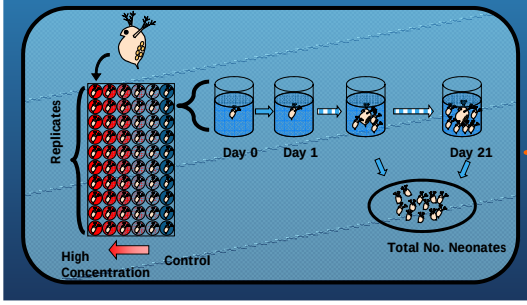


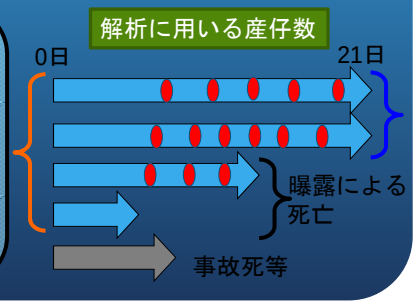
- 容器ごとの平均で代用してしまう（個体ごとのデータがそもそも得られない場合を含む）
  - 観察個体数が（ほぼ）同じであること
  - 個体データが正規分布から多少はずれていても平均は正規性の前提を満たすことが多い（中心極限定理）
- 容器間の効果が*もしもないのであれば*プーリングを行うことができる。
  - 自由度が増え、検出力があがる
  - 容器効果があるにもかかわらずプールすると誤った結論を導くことも

12

OECD TG 211改訂（2012）

- 従来の「試験終了時まで生存した試験個体あたりの産仔数」に、「試験開始時の試験個体あたりの産仔数（ただし事故や予期しない死亡を除く）」が追加された。
  - 生態学的影響、個体群レベルの影響
  - 他の無脊椎動物を用いたTGでは繁殖への影響は試験個体の曝露による死亡の影響を含めて解析を行っている。



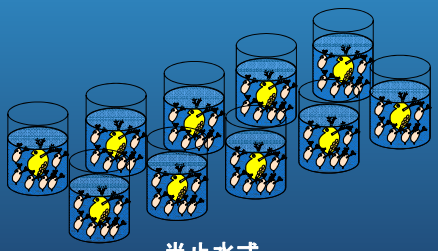


13

試験デザインについて

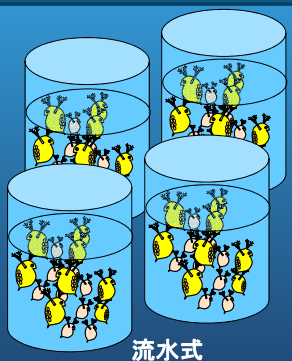
- TG 211の産仔数データ
  - 半止水式曝露
    1個体/容器
  - 流水式曝露
    10個体/容器

他の無脊椎動物を用いた生態毒性試験（繁殖）の多くも個体識別せず、複数個体を容器ごとの平均として扱っている。



半止水式

処理（容器＝個体）



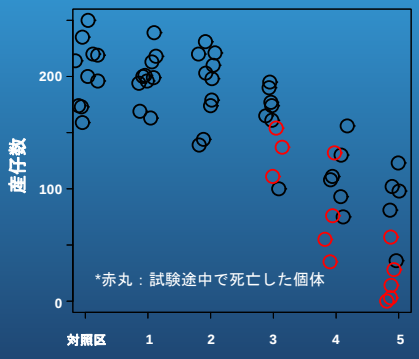
流水式

処理（容器（複数個体））

14

試験個体の死亡と平均産仔数

オオミジンコ繁殖試験
（架空のデータ）



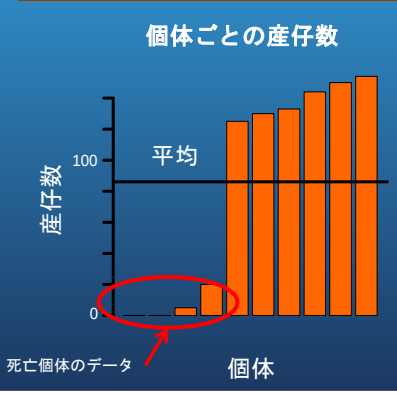
- 新たな応答変数の使用条件
  - 濃度依存的な死亡の確認
  - Cochran-Armitage testなど
    （Exact CAが良いかも）
- 使用した場合には従来の方法による結果と比較して毒性値を選択

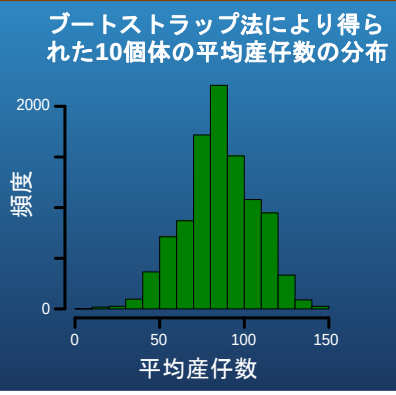
- 試験途中に曝露により死亡した試験個体を解析に含めることにより、
  - 死亡が産仔数低下に反映される
  - サンプルサイズが保たれる
  - 残差の増加による検出力低下
  - 正規性、等分散性からの逸脱によりパラメトリック検定からノンパラメトリック検定へ
- 個体ごとのデータであることの影響
  - 分布の仮定が崩れる

15

平均産仔数の振る舞い

- 試験終了までに死亡した個体のデータを解析に含める
  - 産仔数の分布は正規分布とは見なせない
  - 平均の振る舞いはある程度正規分布に近づく
  - 「容器=個体」と「容器=複数個体」の違い

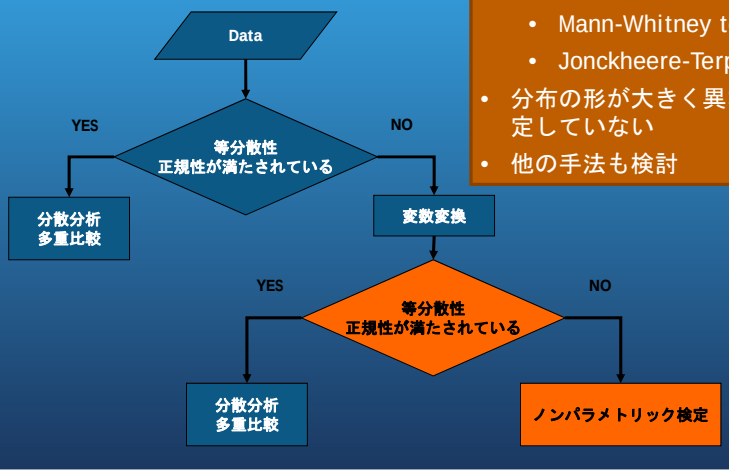




16

死亡個体の産仔数を含めたデータの扱い

- ノンパラメトリック検定
  - Kruskal-Wallis test
  - Mann-Whitney test
  - Jonckheere-Terpstra testなど
- 分布の形が大きく異なることを想定していない
- 他の手法も検討



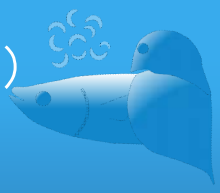
17

新たな応答変数（TG 211）

- TG 211（流水式曝露）やその他の無脊椎動物を用いた繁殖試験では容器には複数個体が配置される。
- TG 211（半止水式曝露）では容器と個体の効果は区別されない。
- TG 211に追加された新たな応答変数では半止水式曝露データの分布に正規性、等分散性を仮定することが難しい。
- 通常用いられるノンパラメトリック検定の手法は、分布の形、広がりがグループ間で大きく異なることを想定していないものが多い。

18

ふ化、死亡データ（TG 210）



- 二値データ
- 処理-容器-個体の入れ子構造
- 観察数が大きく異ならないのであれば、容器を解析の単位として扱い、パラメトリック、ノンパラメトリックの手法により解析
  - パラメトリックな手法では、容器ごとに平均をとり変数変換（アークサイン変換）
- 個体の反応データをそのまま扱う場合には、Cochran-Armitage test
  - 容器の効果がある場合には、Extra binomial variance（過分散）に注意

19

二項分布と過分散

二項分布

- ふ化や死亡（する，しない）
- 性別（オス，メス）

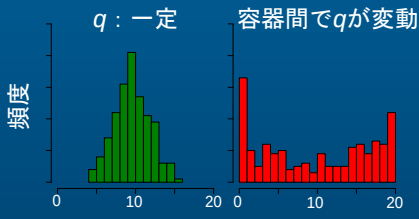
生起確率qである事象がk回の試行によりY回起きる確率

$$p(Y \mid k,q) = \binom{k}{Y} q^{Y}(1-q)^{k-Y}$$

平均 $\mu = kq$

分散 $\sigma^2 = kq(1-q)$

過分散
（extra-binomial variance, overdispersion）



- 1容器に入れた20個体の生死
- 曝露によりq=0.5の死亡率
- 平均死亡個体は10個体

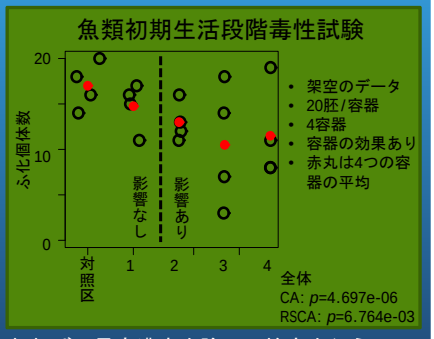
個体差、容器差により生じる過分散を考慮しないと
- モデルの当てはまりが悪い
- 曝露の効果に関して誤った結論を導く

20

Cochran-Armitage test

	対照区：0	1	2	3	4
ふ化	68	59	52	42	46
未ふ化・死亡	12	21	28	38	34

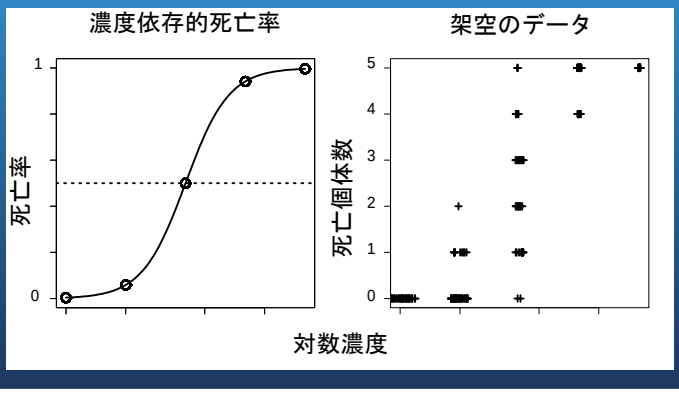
- 変量（濃度）つき分割表
- Chi-square testを、濃度に依存した線形な傾向成分とそこからのズレの成分とに分割
- 容器の効果が見られるか
  - Tarone's C(α) test, Chi-square testを濃度ごとに行う
- 容器効果が見られれば
  - 過分散、容器間の個体数の違いへの対応
  - Rao-Scottの補正付きCochran-Armitage test
- ステップダウン方式
  1. すべてのデータを用いて検定を行う
  2. α=0.05の危険率で減少、増加の傾向が見られれば、最高濃度を除いて検定を行う
  3. 有意な傾向が見られなくなるまでステップ２を繰り返す
  4. 危険率はすべてα=0.05でよい



21

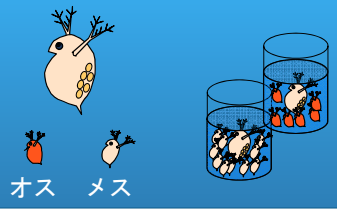
ミジンコ急性遊泳阻害試験

- 各濃度20個体を4つの容器に分けて行う。
- 容器あたり5個体
- 短期の試験
- 過分散は生じにくいのでは

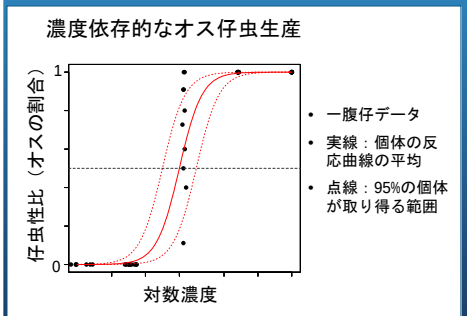


22

仔虫性比（TG 211 Annex 7）



- オス仔虫生産
- 幼若ホルモン様作用
- 一腹仔の性はオス、メスのどちらかに偏ることが多いように思われる
- 過分散の例



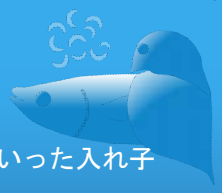
一腹仔の性別

	1	2	3	4	5	6	7	8	9	10
オス	10	10	8	11	8	4	5	1	6	10
メス	1	0	3	0	2	6	5	8	4	1

- 過分散が見られる
  - 1濃度区だけの分割表
  - ロジスティック曲線のあてはめ
- 同一個体の腹仔間にも過分散が見られる
- 性比データのプーリングには注意

23

まとめ



- 多くの生態毒性試験データは、処理-容器-個体といった入れ子構造を持っている。
  - 容器の効果を無視してデータをプールしない
  - 解析の単位を容器として、容器ごとの平均値を用いることは、入れ子構造を扱うタイプの分散分析の部分と見なせる場合もある
  - 個体のデータが正規性、等分散性を満たさない場合にも、容器ごとの平均値を用いることによって分散分析が可能となることもある
- 容器-個体の区別がないミジンコ繁殖試験（半止水式）では、死亡した試験個体の産仔数を解析に含めると、正規性、等分散性を仮定できない。
- 二値データ（ふ化、死亡、性別など）
  - サンプルサイズが容器により大きく異なるときには平均（割合）は用いない
  - Cochran-Armitageの傾向検定
  - 容器間にばらつきがみられる場合（過分散）にはRao-Scottの手法による補正を行う必要がある

生態毒性試験実施にあたっての留意点について

生態影響に関する化学物質審査規制／試験法セミナー
菅谷　芳雄（独）国立環境研究所環境リスク研究センター

化審法ではECxは採用されない？

最近採択されるOECD試験ガイドラインでは、NOECを求めるとともにEC x を算出する例が多くなっています。化審法ではEC x を併用することはありますか、またはXは何%ですか？

- OECD試験ガイドライン２０１，２１８，さらに２１０，２１１の改訂版はEC x （X%影響濃度でNOEC値相当として利用する）も求める試験法です。
- NOECとEC x を求める試験デザイン（曝露濃度や各濃度区の繰り返し数）は異なります。
- 化審法では、NOEC値を求めていますが、リスク評価のための既存情報としてEC10も利用しています。

GLP試験における、外れ値の扱い

生態影響試験の結果、ある試験の１つの数値が極端に他の繰り返しの数値と異なる場合に、どのように判断するのか？

棄却は妥当でしょうか？

- 繰り返しのある試験手順が規定されていますので（NOEC値を求める場合は必須）繰り返し間で著しく異なる結果がでることがあります。
- 曝露条件や試験操作に原因がある場合は、その点を明らかにして対処してください。
- 原因が明らかではない場合で、適切な統計検定で棄却できる場合は、試験責任者の判断で棄却することは許容されます。

3

ミジンコ繁殖試験での産まれた異常個体の扱い

TG211の試験で、孵化していない卵で産まれたり、死亡ではないが、遊泳できない個体で産まれる場合があるが、その扱いは毒性影響とみてよい？

曝露濃度に依存して増加する現象の場合は被験物質の影響と推定されるので、死んでいなくとも正常に遊泳する個体とは区別すべきである（正常個体だけを産仔数とする）。

子ミジンコは産まれた直後から試験溶液中の被験物質に曝露されるが、親ミジンコ世代も同様に曝露を受け影響が出ていたはずであり、その濃度で現れる子ミジンコへの多少の影響は、（その後正常に繁殖できるのであれば）無視できる程度の異常であるかもしれない。

【結論】判断に迷う場合には、正常個体のみを産仔数とした場合と、軽度の異常は考えず生存個体を産仔に数えた場合の2通り産仔数があってよい。その場合は、毒性値も2組だした上で、どちらが妥当であるか試験責任者の判断を示すこと。

4

ミジンコ繁殖試験で毎日の試験溶液交換は必須か？

水溶解度付近の試験では、不溶物の物理的な影響を排除する一方、濃度維持のため半止水式曝露試験を行うが、毎日の換水が求められるのか？

- 換水は少なからずミジンコにストレスを与えるので、作業の手順には工夫が必要です。工程を見直し，それでも週３回程度換水の場合と比べてストレスのため悪影響がでる場合は，必ずしも毎日の換水に固執する必要はありません。
- 毎日換水の悪影響が見られないとするラボもある（系統や試験環境条件の差は考慮）。

5

Elendt の培地組成・・

211(Annex2)では$Na_2MoO_4 \cdot 2H_2O$→1260 mg/L
202(Annex3)では$Na_2MoO_4 \cdot 2H_2O$→1230 mg/L
どちらが正しいのでしょうか？

- 原著論文から判断してTG211の値が正しいようです。

Elendt(1990)では、Mo の濃度は0.025mg/L

$Na_2MoO_4 \cdot 2H_2O$(分子量）　= 241.95

モリブデン（原子量）　= 95.96

M4培地Stock sol. I　希釈倍率　20000

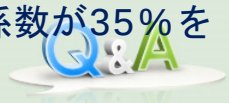
0.025*241.95/95.96*20000=1260.7

6

藻類生長速度の日間変動係数の妥当性クライテリア

藻類生長阻害試験で妥当性基準の１つ日間変動係数35％以下で，この変動係数の算出方法は？

- 化審法ガイドラインでは「対照区の毎日の生長速度の変動係数が暴露期間を通じて35％を超えないこと。」としている。OECD試験ガイドラインでは，繰り返し（ｎ＝６）毎に日間変動係数を求め，それをさらに平均をとった平均変動係数が35％を超えないことと規定している。



7

日間変動係数の算出（例）

藻類密度				平均生長速度	区間生長速度			
0 h	24 h	48 h	72h	0-72h	0-24h	24-48h	48-72h	CV
0.5	4.35	32.1	80.3	**1.69**	2.16	2.00	0.92	0.4
0.5	4.36	29.4	83.9	**1.71**	2.17	1.91	1.05	0.34
0.5	3.99	30.4	86.4	**1.72**	2.08	2.03	1.04	0.34
0.5	3.83	36.55	107	**1.79**	2.04	2.26	1.07	0.35
0.5	4.11	31.35	91.6	**1.74**	2.11	2.03	1.07	0.33
0.5	3.57	27.35	90.7	**1.73**	1.97	2.04	1.20	0.27
			平均値	1.73			日間変動係数（平均）	34 %
			標準偏差	0.03				
			繰返間の変動係数(CV値)	2 %				

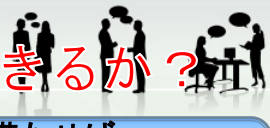
短期試験の場合の被験物質サンプルの保管（化学物質ＧＬＰ）

化審法の新規化学物質審査の際に求められるＧＬＰ試験では，被験物質の試料保管の義務はあるのでしょうか？

- 急性試験（藻類・ミジンコ・魚類）の各試験法においては，OECD-GLP原則やガイダンス文書の規定により被験物質の保管は必須ではありません。
- 保管した被験物質は，後に不純物の存在，多成分物質の場合は構成比，分子量分布の確認のために使用される可能性があるが，すべての試験で必要となるものではない（管理当局の意見を参考にスポンサーが判断）。

9

Pooled controlは利用できるか？



OECD-TG 211（改訂版）では、条件を満たせば“Pooled control”を利用してもよいとしているが、どのように理解すればよいのか？

59. To determine significant differences between the controls (control and solvent or dispersant control), the replicates of each control can be tested as described for the limit test. If these tests do not detect significant differences, all control and solvent control replicates may be pooled. Otherwise all treatments should be compared with the solvent control.

- OECDガイダンス文書No.54(2006)では、陰性対照区と助剤対照区間に有意な差がないとの判定でも、同じであることの証明ではないので慎重に対処すべき（科学的判断）。
- 判断が普遍的・統一的であるべき（行政的判断）

10

最終報告書のまとめに当たって

化審法TG／テストガイドラインでは，最終報告書に記載する事項を指定している

ただし，重要度には強弱あり・・・・

毒性値そのものに関係する要素

毒性値で政策判断をするため重要．

【妥当な科学的判断】

毒性発現に関係する要素

ある物質では試験環境により．毒性発現は異なる

【特に試験困難物質】

試験の信頼性に関係する要素

Validである事を示し，行政（規制）判断の根拠となる．

生態毒性QSARモデル「KATE」について

独立行政法人国立環境研究所
環境リスク研究センター　蓮沼　和夫

H25年度化審法セミナー

生態毒性QSARの状況

2

代表的な生態毒性QSAR

- 代表的なQSARの特徴

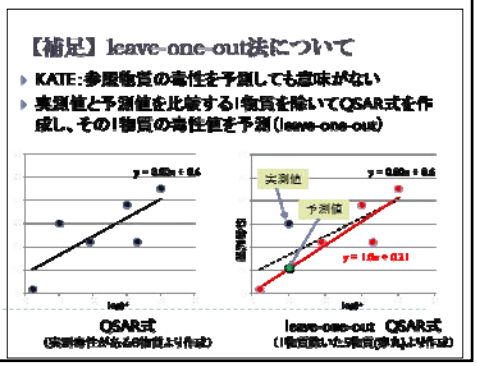
名称	開発元	記述子	予測エンドポイント	その他
KATE	環境省、 国立環境研究所	logP （水-オクタノール 分配係数）	魚類・甲殻類急性毒性 （魚類・甲殻類慢性、藻類は開発中）	**ドメイン判定：構造、 記述子**
TIMES	ブルガリア ブルガス大学	logBCFtox、 LUMO等	魚類・甲殻類急性毒性等 *（Rana japonica, Lymnaea stagnalis , Carassius auratus, Oryzias latipes , Leuciscus idus, Pimephales promelas , Daphnia magna, Daphnia pulex, Ceriodaphnia dubia , Escherichia coli, Bacillus subtilis, Tetrahymena pyriformis 等 ）*	・ドメイン判定：構造 ・有償（約150万/年）
ECOSAR	米国EPA	主にlogP	魚類・甲殻類・藻類急性毒性 魚類・甲殻類・藻類ChV (ChV：Chron c Value: NOECとLOECの幾何平均)	ドメイン判定： 記述子
OECD QSAR Too box	OECD、EU	任意（ユーザがlogPやpKa等から選択）	任意	・ドメイン判定：ユーザが判断 ・1物質毎にユーザがQSAR式を構築する必要あり

3

代表的な生態毒性QSARの予測精度の現状

- 実測毒性値とその予測値を比較
  - 三省合同審議会※に予測結果を提出しているKATE、TIMES、ECOSARを使用 （KATEは、Looを実施）
  - ドメイン外の予測結果は除外し検討
    - ・KATE：logP判定×、構造C判定：×
    - ・ECOSAR：logP判定×
    - ・TIMES：Reactive Unspecifiedクラス、構造ドメイン外
- 検証に用いた実測毒性
  - KATE2011年3月版の参照物質

※：薬事・食品衛生審議会薬事分科会化学物質安全対策部会化学物質調査会、化学物質審議会審査部会、中央環境審議会環境保健部会化学物質審査小委員会



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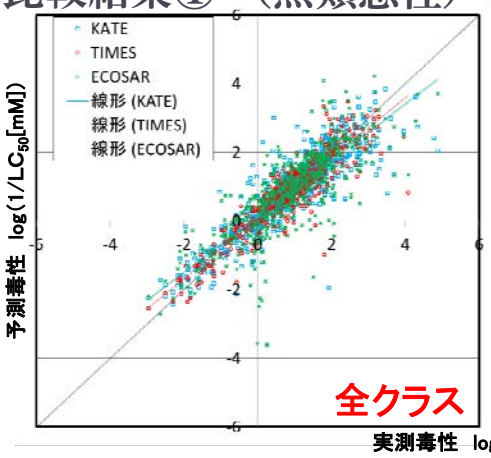
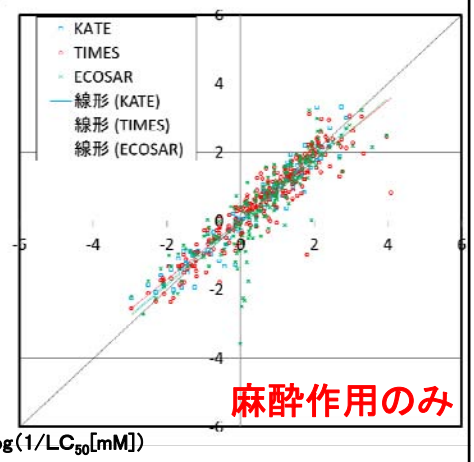
比較結果①　（魚類急性）





実測毒性　log(1/LC50[mM])

麻酔作用のみ：麻酔作用で説明できるクラスのみ予測

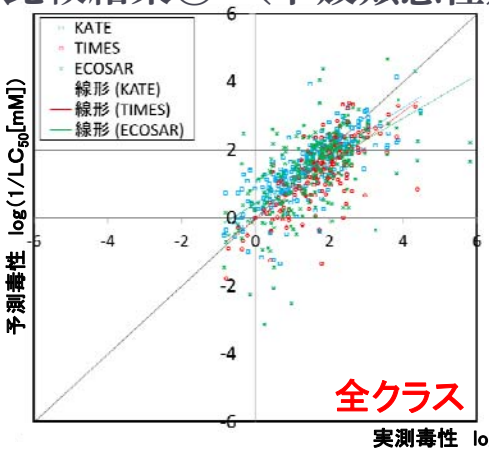
- **KATE: Neutral Organics**
- **TIMES: basesurface narcotics, narcotic amines**
- **ECOSAR: Neutral Organics , Neutral Organics-acid**

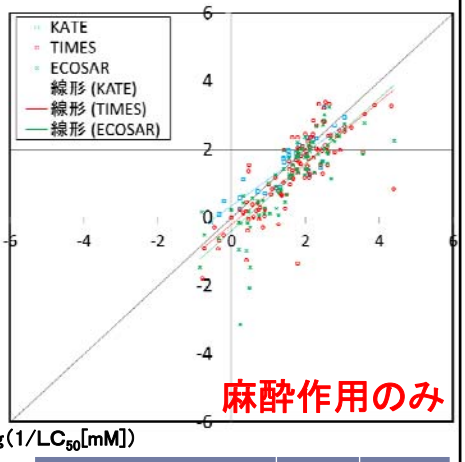
モデル		R^2adj	RMSE
KATE	**全クラス**	**0.78**	**0.60**
	麻酔作用	**0.91**	**0.41**
T MES	全クラス	0.83	0.49
	麻酔作用	0.85	0.50
ECOSAR	全クラス	0.64	1.1
	麻酔作用	0.77	0.79

5

- RMSE:平均二乗誤差
- R^2adj: 自由度調整済決定係数

比較結果②　（甲殻類急性）





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- **ECOSAR: Neutral Organics , Neutral Organics-acid**

モデル		R^2adj	RMSE
KATE	**全クラス**	**0.60**	**0.62**
	麻酔作用	**0.76**	**0.46**
T MES	全クラス	0.35	0.53
	麻酔作用	0.65	0.79
ECOSAR	全クラス	0.36	1.4
	麻酔作用	0.68	0.46

6

- RMSE:平均二乗誤差
- R^2adj: 自由度調整済決定係数

KATE(KAshinhou Tool for Ecotoxicity)概要

7

予測の対象となる化学物質

- 一般工業化学物質
  - 特定の生物活性がある物質は予測不可（例：農薬、医薬品）
- 単一の構造を持つ物質
- 分子量1000未満の物質
- 水環境中で安定な物質
- 水環境中で溶存態として溶解している物質
- 有機化学物質（有機金属は除く）
  - 無機化合物は予測不可
- 塩の場合は酸で代替
  - 例：カルボン酸ナトリウム⇒カルボン酸で予測
- タンパク質等との反応性を有しない物質

上記物質でも、ドメイン外となった予測結果は利用不可

8

KATE開発の経緯

2012年4月　改正化審法2段階目施行（ｽｸ評価・ﾘｽｸ評価）

2011年3月　KATE 2011公開　（PASは開発休止中）

2009年3月　インターネット版に加え スタンドアロン(Windows)版を公開

2008年1月　試用版 (KATE Ver0.1) 公開

2007年7月　三省合同審議会に対し、予測結果の提供を開始

2004年4月　環境省の請負業務として研究・開発開始（2004年度～2012年度）

2003年　化審法改正（生態）

有害性ｸﾗｽ
1：PNEC≦0.001 mg/L
2：0.001<PNEC≦ 0.01 mg/L
3： 0.01<PNEC≦0.1 mg/L
4： 0. 1<PNEC≦ 1 mg/L**外**：PNEC>1 mg/L

第三種監視化学物質
NOEC≦0.1 mg/L

9

生態毒性予測システムKATE

Knowledge-Driven QSAR Model
Quantitative Structure-Activity Relationship (QSAR)
定量的構造活性相関

化学物質の構造上の特徴から事前にクラスに分類する

★Multiple QSAR models
★battery QSAR models
★combine classes
★check the fragments in the training set of model

相関

ULR
MLR
logP等

化学物質規制で要求される毒性値

・魚類毒性値

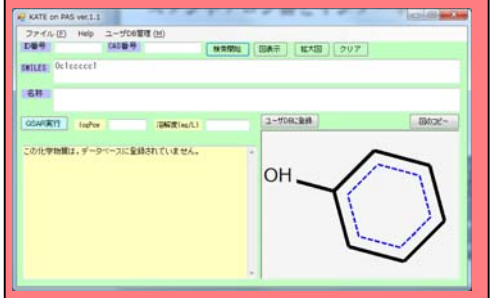
・ミジンコ毒性値

・藻類毒性値

（・ユスリカ毒性値）

10

スタンドアロン版とインターネット版



KATE on PAS
（スタンドアロン版）

KATE on NET
（インターネット版）

FITS（Fragment Identification by Tree Structure）

11

KATEシステム

曖昧でないアルゴリム

QSAR予測

化学物質の構造(SMILES)の入力

- SMILES：化合物の分子構造を線形表記した識別子
  - c1ccccc1O
- FITS：部分構造の取得
  - Aromatic c-OH
- QSAR式の選択
  - amines aromatic or phenols4
  - log(1/LC50) = 0.72logP -0.72
- 記述子の取得
  - ・実測値
  - ・スタンドアロン版：U.S. EPA KOWWIN
  - ・NET版：BioByte社 ClogP

部分構造に分解

モデル開発

計画（plan）

実行（do）

構造分類
・部分構造の定義
・分類ルール
（部分構造の組合せとその優先順位）

参照物質データ
・環境省生態毒性試験結果
・U.S. EPA ファットヘッド ミノー データベース

評価（check）

改善（act）

検証データ
・環境省既存点検結果
・新規届出
既知見（反応性）

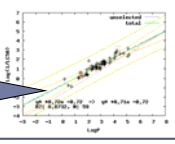
QSAR式を構成する参照物質の部分構造と比較

（部分構造） ○ 適応可能領域（記述子） ○

定義されたエンドポイント

Fish LC50 = 44 (mg/L)

当てはまりの良さ、予測性の指標

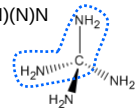


PASとは

- PAS (Platform for Assessment from Structure)注は・・・
  - 構造分類に基づく物性や毒性を予測するための独自のシステム
  - 部分構造の取得プログラム（FITS；Fragment Identification by Tree Structure）、構造図の表示・入力プログラムなどからなる統合システム
- FITSは部分構造の規定に独自の記号を使用
  - 主体部分は，1次元構造を基本としたFITS記述です。
    F/01211/C=CNC=C/1JnC=O,3V3,3B3,2Cy,3Cy,4Cy,2Rs4,/|

例: NC(N)(N)Nの構造でNCNの構造の数を、目的に応じて1－6個まで定義できます。

SMILES: NC(N)(N)N



注：PASの開発は、2000～2002年度(H12～14)環境省環境研究総合促進費「環境中の複合化学物質による次世代影響リスクの評価とリスク支援に関する研究」の一環として大分大学で実施。また、「環境データの解析と環境中生物影響評価に関する研究」として、2005～2008年度(H17～20)には（独）国立環境研究所と大分大学との委託・共同研究として実施。

Presence of Substructures

- 生物学的活性の可能性を示す部分構造と、その有無に基づいたQSARsが開発されている。
- 生態毒性では部分構造の評価が可能な大きなデータベースが入手可能であり、分類することにより、予測の誤差を減らす可能性がある。
- 一方、部分構造の取り扱いは困難。例えば、部分構造間の電子の相互作用を見込むことはできない。
- 予測する化学物質に新規な部分構造がある場合は、元のデータベースに存在していないため適切に評価できているか保証がない。

⇩

- 参照物質との比較によるドメイン判定が必要（trade off）

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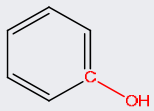
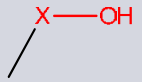
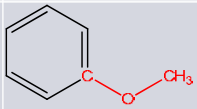
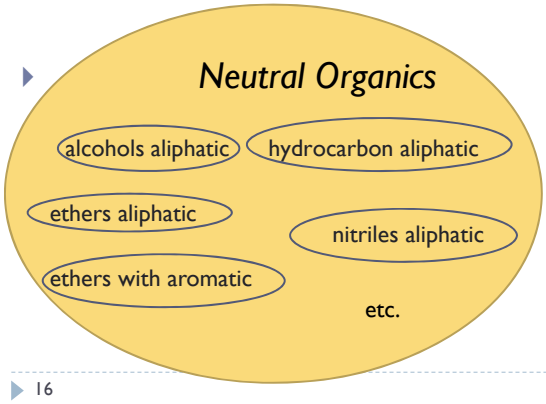
参照物質との構造比較に用いている部分構造の例

ID	説明	FITS	部分構造イメージ
5011	脂肪族C-OH	F/11/CO/2H1,/|	
5012	芳香族c-OH	F/11/cO/2H1,/|	
5013	X(炭素以外)-OH	F/11/?O/1?!;C;c;,2H1,/|	
5014	脂肪族C-OMe	F/111/COC/3H3,/|	
5015	芳香族c-OMe	F/111/cOC/3H3,/|	
:	:	:	:

combine classes

- Neutral Organicsクラス：脂肪族炭化水素、脂肪族・芳香族エーテル、脂肪族・芳香族ケトン、アルコールといった単純な麻酔作用のみで毒性が説明できると考えられるクラスの物質を統合



Neutral Organics
⇒本クラスの参照物質の部分構造は反応性の毒性に寄与しないとし、他のクラスの構造C判定においても参照物質に含める。

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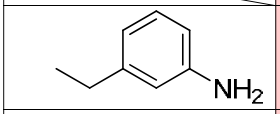
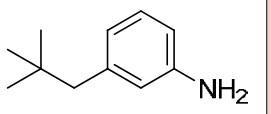
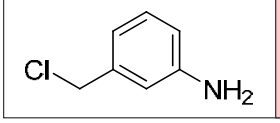
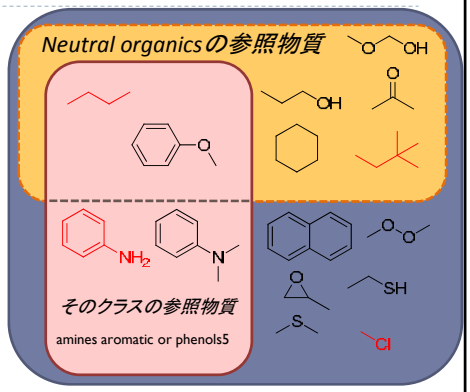
部分構造によるクラス分類の適用範囲

	C(1)	C(2)
	YES 構造C判定：○	―
	NO 構造C判定：△	YES
	NO 構造C判定：×	NO



構造C 判定

参照物質の部分構造のリスト

C(1): 予測した化学物質の全ての部分構造が、「*そのクラス*」の参照物質の部分構造リストに含まれるか。

C(2): 予測した化学物質の全ての部分構造が、「*そのクラス*」又は*Neutral Organics*の参照物質の部分構造リストに含まれるか。

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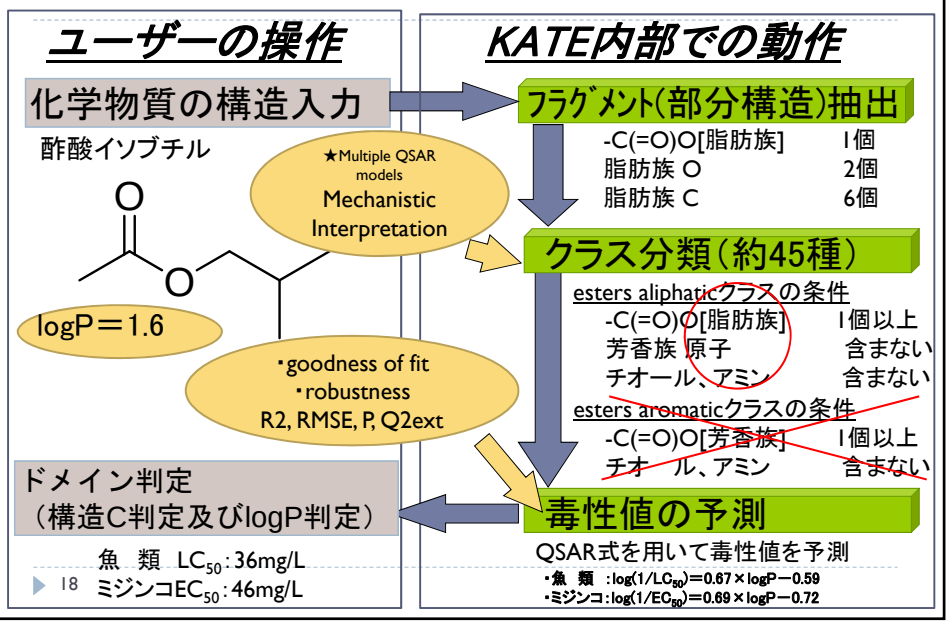
KATEの予測（Battery QSAR models）



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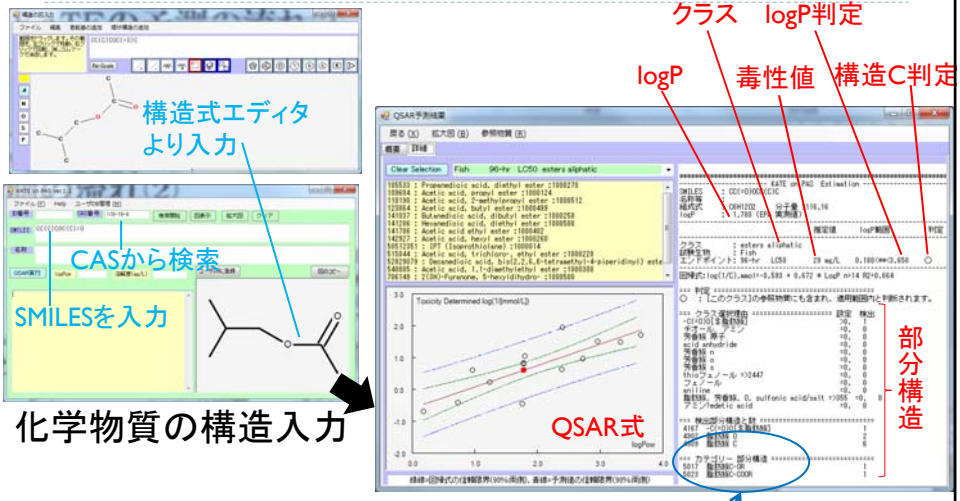
KATE on PAS(スタンドアロン版)



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Battery QSAR models（Score）

予測不可

構造または作用機序

Name	Score	Name	Score
N or P cations	37	thiols or disulfides	18
metals	36	halides low-reactive	17
Acrylate / acrylic acids	35	esters aliphatic	16
conjugated systems1	34	ethers with aromatic	15
A-group	33	amides or imides	14
amines aromatic or phenols	32	aldehydes	13
halides reactive	31	secondary or tertiary amines	12
dinitrobenzenes	30	esters aromatic	11
epoxides	29	ethers aliphatic	10
pyrethroids	28	alcohols aliphatic	9
carbamates	27	hydrocabons aliphatic	8
conjugated systems2	26	sulfides	7
barbitals	25	nitrobenzenes	6
hydrazines or polyamines	24	hydrocarbons aromatic	5
phenols	23	ketones	4
primary amines aliphatic or aromatic	22	phosphates	3
amines aromatic	21	nitriles aliphatic	2
methacrylates	20	acids	1
esters phosphate	19	Unclassified	0

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一般化学物質の有害性情報を活用した外部バリデーション結果

- バリデーションに用いたQSAR式
  - KATE2011年公開版
  - 精度が高いと考えられるQSAR式のみ使用（r2>0.7、p(slope)<0.05）
- バリデーションに用いた毒性情報
  - KATE2011公開後に報告された環境省が実施した生態毒性試験結果（魚類急性毒性試験、ミジンコ遊泳阻害試験）
    http://www.env.go.jp/chemi/sesaku/02e.pdf
  - H25.07.19　化学物質審査小委員会（第135回）の「資料4-2　生態影響に関する優先度判定案」における魚類・ミジンコ類急性毒性値のうちKATE2011の参照物質として使用されていない物質
    http://www.env.go.jp/council/05hoken/y051-135-1/mat04_2.pdf

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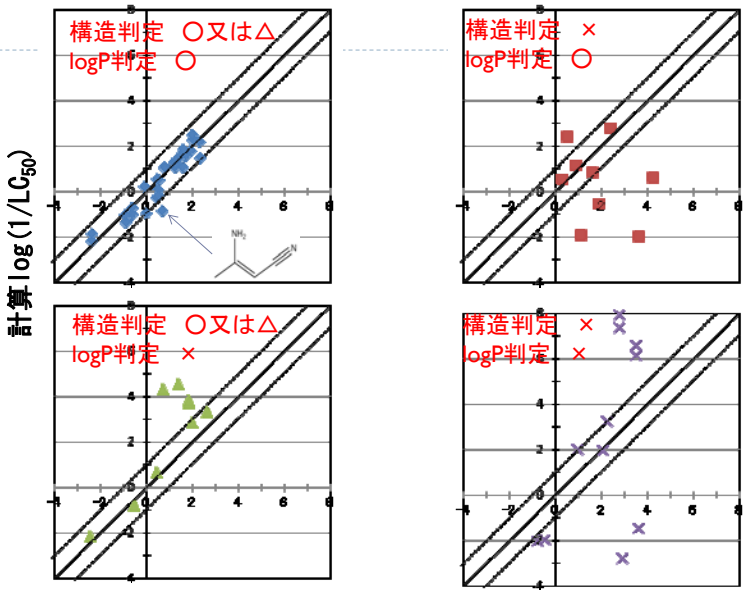
魚類バリデーション結果(n=62)



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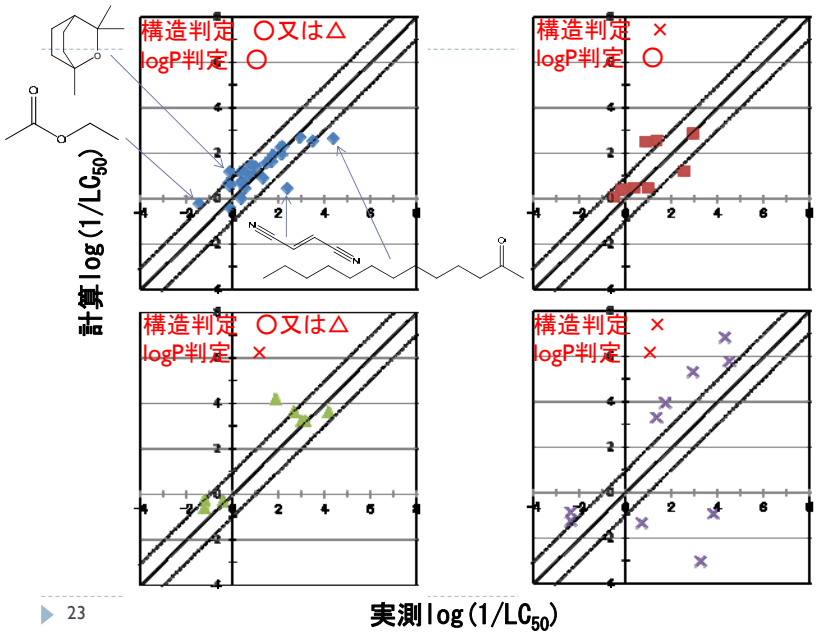
甲殻類バリデーション結果(n=53)



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KATEの活用検討状況

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化審法スクリーニング評価におけるQSAR、カテゴリーアプローチの活用検討

- QSAR やカテゴリーアプローチの導入については、スクリーニング評価作業の中のどのような場面で活用可能か等を早急に検討し、活用可能と考えられる部分については、一般化学物質のスクリーニング評価の実施に合わせて試行することを目指すとしたが、より具体的な検討ステップは以下のとおり考える。

- 1. スクリーニング評価において、どのような場面でQSAR やカテゴリーアプローチが活用可能かを検討する。その際、①有害性を過小に評価しないこと。②効率的で低負荷なスクリーニング評価の実施に貢献すること等を念頭に検討を行う。
- 2. 人健康、生態に対する候補QSAR モデルについて、新規化学物質、既存点検等用いた試験データとの検証を進めてきた推計成績（正解率、統計データ）をまとめる。
- 3. 1. で活用すべきと判断した場面において、2. の推計成績を加味し、利用可能なQSAR モデルやカテゴリーを利用するものとする。具体的に想定される活用事例としては、「有害性情報が得られない場合の代用」、「評価を行う順序付け」等が挙げられる。なお、後者の活用例等は、推計成績が必ずしも高い必要がないと考えられることから、積極的なQSAR やカテゴリーアプローチの活用を行う。
- 4. このような実績を積み上げながら、国際動向や国内外のQSAR モデルの開発動向やカテゴリーアプローチの活用動向も注視し、一層、適用範囲を広げていく。
- なお、1～4の検討については別途3省で毒性等の専門家の意見を踏まえつつ検討を進め、一般化学物質のスクリーニングの開始までに3省の審議会で具体的な利用方法をとりまとめるものとする。

スクリーニング評価の基本的な考え方における（三省合同審議会、平成22年10月8日）より

25

化審法スクリーニング評価における検討事例

生態影響に係る優先度「中」区分からの優先評価化学物質選定について（抜粋）

物質名	有害性クラス	PNEC (mg/L) (A)／(B)	最小値 (mg/L) (A)	Ufs (B)	藻類(mg/L) 急性毒性値 (EC50)	藻類(mg/L) EC50/ACR	藻類(mg/L) 慢性毒性値 (NOEC)	藻類(mg/L) NOEC/UF (種間外挿)	ミジンコ類(mg/L) 急性毒性値 (EC50)	ミジンコ類(mg/L) EC50/ACR	ミジンコ類(mg/L) 慢性毒性値 (NOEC)	ミジンコ類(mg/L) NOEC/UF(種間外挿)	魚類(mg/L) 急性毒性値 (LC50)	魚類(mg/L) LC50/ACR	魚類(mg/L) 慢性毒性値 (NOEC)	魚類(mg/L) NOEC/UF(種間外挿)
オクタデシルアミン	1	0.000013	0.13	10000	0.12	0.006	0.029	0.0029	0.13	0.0013						
N, N－ジメチルドデカン－1－イルアミン	1	＜ 0.000052	＜0.0026	50	0.014	0.0007	＜ 0.0026	＜ 0.00052	0.083	0.00083	0.036	0.0072	0.57	0.0057		
ココアルキルアミン	1	0.000045	0.045	1000	0.17	0.0085	0.071	0.0071	0.045	0.00045			0.16	0.0016		

三省合同審議会（平成25年1月19日）より

魚類急性毒性：ミジンコよりも毒性弱い可能性？
魚類急性毒性ありと見なす場合（エキスパートジャッジ）
- UF：10000（急性二種）⇒1000（急性三種）
- PNEC：0.000013 mg/L⇒ 0.00013 mg/L
- スクリーニング評価結果：優先評価化学物質相当⇒一般化学物質相当

オクタデシルアミンKATE予測結果：
- ミジンコ48時間EC_{50}：0.00091 mg/L
- 魚類96時間LC_{50}：0.037 mg/L
- ドメイン判定(構造)：内
- ドメイン判定(logP)：外
  （本物質logP：7.7，参照物質log上限：5.4）

⇒ 今回はドメイン外であり活用不可
次回以降のスクリーニング評価で
活用可能か、引き続き検討を実施

26

化学物質の環境リスク初期評価における活用：検討中

- QSARの生態リスク初期評価への活用方法について、活用の限界等について論点を整理した上でルール化について検討中。

表　水生生物に対する毒性値の概要

生物群	急性	慢性	毒：性値 [μg/L]	生物名	生物分類／和名	エンドポイント／影響内容	ばく露期間[日]	試験の信頼性	採用の可能性	文献No.
藻 類	○		640	Pseudokirchneriella subcapitata	緑藻類	EC_{50} GRO(RATE)	2	B	B	1)-106416
	○		7,100	Skeletonema costatum	珪藻類	EC_{50} GRO	4	D	C	1)-9607
	○		52,900	Pseudokirchneriella subcapitata	緑藻類	EC_{50} GRO	4	D	C	1)-9607
甲殻類	○		1,480	Americamysis bahia	アミ科	LC_{50} MOR	4	D	C	1)-9607
	○		>530,000	Daphnia magna	オオミジンコ	LC_{50} MOR	2	C	C	1)-5184
魚 類			69	Jordanella floridae	キプリノドン科（仔魚）	NOEC GRO	28	B	C	1)-140
	○		>89	Pimephales promelas	ファットヘッドミノー	LC_{50} MOR	4 (止水式)	C	C	1)-17138
		○	90	Cyprinodon variegatus	キプリノドン科（胚）	NOEC MOR（仔魚）	～ふ化後28	B	B	1)-9953
	○		305	Pimephales promelas	ファットヘッドミノー	LC_{50} MOR	4 (流水式)	D	C	4)-2012253
：	：	：	：	：	：	：	：	：	：	：

化学物質の環境リスク評価　第11巻　1,2,4,5-テトラクロロベンゼンより

http://www.env.go.jp/chemi/risk/index.html

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ご静聴ありがとうございました

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1. A. Furuhama, T. Toida, N. Nishikawa, Y. Aoki, Y. Yoshioka, H. Shiraishi, Development of an ecotoxicity QSAR model for the KAshinhou Tool for Ecotoxicity (KATE) system, March 2009 version, SAR QSAR Environ. Res., 21 (2010), pp. 403-413.
2. A. Furuhama, K. Hasunuma, Y. Aoki, Y. Yoshioka and H. Shiraishi, Application of chemical reaction mechanistic domains to an ecotoxicity QSAR model, KAshinhou Tool for Ecotoxicity (KATE), SAR QSAR Environ. Res., 22 (2011), 505-523.
3. A. Furuhama, Y. Aoki, and H. Shiraishi, Development of ecotoxicity QSAR models based on partial charge descriptors for acrylate and related compounds, SAR QSAR Environ. Res., in press.
4. 吉岡義正「QSAR用プラットホーム(PAS)作成のノウハウ-1 SMILES式からの要素の抽出-」環境毒性学会誌 Vol. 11 (2008), pp. 33-40 .
5. 吉岡義正「QSAR用プラットホーム(PAS)作成のノウハウ-2 構造図の描画-」環境毒性学会誌 Vol. 12 (2009) , pp. 107-112.
6. 吉岡義正「QSAR用プラットホーム(PAS)作成のノウハウ-3 部分構造の取得法-」環境毒性学会誌 Vol. 12 (2009), pp.113-122.
7. 古濱彩子、白石寛明「化学物質の生態毒性予測システムKATEとQSAR」日本化学会情報化学部会誌 Vol. 30 (2012), pp.42-45.

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KATEの詳細・ダウンロード先：https://kate.nies.go.jp

OECD Guidelines for the Testing of Chemicals 210 Fish, Early-life Stage Toxicity Test 1992-2013 改定版比較表 （仮）

1992	2013
INTRODUCTION	**INTRODUCTION**
1. Tests with the early-life stages of fish are intended to define the lethal and sub-lethal effects of chemicals on the stages and species tested. They yield information of value for the estimation of the chronic lethal and sub-lethal effects of the substance on other fish species.	1. Tests with the early-life stages of fish are intended to define the lethal and sub-lethal effects of chemicals on the stages and species tested. They yield information of value for the estimation of the chronic lethal and sub-lethal effects of the chemical on other fish species.
2. This guideline is based on a proposal from the United Kingdom which was discussed at a meeting of OECD experts convened at Medmenham (United Kingdom) in November 1988.	2. This Test Guideline 210 is based on a proposal from the United Kingdom which was discussed at a meeting of OECD experts convened at Medmenham (United Kingdom) in November 1988 and further updated in 2013 to reflect experience in using the test and recommendations from an OECD workshop on fish toxicity testing, held in September 2010 (1).
PRINCIPLE OF THE TEST	**PRINCIPLE OF THE TEST**
3. The early-life stages of fish are exposed to a range of concentrations of the test substance dissolved in water, preferably under flow-through conditions, or where appropriate, semi-static conditions. The test is begun by placing fertilised eggs in the test chambers and is continued at least until all the control fish are free-feeding. Lethal and sub-lethal effects are assessed and compared with control values to determine the lowest observed effect concentration and hence the no observed effect concentration (see Annex 1 for definitions).	3. The early-life stages of fish are exposed to a range of concentrations of the test chemical dissolved in water. Flow-through conditions are preferred; however, if it is not possible semi-static conditions are acceptable. For details the OECD Guidance Document No. 23 on aquatic toxicity testing of difficult substances and mixtures should be consulted (2). The test is initiated by placing fertilised eggs in test chambers and is continued for a species-specific time period that is necessary for the control fish to reach a juvenile life-stage. Lethal and sub-lethal effects are assessed and compared with control values to determine the lowest observed effect concentration (LOEC) in order to determine the (i) no observed effect concentration (NOEC) and/or (ii) ECx (e.g. EC10, EC20) by using a regression model to estimate the concentration that would cause a x % change in the effect measured. Reporting of relevant effect concentrations and parameters may depend upon the regulatory framework. The test concentrations should bracket the ECx so that the ECx comes from interpolation rather than extrapolation (see Annex 1 for definitions).
INFORMATION ON THE TEST SUBSTANCE	**INFORMATION ON THE TEST CHEMICAL**
4. Results of an acute toxicity test (see Guideline 203), preferably performed with the species chosen for this test, should be available. This implies that the water solubility and the vapour pressure of the test substance are known and a reliable analytical method for the quantification of the substance in the test solutions with known and reported accuracy and limit of detection is available.	4. Test chemical refers to what is being tested. The water solubility (see Guideline 105) and the vapour pressure (see Guideline 104) of the test chemical should be known and a reliable analytical method for the quantification of the chemical in the test solutions with known and reported accuracy and limit of quantification should be available. Although not necessary to conduct the test, results from an acute toxicity test (see Guideline 203 or Guideline 236), preferably v performed with the species chosen for this test, may provide useful information.
	5. If the Test Guideline is used for the testing of a mixture, its composition should as far as possible be characterised, e.g., by the chemical identity of its constituents, their

1992	2013
	quantitative occurrence and their substance-specific properties (like those mentioned above). Before use of the Test Guideline for regulatory testing of a mixture, it should be considered whether it will provide acceptable results for the intended regulatory purpose.
5. Useful information includes the structural formula, purity of the substance, stability in water and light, pKa, Pow and results of a test for ready biodegradability (see Guideline 301).	6. Useful information includes the structural formula, purity of the substance, water solubility, stability in water and light, pKa, Pow and results of a test for ready biodegradability (e.g., Guideline 301 or Guideline 310).
VALIDITY OF THE TEST	**VALIDITY OF THE TEST**
6. For a test to be valid the following conditions apply:	7. For a test to be valid the following conditions apply:
- the dissolved oxygen concentration must be between 60 and 100 per cent of the air saturation value throughout the test; - the water temperature must not differ by more than + 1.5oC between test chambers or between successive days at any time during the test, and should be within the temperature ranges specified for the test species (Annexes 3 and 6); ~~- evidence must be available to demonstrate that the concentrations of the test substance in solution have been satisfactorily maintained within + 20% of the mean measured values;~~ - overall survival of fertilised eggs in the controls and, where relevant, in the solvent ~~only~~ controls must be greater than or equal to the limits defined in Annexes 3 and 6; ~~- when a solubilising agent is used it must have no significant effect on survival nor produce any other adverse effects on the early-life stages as revealed by a solvent-only control.~~	・ the dissolved oxygen concentration should be >60% of the air saturation value throughout the test; ・ the water temperature should not differ by more than + 1.5oC between test chambers or between successive days at any time during the test, and should be within the temperature ranges specified for the test species (Annex 2); ・the analytical measure of the test concentrations is compulsory. ・ overall survival of fertilised eggs and post-hatch success in the controls and, where relevant, in the solvent controls should be greater than or equal to the limits defined in Annex 2.
	8. If a minor deviation from the validity criteria is observed, the consequences should be considered in relation to the reliability of the test data and these considerations should be included in the report. Effects on survival, hatch or growth occurring in the solvent control, when compared to the negative control, should be reported and discussed in the context of the reliability of the test data.
DESCRIPTION OF THE METHOD	**DESCRIPTION OF THE METHOD**
Test chambers	**Test chambers**
7. Any glass, stainless steel or other chemically inert vessels can be used. The dimensions of the vessels should be large enough to allow compliance with loading rate criteria given below. It is desirable that test chambers be randomly positioned in the test area. A randomised block design with each treatment being present in each block	9. Any glass, stainless steel or other chemically inert vessels can be used. As silicone is known to have a strong capacity to absorb lipophilic substances, the use of silicone tubing in flow-through studies and use of silicone seals in contact with water should be minimised by the use of e.g. monoblock glass aquaria. The dimensions of the vessels should be large enough to allow proper growth in the control, maintenance of dissolved

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is preferable to a completely randomised design. The test chambers should be shielded from unwanted disturbance.	oxygen concentration (e.g. for small fish species, a 7 L tank volume will achieve this) and compliance with the loading rate criteria given in paragraph 19. It is desirable that test chambers be randomly positioned in the test area. A randomised block design with each treatment being present in each block is preferable to a completely randomised design. The test chambers should be shielded from unwanted disturbance. The test system should preferably be conditioned with concentrations of the test chemical for a sufficient duration to demonstrate stable exposure concentrations prior to the introduction of test organisms.
Selection of species	**Selection of species**
8. Recommended fish species are given in Table 1~~a~~. This does not preclude the use of other species (~~and examples are given in Table 1b~~), but the test procedure may have to be adapted to provide suitable test conditions. The rationale for the selection of the species and the experimental method should be reported in this case.	10. Recommended fish species are given in Table 1. This does not preclude the use of other species, but the test procedure may have to be adapted to provide suitable test conditions. The rationale for the selection of the species and the experimental method should be reported in this case.
Holding of the brood fish	**Holding of the brood fish**
9. Details on holding the brood stock under satisfactory conditions may be found in Annex 2 and the references cited (1)(2)(3).	11. Details on holding the brood stock under satisfactory conditions may be found in Annex 3 and the references cited (3)(4)(5).
Handling of embryos and larvae	**Handling of fertilised eggs, embryos and larvae**
10. Initially, embryos and larvae may be exposed within the main vessel in smaller glass or stainless steel vessels, fitted with mesh sides or ends to permit a flow of test solution through the vessel. Non-turbulent flow through these small vessels may be induced by suspending them from an arm arranged to move the vessel up and down but always keeping the organisms submerged. Fertilised eggs of salmonid fishes can be supported on racks or meshes with apertures sufficiently large to allow larvae to drop through after hatching.	12. Initially, fertilised eggs, embryos and larvae may be exposed within the main vessel in smaller glass or stainless steel vessels, fitted with mesh sides or ends to permit a flow of test solution through the vessel. Non-turbulent flow-through in these small vessels may be induced by suspending them from an arm arranged to move the vessel up and down but always keeping the organisms submerged. Fertilised eggs of salmonid fishes can be supported on racks or meshes with apertures sufficiently large to allow larvae to drop through after hatching.
11. Where egg containers, grids or meshes have been used to hold eggs within the main test vessel, these restraints should be removed after the larvae hatch, according to the advice in Annex 2, except that meshes should be retained to prevent the escape of the fish. If there is a need to transfer the larvae, they should not be exposed to the air and nets should not be used to release fish from egg containers. The timing of this transfer varies with the species and transfer may not always be necessary.	13. Where egg containers, grids or meshes have been used to hold eggs within the main test vessel, these restraints should be removed after the larvae hatch, according to the guidance in Annex 3, except that meshes should be retained to prevent the escape of the larvae. If there is a need to transfer the larvae, they should not be exposed to the air and nets should not be used to release larvae from egg containers. The timing of this transfer varies with the species and should be documented in the report. However, a transfer may not always be necessary.
Water	**Water**
12. Any water in which the test species shows control survival at least as good as that described in Annexes 3 and 6 is suitable as a test water. It should be of constant quality during the period of the test. In order to ensure that the dilution water will not unduly influence the test result (for example by complexation of test substance) or	14. Any water in which the test species shows suitable long-term survival and growth may be used as test water (see Annex 4). It should be of constant quality during the period of the test. In order to ensure that the dilution water will not unduly influence the test result (for example by complexation of test chemical), or adversely affect the

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adversely affect the performance of the brood stock, samples should be taken at intervals for analysis. Measurements of heavy metals (e.g. Cu, Pb, Zn, Hg, Cd, Ni), major anions and cations (e.g. Ca, Mg, Na, K, Cl, SO_4), pesticides, total organic carbon and suspended solids should be made, for example every three months where a dilution water is known to be relatively constant in quality. Some chemical characteristics of an acceptable dilution water are listed in Annex 4.	performance of the brood stock, samples should be taken at intervals for analysis. Measurements of heavy metals (e.g. Cu, Pb, Zn, Hg, Cd, Ni), major anions and cations (e.g. Ca, Mg, Na, K, Cl, SO_4), ammonia, total residual chlorine pesticides, total organic carbon and suspended solids should be made, for example, on a bi-annual basis where a dilution water is known to be relatively constant in quality. If the water is known to be of variable quality the measurements have to be conducted more often; the frequency is dependent of how variable the quality is. Some chemical characteristics of an acceptable dilution water are listed in Annex 4.
Test solutions	**Test solutions**
13. For flow-through tests, a system which continually dispenses and dilutes a stock solution of the test substance (eg metering pump, proportional diluter, saturator system) is required to deliver a series of concentrations to the test chambers. The flow rates of stock solutions and dilution water should be checked at intervals during the test and should not vary by more than 10% throughout the test. A flow rate equivalent to at least five test chamber volumes per 24 hours has been found suitable (1).	15. For flow-through tests, a system which continually dispenses and dilutes a stock solution of the test chemical (e.g. metering pump, proportional diluter, saturator system) is required to deliver a series of concentrations to the test chambers. The flow rates of stock solutions and dilution water should be checked at intervals during the test and should not vary by more than 10% throughout the test. A flow rate equivalent to at least five test chamber volumes per 24 hours has been found suitable (3). However, if the loading rate specified in paragraph 18 is respected, a lower flow rate of e.g. 2-3 test chamber volumes is possible to prevent quick removal of food.
~~14. The use of solvents or dispersants (solubilising agents) may be required in some cases in order to produce a suitably concentrated stock solution.~~	
	16. Test solutions of the chosen concentrations are prepared by dilution of a stock solution. The stock solution should preferably be prepared by simply mixing or agitating the test chemical in dilution water by using mechanical means (e.g. stirring and/or ultrasonication). Saturation columns (solubility columns) or passive dosing methods (6) can be used for achieving a suitable concentrated stock solution. The use of a solvent carrier is not recommended. However, in case a solvent is necessary, a solvent control should be run in parallel, at the same solvent concentration as the chemical treatments; i.e. the solvent level should preferably be equal across all concentrations as well as the solvent control. For some diluter systems this might be technically difficult; here the solvent concentration in the solvent control should be equal to the highest solvent concentration in the treatment group. For difficult to test substances, the OECD Guidance Document No. 23 on aquatic toxicity testing of difficult substances and mixtures should be consulted (2). If a solvent is used, the choice of solvent will be determined by the chemical properties of the substance. The OECD Guidance Document No. 23 recommends a maximum concentration of 100 µl/L. To avoid potential effect of the solvent on endpoints measured (7), it is recommended to keep solvent concentration as low as possible.

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15. For the semi-static technique, two different renewal procedures may be followed. Either new test solutions are prepared in clean vessels and survivings eggs and larvae gently transferred into the new vessels, or the test organisms are retained in the test vessels whilst a proportion (at least two thirds) of the test water is changed.	17. For a semi-static test, two different renewal procedures may be followed. Either new test solutions are prepared in clean vessels and surviving eggs and larvae gently transferred into the new vessels, or the test organisms are retained in the test vessels whilst a proportion (at least two thirds) of the test solution / control volume is changed.
PROCEDURE	**PROCEDURE**
~~16. Useful information on the performance of fish early-life stage tests is available in the literature, some examples of which are included in the literature section of this text (1)(4)(5)(6)(7)(8).~~	
Conditions of Exposure	**Conditions of Exposure**
Duration	**Duration**
17. The test should start as soon as possible after the eggs have been fertilised,~~the embryos~~ preferably being immersed in the test solutions before cleavage of the blastodisc commences, or as close as possible after this stage. ~~The test should continue at least until all the control fish have been free-feeding.~~ Test duration will depend upon the species used. Some recommended durations are given in Annexes 3 and 6.	18. The test should start as soon as possible after the eggs have been fertilised and preferably being immersed in the test solutions before cleavage of the blastodisc commences, or as close as possible after this stage. The test duration will depend upon the species used. Some recommended durations are given in Annex 2.
Loading	**Loading**
18. The number of fertilised eggs at the start of the test should be sufficient to meet statistical requirements. They should be randomly distributed among treatments, and at least 60 eggs, divided equally between at least two replicate test chambers, should be used per concentration. The loading rate (biomass per volume of test solution) should be low enough in order that a dissolved oxygen concentration of at least 60% of the air saturation value ~~(ASV)~~ can be maintained without aeration. For flow-through tests, a loading rate not exceeding 0.5 g/l per 24 hours and not exceeding 5 g/l of solution at any time has been recommended (1).	19. The number of fertilised eggs at the start of the test should be sufficient to meet statistical requirements. They should be randomly distributed among treatments, and at least 80 eggs, divided equally between at least four replicate test chambers, should be used per concentration. The loading rate (biomass per volume of test solution) should be low enough in order that a dissolved oxygen concentration of at least 60% of the air saturation value can be maintained without aeration during the egg and larval stage. For flow-through tests, a loading rate not exceeding 0.5 g/L wet weight per 24 hours and not exceeding 5 g/L of solution at any time has been recommended (3).
Light and temperature	**Light and temperature**
19. The photoperiod and water temperature should be appropriate for the test species (see Annex 3).	20. The photoperiod and water temperature should be appropriate for the test species (see Annex 2).
Feeding	**Feeding**
20. Food and feeding are critical, and it is essential that the correct food for each stage ~~should~~ be supplied from an appropriate time and at a level sufficient to support normal growth. Feeding should be ad libitum whilst minimising the surplus. Surplus food and faeces should be removed as necessary to avoid accumulation of waste. Detailed feeding regimes are given in Annex 2 but, as experience is gained, food and feeding regimes are continually being refined to improve survival and optimise growth. ~~Effort should therefore be made to confirm the proposed regime with acknowledged experts.~~	21. Food and feeding are critical, and it is essential that the correct food for each life-stage is supplied from an appropriate time and at a level sufficient to support normal growth. Feeding should be approximately equal across replicates unless adjusted to account for mortality. Surplus food and faeces should be removed as necessary, to avoid accumulation of waste. Detailed feeding regimes are given in Annex 3 but, as experience is gained, food and feeding regimes are continually being refined to improve survival and optimise growth. Live food provides a source of environmental enrichment

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	and therefore should be used in place of or in addition to dry or frozen food whenever appropriate to the species and life stage.
Test concentrations	**Test concentrations**
21. Normally five concentrations of the test substance spaced by a constant factor not exceeding 3.2 are required. The curve relating LC50 to period of exposure in the acute study should be considered when selecting the range of test concentrations. The use of fewer than five concentrations, for example in limit tests, and a narrower concentration interval may be appropriate in some circumstances. Justification should be provided if fewer than five concentrations are used. Concentrations of the substance higher than the 96 hour LC50 or 10 mg/l, whichever is the lower, need not be tested.	22. Normally five concentrations of the test chemical, with a mimimum of four replicates per concentration, spaced by a constant factor not exceeding 3.2 are required. If available, information on the acute testing, preferable with the same species and/or a range finding test should be considered (1) when selecting the range of test concentrations. However, all sources of information should be considered when selecting the range of test concentrations, including sources like e.g., read across, fish embryo acute toxicity test data. A limit test, or an extended limit test, with fewer than five concentrations as the definitive test may be acceptable where empirical NOECs only are to be established. Justification should be provided if fewer than five concentrations are used. Concentrations of the test chemical higher than the 96 hour LC50 or 10 mg/L, whichever is the lower, need not be tested.
~~22. Where a solubilising agent is used its concentration should not be greater than 0.1 ml/l and should be the same in all test vessels. However, every effort should be made to avoid the use of such materials.~~	
Controls	**Controls**
23. One dilution-water control and ~~also~~, if relevant, one control containing the solubilising agent should be run in addition to the test series.	23. A dilution-water control and, if needed, a solvent control containing the solvent carrier only should be run in addition to the test chemical concentration series (see paragraph 16).
Frequency of Analytical Determinations and Measurements	**Frequency of Analytical Determinations and Measurements**
24. During the test, the concentrations of the test substance are determined at regular intervals to check compliance with the validity criteria. A minimum of five determinations is necessary. In studies lasting more than one month determinations should be made at least once a week. Samples may need to be filtered (e.g. using a 0.45 m pore size) or centrifuged to ensure that the determinations are made on the substance in true solution.	24. Prior to initiation of the exposure period, proper function of the chemical delivery system across all replicates should be ensured (for example, by measuring test concentrations). Analytical methods required should be established, including an appropriate limit of quantification (LOQ) and sufficient knowledge on the substance stability in the test system. During the test, the concentrations of the test chemical are determined at regular intervals to characterise exposure. A minimum of five determinations is necessary. In flow-through systems, analytical measurements of the test chemical in one replicate per concentration should be made at least once a week changing systematically amongst replicates. Additional analytical determinations will often improve the quality of the test outcome. Samples may need to be filtered to remove any particulate matter (e.g. using a 0.45 μm pore size) or centrifuged to ensure that the determinations are made on the chemical in true solution. In order to reduce adsorption of the test chemical, the filters should be saturated before the use. When the measured concentrations do not remain within 80-120% of the nominal

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	concentration, the effect concentrations should be determined and expressed relative to the arithmetic mean concentration for flow-through tests (see Annex 6 of the Test Guideline 211 for the calculation of the arithmetic mean (8)), and expressed relative to the geometric mean of the measured concentrations for semi-static tests (see Chapter 5 in the OECD Guidance Document No. 23 on aquatic toxicity testing of difficult substances and mixtures (2)).
25. During the test, dissolved oxygen, pH, ~~total hardness and salinity (if relevant)~~ and temperature should be measured in all test vessels. ~~As a minimum, dissolved oxygen, salinity (if relevant) and temperature should be measured weekly, and pH and hardness at the beginning and end of the test.~~ Temperature should preferably be monitored continuously in at least one test vessel.	25. During the test, dissolved oxygen, pH, and temperature should be measured in all test vessels, at least weekly, and salinity and hardness, if warranted, at the beginning and end of the test. Temperature should preferably be monitored continuously in at least one test vessel.
Observations	**Observations**
26. Stage of embryonic development: the embryonic stage at the beginning of exposure to the test substance should be verified as precisely as possible. This can be done using a representative sample of eggs suitably preserved and cleared.	26. Stage of embryonic development: the embryonic stage at the beginning of exposure to the test chemical should be verified as precisely as possible. This can be done using a representative sample of eggs suitably preserved and cleaned.
27. Hatching and survival: observations on hatching and survival should be made at least once daily and numbers recorded. Dead embryos, larvae and juvenile fish should be removed as soon as observed since they can decompose rapidly and may be broken up by the actions of the other fish. Extreme care should be taken when removing dead individuals not to ~~knock or~~ physically damage adjacent eggs/larvae, ~~these being extremely delicate and sensitive. Criteria for death vary according to life stage:~~	27. Hatching and survival: observations on hatching and survival should be made at least once daily and numbers recorded. If fungus on eggs is observed early in embryonic development (e.g., at day one or two of test), those eggs should be counted and removed. Dead embryos, larvae and juvenile fish should be removed as soon as observed since they can decompose rapidly and may be broken up by the actions of the other fish. Extreme care should be taken when removing dead individuals not to physically damage adjacent eggs/larvae. Signs of death vary according to species and life stage. For example:
– for eggs: particularly in the early stages, a marked loss of translucency and change in colouration, caused by coagulation and/or precipitation of protein, leading to a white opaque appearance; – for embryos: absence of body movement and/or absence of heart-beat; – for larvae and juvenile fish: immobility and/or absence of respiratory movement and/or absence of heart-beat and/or ~~white opaque colouration of central nervous system~~ and/or lack of reaction to mechanical stimulus.	・for fertilised eggs: particularly in the early stages, a marked loss of translucency and change in colouration, caused by coagulation and/or precipitation of protein, leading to a white opaque appearance; ・for embryos, larvae and juvenile fish: immobility and/or absence of respiratory movement and/or absence of heartbeat and/or lack of reaction to mechanical stimulus.
28. Abnormal appearance: the number of larvae or fish showing abnormality of body form should be recorded at adequate intervals depending on the duration of the test and the nature of the abnormality described. It should be noted that abnormal embryos and larvae occur naturally and can be of the order of several percent in the control(s) in some species. ~~Abnormal animals should only be removed from the test vessels on death.~~	28. Abnormal appearance: the number of larvae or juvenile fish showing abnormality of body form should be recorded at adequate intervals depending on the duration of the test and the nature of the abnormality described. It should be noted that abnormal larvae and juvenile fish occur naturally and can be of the order of several percent in the control(s) in some species. Where deformities and associated abnormal behaviour are considered so severe that there is considerable suffering to the organism, and it

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	has reached a point beyond which it will not recover, it may be removed from the test. Such animals should be euthanised and treated as mortalities for subsequent data analysis. Normal embryonic development has been documented for most species recommended in this Guideline (9) (10) (11) (12).
29. Abnormal behaviour: abnormalities, e.g. hyperventilation, unco-ordinated swimming, atypical quiescence and atypical feeding behaviour should be recorded at adequate intervals depending on the duration of the test. These effects, although difficult to quantify, can, when observed, aid in the interpretation of mortality data ~~and influence a decision to extend the exposure period beyond the recommended duration.~~	29. Abnormal behaviour: abnormalities, e.g. hyperventilation, uncoordinated swimming, atypical quiescence and atypical feeding behaviour should be recorded at adequate intervals depending on the duration of the test (e.g. once daily for warm water species). These effects, although difficult to quantify, can, when observed, aid in the interpretation of mortality data.
30. Weight: at the end of the test all surviving fish must be weighed. ~~Individual weights are preferred but, if the fish are especially small, they may be weighed in groups by test vessel. Dry weights (24 hours at 60°C) are preferable to wet weights (blotted dry).~~	30. Weight: at the end of the test, all surviving fish are weighed at least on a replicate basis (reporting the number of animals in the replicate and the mean weight per animal): wet weight ? (blotted dry) is preferred, however, dry weight data may also be reported (13).
31. Length: at the end of the test, measurement of individual lengths is recommended; ~~standard, fork or total length may be used.~~ If however, caudal fin rot or fin erosion occurs, standard lengths should be used.	31. Length: at the end of the test, individual lengths are measured. Total length is recommended, if however, caudal fin rot or fin erosion occurs, standard lengths can be used. The same method should be used for all fish in a given test. Individual length can be measured either by e.g. callipers, digital camera, or calibrated ocular micrometer. Typical minimum lengths are defined in Annex 2.
~~32. These observations will result in some or all of the following data being available for statistical analysis:~~ ~~- cumulative mortality;~~ ~~- numbers of healthy fish at end of test;~~ ~~- time to start of hatching and end of hatching;~~ ~~- numbers of larvae hatching each day;~~ ~~- length and weight of surviving animals;~~ ~~- numbers of deformed larvae;~~ ~~- numbers of fish exhibiting abnormal behaviour.~~	
DATA AND REPORTING	**DATA AND REPORTING**
Treatment of results	**Treatment of results**
33. It is recommended that ~~a statistician be involved in both the design and analysis of the test since this Test Guideline allows for considerable variation in experimental design as, for example, in the number of test chambers, number of test concentrations, starting number of fertilised eggs and in the parameters measured.~~	32. It is recommended that the design of the experiment and selection of statistical test permit adequate power (80% or higher) to detect changes of biological importance in endpoints where a NOEC is to be reported. Reporting of relevant effect concentrations and parameters may depend upon the regulatory framework. If an ECx is to be reported, the design of the experiment and selection of regression model should permit estimation of ECx so that (i) the 95% confidence interval reported for ECx does not contain zero and is not overly wide, (ii) the 95% confidence interval for the predicted

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	mean at ECx does not contain the control mean (iii) there is no significant lack-of-fit of regression model to the data. Either approach requires the identification of the percent change in each endpoint that is important to detect or estimate. The experimental design should be tailored to allow that. When the above conditions for determining the ECx are not satisfied, the NOEC approach should be used. It is not likely that the same percent change applies to all endpoints, nor is it likely that a feasible experiment can be designed that will meet these criteria for all endpoints, so it is important to focus on the endpoints, which are important for the respective experiment in designing the experiment appropriately. Statistical flow diagrams and guidance for each approach are available in Annexes 5 and 6 to guide in the treatment of data and in the choice of the most appropriate statistical test or model to use. Other statistical approaches may be used, provided they are scientifically justified.
34. ~~In view of the options available in test design, specific guidance on statistical procedures is not given here. However~~ it will be necessary for variations to be analysed within each set of replicates using analysis of variance or contingency table procedures. In order to make a multiple comparison between the results at the individual concentrations and those for the controls, ~~Dunnett's method may be found useful (9)(10). However,~~ care must be taken where applying such a method to ensure that chamber to chamber variability is estimated and ~~is acceptably low. Other useful examples are also available (1)(6)(11).~~	33. It will be necessary for variations to be analysed within each set of replicates using analysis of variance or contingency table procedures and appropriate statistical analysis methods be used based on this analysis. In order to make a multiple comparison between the results at the individual concentrations and those for the controls, the step-down Jonckheere-Terpstra or Williams' test is recommended for continuous responses and a step-down Cochran-Armitage test for quantal responses that are consistent with a monotone concentration-response and with no evidence of extra-binomial variance (14). When there is evidence of extra-binomial variance, the Rao-Scott modification of the Cochran-Armitage test is recommended (15) (16) or Williams or Dunnett's (after an arcsin-square-root transform) or Jonckheere-Terpstra test applied to replicate proportions. Where the data are not consistent with a monotone concentration-response, Dunnett's or Dunn's or the Mann-Whitney method may be found useful for continuous responses and Fisher's Exact test for quantal responses (14) (17) (18). Care should be taken where applying any statistical method or model to ensure that the requirements of the method or model are satisfied (e.g. chamber to chamber variability is estimated and accounted for in the experimental design and test or model used). Data are to be evaluated for normality and Annex 5 indicates what should be done on the residuals from an ANOVA. Annex 6 discusses additional considerations for the regression approach. Transformations to meet the requirements of a statistical test should be considered. However, transformations to enable the fitting of a regression model require great care, as, for example, a 25% change in the untransformed response does not correspond to a 25% change in a transformed response. In all analyses, the test chamber, not the individual fish, is the

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	unit of analysis and the experimental unit and both hypothesis tests and regression should reflect that (3) (14) (19) (20).
~~**Interpretation of results**~~	
~~35. The results should be interpreted with caution where measured toxicant concentrations in test solutions occur at levels near the detection limit of the analytical method.~~	
Test report	**Test report**
36. The test report must include the following information:	34. The test report should include the following information:
Test substance:	**Test chemical:**
	Mono-constituent substance
– physical nature and, where relevant, physicochemical properties; – chemical identification data.	– physical appearance, water solubility, and additional relevant physicochemical properties; – chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc. (including the organic carbon content, if appropriate.
	Multi-constituent substance, UVBCs and mixtures:
	– characterised as far as possible, e.g., by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents
Test species:	**Test species:**
– scientific name, strain, source and method of collection of the fertilised eggs and subsequent handling.	– scientific name, strain, source and method of collection of the fertilised eggs and subsequent handling.
Test conditions:	**Test conditions:**
– test procedure used (e.g. semi-static or flow-through, loading); – photoperiod(s); – test design (e.g. number of test chambers and replicates, number of embryos per replicate); – method of preparation of stock solutions and frequency of renewal (the solubilizing agent and its concentration must be given, when used); – the nominal test concentrations, the means of the measured values and their standard deviations in the test vessels and the method by which these were attained and evidence that the measurements refer to the concentrations of the test substance in true solution;	– test procedure used (e.g. semi-static or flow-through, loading); – photoperiod(s); – test design (e.g. number of test chambers and replicates, number of eggs per replicate, material and size of the test chamber (height, width, volume), water volume per test chamber); – method of preparation of stock solutions and frequency of renewal (the solubilising agent and its concentration should be given, when used); – method of dosing the test chemical (e.g. pumps, diluting systems) – the recovery efficiency of the method and the nominal test concentrations, the limit of quantification, the means of the measured values and their standard deviations in the test vessels and the method by which these were attained and evidence that the measurements refer to the concentrations of

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– dilution water characteristics: pH, hardness, temperature, dissolved oxygen concentration, residual chlorine levels (if measured), total organic carbon, suspended solids, salinity of the test medium (if measured) and any other measurements made; – water quality within test vessels, pH, hardness, temperature and dissolved oxygen concentration; – detailed information on feeding (e.g. type of food(s), source, amount given and frequency).	the test chemical in true solution; – dilution water characteristics: pH, hardness, temperature, dissolved oxygen concentration, residual chlorine levels (if measured), total organic carbon (if measured), suspended solids (if measured), salinity of the test medium (if measured) and any other measurements made; – water quality within test vessels, pH, hardness, temperature and dissolved oxygen concentration; – detailed information on feeding (e.g. type of food(s), source, amount given and frequency).
Results:	**Results reported individually (or on a replicate basis) and as mean and coefficient of variation, as appropriate, for the following endpoints:**
– evidence that controls met the overall survival acceptability standard of the test species (Annexes 3 and 6); – data on mortality~~/survival~~ at embryo, larval and juvenile stages and overall mortality~~/survival~~; – days to hatch and numbers hatched; – data for length and weight; – incidence and description of morphological abnormalities, if any; – incidence and description of behavioural effects, if any; – statistical analysis and treatment of data; – no observed effect concentration for each response assessed (NOEC); – lowest observed effect concentration (at p = 0.05) for each response assessed (LOEC); ~~– any concentration–response data and curves available.~~	– evidence that controls met the overall survival acceptability standard of the test species (Annex 2); – data on mortality at each stage (embryo, larval and juvenile) and cumulative mortality; – days to hatch, numbers of larvae hatched each day, and end of hatching; – number of healthy fish at end of test; – data for length (specify either standard or total) and weight of surviving animals; – incidence, description and number of morphological abnormalities, if any; – incidence, description and number of behavioural effects, if any; – approach for the statistical analysis (regression analysis or analysis of the variance) and treatment of data (statistical test or model used); – no observed effect concentration for each response assessed (NOEC); – lowest observed effect concentration (at p = 0.05) for each response assessed (LOEC); – ECx for each response assessed, if applicable, and confidence intervals (e.g. 90% or 95%) and a graph of the fitted model used for its calculation, the slope of the concentration–response curve, the formula of the regression model, the estimated model parameters and their standard errors.
	Any deviation from the Test Guideline.
Discussion of the results.	Discussion of the results, including any influence of deviations from the Guideline on the outcome of the test.

TABLE 1~~A~~: FISH SPECIES RECOMMENDED FOR TESTING

1992	2013	1992	2013
FRESHWATER	FRESHWATER	SALTWATER	ESTUARINE and MARINE
Oncorhynchus mykiss (Rainbow trout) *Pimephales promelas* (Fathead minnow) *Brachydanio rerio* (Zebra fish) *Oryzias latipes* (Ricefish)	*Oncorhynchus mykiss* (Rainbow trout) *Pimephales promelas* (Fathead minnow) *Danio rerio* (Zebrafish) *Oryzias latipes* (Japanese ricefish or Medaka)	*Cyprinodon variegatus* (Sheepshead minnow)	*Cyprinodon variegatus* (Sheepshead minnow) *Menidia* sp. (Silverside)

ANNEX 2(1992) 3(2013) FEEDING AND HANDLING REQUIREMENTS OF BROOD AND TEST ANIMALS OF RECOMMENDED SPECIES

SPECIES	adapted	FOOD*					POST-HATCH TRANSFER TIME ~~(if applicable)~~	TIME TO FIRST FEEDING
		Brood fish	Newly-hatched larvae	Juveniles				
				Type	~~Amount~~	Frequency		
Freshwater:								
Oncorhynchus mykiss (Rainbow trout)	1992	trout food	none(a)	trout starter	~~4% body wt per day~~	2-4 feeds per day	14-16 days post-hatch or at swim-up (not essential)	19 days post-hatch or at swim-up
	2013	trout food	None [(a)]	trout starter BSN		2-4 feeds per day	14-16 days post-hatch or at swim-up (not essential)	19 days post hatch or at swim-up
Pimephales promelas (Fathead minnow)	1992	FBS	BSN	BSN48		ad lib.	once hatching is 90%	within 2 days of hatching
	2013	BSN, flake food FBS	BSN	BSN48, flake food		2-3 times a day	once hatching is 90%	2 day post hatch
Brachydanio rerio(1992) *Danio rerio*(2013) (Zebra fish)	1992	BSN48, flake food	protozoa(b), protein(c)	BSN48			not necessary	6-7 days after spawning
	2013	BSN, flake food	Commercial larvae food, protozoa[(b)], protein[(c)]	BSN48, flake food,		BSN once daily; flake food twice daily	once hatching is 90%	2 days post hatch
Oryzias latipes	1992	flake food	BSN, flake food	BSN48, flake food		BSN once daily;	from hatch to	within 24h of

SPECIES	adapted	FOOD*					POST-HATCH TRANSFER TIME ~~(if applicable)~~	TIME TO FIRST FEEDING
		Brood fish	Newly-hatched larvae	Juveniles				
				Type	~~Amount~~	Frequency		
(Ricefish(1992) Japanese ricefish or Medaka(2013))			(or protozoa or rotifers)	(or rotifers)		flake food twice daily or flake food and rotifers once daily	swim-up	hatch/swim-up
	2013	flake food	BSN, flake food (or protozoa or rotifers)	BSN48, flake food (or rotifers)		BSN once daily; flake food twice daily or flake food and rotifers once daily	not applicable	6-7 days post spawn
SALTWATER:								
Cyprinodon variegatus (Sheepshead minnow)	1992	FBS or flake food	BSN	BSN48		2-3 feeds per day	not applicable	~~within~~ 1 day frist hatch
	2013	BSN, flake food, FBS	BSN	BSN48		2-3 feeds per day	not applicable	1 day post hatch/swim-up
Menidia sp. (Silverside)	2013	BSN48, flake food	BSN	BSN48		2-3 feeds per day	not applicable	1 day post hatch/swim-up

key

1992	2013
	*Food should be given to satiation. Surplus food and faeces should be removed, as necessary to avoid accumulation of waste
FBS frozen brine shrimps; adults Artemia sp	FBS frozen brine shrimps; adults Artemia sp
BSN brine shrimp nauplii; newly hatched	BSN brine shrimp nauplii; newly hatched
BSN48 brine shrimp nauplii; 48 hours old	BSN48 brine shrimp nauplii; 48 hours old
(a) yolk-sac larvae require no food	(a) yolk-sac larvae require no food
(b) filtered from mixed culture	(b) filtered from mixed culture
(c) granules from fermentation process	(c) granules from fermentation process

ANNEX 3(1992) 2(2013) TEST CONDITIONS, DURATION AND SURVIVAL CRITERIA FOR RECOMMENDED SPECIES

SPECIES	adapted	TEST CONDITIONS			RECOMMENDED DURATION OF TEST	Typical minimum mean total length of control fish at the end of the study (mm)(1)	SURVIVAL OF CONTROLS (minimum)	
		Temperature (℃)	Salinity (‰)	Photoperiod (hrs)			Hatching success	Post-hatch success
Freshwater:								
Oncorhynchus mykiss (Rainbow trout)	1992	10 ± 2 (a) 12 ± 2 (b)(1)		(c)	2 weeks after controls are free-feeding (or 60 days post-hatch)		>66%	70%
	2013	10 ± 1.5(2)		12 – 16(3)	2 weeks after controls are free-feeding (or 60 days post-hatch)	40	75%	75%
Pimephales promelas (Fathead minnow)	1992	25 ± 2		16	32 days from start of test (or 28 days posthatch)		>66%	70%
	2013	25 ± 1.5		16	32 days from start of test (or 28 days post-hatch)	18	70%	75%
Brachydanio rerio(1992) *Danio rerio*(2013) (Zebra fish)	1992	25 ± 2		12 – 16(4)	30 days post-hatch			70
	2013	26 ± 1.5		12 – 16(4)	30 days post-hatch	11	70%	75
Oryzias latipes (Ricefish(1992) Japanese ricefish or Medaka(2013))	1992	24 ± 1 (a) 23 ± 2(b)(2)		12 – 16(4)	30 days post-hatch			80%
	2013	25 ± 2		12 – 16(4)	30 days post-hatch	17	80%	80%
SALTWATER:								
Cyprinodon variegatus (Sheepshead minnow)	1992	25 ± 2	15–30(3)	12 – 16(4)	32 days from start of test (or 28 days posthatch)		≥75%	80%
	2013	25 ± 1.5	15–35(5)	12 – 16(4)	32 days from start of test (or 28 days post-hatch)	17	75%	80%
Menidia sp. (Silverside)	2013	22 – 25	15–35(5)	13	28 days	20	80%	60%

key

1992	2013
~~(a) for embryos.~~	
~~(b) for larvae and juvenile fish.~~	
(c) darkness for larvae until one week after hatching except when they are being inspected, then subdued lighting throughout test (12–16 hour photoperiod (4)).	(3) Darkness for larvae until one week after hatching except when they are being inspected, then subdued lighting throughout test (12–16 hour photoperiod)(4).
(1) the particular strain of rainbow trout tested may necessitate the use of other temperatures. Brood stock must be held at the same temperature as that to be used for the eggs.	(2) The particular strain of rainbow trout tested may necessitate the use of other temperatures. Brood stock must be held at the same temperature as that to be used for the eggs. After receipt of eggs from a commercial breeder, a short adaptation (*e.g.* 1–2 h) to test temperature after arrival is necessary.
~~(2) this supersedes the requirement for temperature control given earlier on in the test.~~	
(3) for any given test this shall be performed to ±2‰.	(5) For any given test this shall be performed to ±2‰.
(4) for any given test conditions, light regime should be constant.	(4) For any given test conditions, light regime should be constant.
	(1) Typical minimum mean total length is not a validity criterion but deviations below the figure indicated should be carefully examined in relation to the sensitivity of the test. The minimum mean total length is derived from a selection of data available at the current time.

ANNEX 4 SOME CHEMICAL CHARACTERISTICS OF AN ACCEPTABLE DILUTION WATER

SUBSTANCE	adapted	CONCENTRATIONS
		Limit concentration
Particular matter	1992	<20 mg/l
	2013	5mg/L
Total organic carbon	1992	<2 mg/l
	2013	2mg/L
Unionised ammonia	1992	<1 ug/l
	2013	1 μg/L
Residual chlorine	1992	<10 ug/l
	2013	10 μg/L
Total organophosphorus pesticides	1992	<50 ng/l
	2013	50 ng/L
Total organochlorine pesticides plus polychlorinated biphenyls	1992	<50 ng/l
	2013	50 ng/L
Total organic chlorine	1992	<25 ng/l
	2013	25 ng/L
Aluminium	2013	1 μg/L

SUBSTANCE	adapted	CONCENTRATIONS
		Limit concentration
Arsenic	2013	1 μg/L
Chromium	2013	1 μg/L
Cobalt	2013	1 μg/L
Copper	2013	1 μg/L
Iron	2013	1 μg/L
Lead	2013	1 μg/L
Nickel	2013	1 μg/L
Zinc	2013	1 μg/L
Cadmium	2013	100 ng/L
Mercury	2013	100 ng/L
Silver	2013	100 ng/L

※他の Annex は省略

OECD Guidelines for the Testing of Chemicals 211 *Daphnia magna* Reproduction Test 2008-2012 改定版比較表 （仮）

2008	2012
INTRODUCTION	**INTRODUCTION**
1. OECD Test Guidelines for Testing of Chemicals are periodically reviewed in the light of scientific progress. With respect to Guideline 202, Part II, *Daphnia* sp Reproduction Test (adopted April 1984), it had generally been acknowledged that data from tests performed according to this Guideline could be variable. This led, ~~in recent years~~, to considerable effort being devoted to the identification of the reasons for this variability with the aim of producing a better test method. This updated Guideline is based on the outcome of these research activities and ring-tests performed in 1992 (1) and 1994 (2).	1. OECD Test Guidelines for Testing of Chemicals are periodically reviewed in the light of scientific progress. With respect to Guideline 202, Part II, *Daphnia* sp. Reproduction Test (adopted April 1984), it had generally been acknowledged that data from tests performed according to this Guideline could be variable. This led, to considerable effort being devoted to the identification of the reasons for this variability with the aim of producing a better test method. This Test Guideline (TG) is based on the outcome of these research activities, ring-tests and validation studies performed in 1992 (1), 1994 (2) and 2008 (3).
2. The main differences between the initial version (1984) and the second version (1998) of the Guideline are:	2. The main differences between the initial version (1984), and second version (1998) and this version of the Guideline are:
(a) the species to be used is *Daphnia magna;* (b) the test duration is 21 days; (c) for semi-static tests, the number of animals to be used at each test concentration has been reduced from at least 40, preferably divided into four groups of 10 animals, to at least 10 animals held individually (although different designs can be used for flow-through tests); (d) more specific recommendations have been made with regard to test medium and feeding conditions. The main difference between the second version (1998) and this version is: (e) Annex 7 has been added to describe procedures for the identification of neonate sex if required. In line with previous versions of this guideline sex ratio is an optional endpoint.	(a) the recommended species to be used is *Daphnia magna;* (b) the test duration is 21 days; (c) for semi-static tests, the number of animals to be used at each test concentration has been reduced from at least 40, preferably divided into four groups of 10 animals, to at least 10 animals held individually (although different designs can be used for flow-through tests); (d) more specific recommendations have been made with regard to test medium and feeding conditions. The main differences between the second version (1998) and this version are: (e) In 2008, Annex 7 has been added to describe procedures for the identification of neonate sex if required. In line with previous versions of this TG sex ratio is an optional endpoint; (f) In 2012, the response variable number of living offspring produced per surviving parental animal has been supplemented with an additional response variable for Daphnia reproduction ,i.e. the total number living offspring produced at the end of the test per parent daphnia at the start of the test excluding from the analysis parental accidental and/or inadvertent mortality. The purpose of the added response variable is to align this response variable with other OECD reproduction Test Guidelines on invertebrates. Furthermore, in relation to this response variable, it is possible, in this TG, to remove a source of error, namely the effect of inadvertent and/or accidental parental mortality, should that occur during the exposure period. (g) Additional statistical guidance for test design and for treatment of results has been included both for ECx (e.g. EC10 or EC50) and for NOEC/LOEC approach.

2008	2012
	(h) A limit test is introduced.
3. Definitions used are given in Annex 1.	3. Definitions used are given in Annex 1.
PRINCIPLE OF THE TEST	**PRINCIPLE OF THE TEST**
4. The primary objective of the test is to assess the effect of chemicals on the reproductive output of *Daphnia magna*. To this end, young female *Daphnia* (the parent animals), aged less than 24 hours at the start of the test, are exposed to the test substance added to water at a range of concentrations. The test duration is 21 days. At the end of the test, the total number of living offspring produced ~~per parent animal alive~~ at the end of the test is assessed. ~~This means that juveniles produced by adults that die during the test are excluded from the calculations.~~ Reproductive output of the parent animals can be expressed in otherways (e.g. number of living offspring produced per animal per day from the first day offspring were observed) but these should be reported in addition to the total number of juveniles produced ~~per parent alive~~ at the end of the test. ~~The reproductive output of the animals exposed to the test substance is compared to that of the control(s) in order to determine the lowest observed effect concentration (LOEC) and hence the no observed effect concentration (NOEC).~~ In addition, and as far as possible, the data are analysed using a regression model in order to estimate the concentration that would cause ~~a~~ x % reduction in reproductive output, i.e. ECx (e.g. EC50, EC20 or EC10).	4. The primary objective of the test is to assess the effect of chemicals on the reproductive output of *Daphnia magna.* To this end, young female *Daphnia* (the parent animals), aged less than 24 hours at the start of the test, are exposed to the test substance added to water at a range of concentrations. The test duration is 21 days. At the end of the test, the total number of living offspring produced is assessed. Reproductive output of the parent animals can be expressed in other ways (e.g. number of living offspring produced per animal per day from the first day offsprings were observed) but these should be reported in addition to the total number of living offspring produced at the end of the test. Because of the particular design of the semi-static test compared to other OECD invertebrate reproduction Test Guidelines, it is also possible to count the number of living offspring produced by each individual parent animal. This enables that, contrary to other OECD invertebrate reproduction tests, if the parent animal dies accidentally and/or inadvertently during the test period, its offspring production can be excluded from data assessment. Hence, if parental mortality occurs in exposed replicates, it should be considered whether or not the mortality follows a concentration-response pattern, e.g. if there is a significant regression of the response versus concentration of the test substance with a positive slope (a statistical test like the Cochran-Armitage trend test may be used for this). If the mortality does not follow a concentration-response pattern, then those replicates with parental mortality should be excluded from the analysis of the test result. If the mortality follows a concentration-response pattern, the parental mortality should be assigned as an effect of the test substance and the replicates should not be excluded from the analysis. If the parent animal dies during the test i.e. accidentally from mishandling or accident, or inadvertently due to unexplained incident not related to the effect of the test substance or turns out to be male, then the replicate is excluded from the analysis (see more in paragraph 51). The toxic effect of the test substance on reproductive output is expressed as ECx by fitting the data to an appropriate model by non-linear regression to estimate the concentration that would cause x % reduction in reproductive output, respectively, or alternatively as the NOEC/LOEC value (4). The test concentrations should preferably bracket the lowest of the used effect concentrations (e.g. EC10) which means that this value is calculated by interpolation and not extrapolation.

2008	2012
5. The survival of the parent animals and time to production of first brood must also be reported. Other substance-related effects on parameters such as growth (e.g. length), and possibly intrinsic rate of increase, may also be examined.	5. The survival of the parent animals and time to production of first brood should also be reported. Other substance-related effects on parameters such as growth (e.g. length), and possibly intrinsic rate of population increase, can also be examined (see paragraph 44).
INFORMATION ON THE TEST SUBSTANCE	**INFORMATION ON THE TEST SUBSTANCE**
6. Results of an acute toxicity test (see Guideline 202: *Daphnia* sp. Acute Immobilisation Test) performed with *Daphnia magna* ~~should be available. The result~~ may be useful in selecting an appropriate range of test concentrations in the reproduction tests. The water solubility and the vapour pressure of the test substance should be known and a reliable analytical method for the quantification of the substance in the test solutions with reported recovery efficiency and limit of determination should be available.	6. Results of an acute toxicity test (see Guideline 202: *Daphnia* sp. Acute Immobilisation Test) performed with *Daphnia magna* may be useful in selecting an appropriate range of test concentrations in the reproduction tests. The water solubility and the vapour pressure of the test substance should be known and a reliable analytical method for the quantification of the substance in the test solutions with reported recovery efficiency and limit of determination should be available.
7. Information on the test substance which may be useful in establishing the test conditions includes the structural formula, purity of the substance, stability in light, stability under the conditions of the test, pKa, Pow and results of a test for ready biodegradability (see Guideline 301).	7. Information on the test substance which may be useful in establishing the test conditions includes the structural formula, purity of the substance, stability in light, stability under the conditions of the test, pKa, Pow and results of a test for ready biodegradability (see Test Guidelines 301 and 310).
VALIDITY OF THE TEST	**VALIDITY OF THE TEST**
8. For a test to be valid, the following performance criteria should be met in the control(s):	8. For a test to be valid, the following performance criteria should be met in the control(s):
– the mortality of the parent animals (female *Daphnia*) does not exceed 20% at the end of the test; – the mean number of live offspring produced per parent animal surviving at the end of the test is ≥60.	– the mortality of the parent animals (female *Daphnia*) does not exceed 20% at the end of the test; – the mean number of living offspring produced per parent animal surviving at the end of the test is ≥60.
	Note: The same validity criterion (20%) can be used for accidental and inadvertent parental mortality for the controls as well as for each of the test concentrations.
DESCRIPTION OF THE METHOD	**DESCRIPTION OF THE METHOD**
Apparatus	**Apparatus**
9. Test vessels and other apparatus which will come into contact with the test solutions should be made entirely of glass or other chemically inert material. The test vessels will normally be glass beakers.	9. Test vessels and other apparatus, which will come into contact with the test solutions should be made entirely of glass or other chemically inert material. The test vessels will normally be glass beakers.
10. In addition some or all of the following equipment will be required:	10. In addition some or all of the following equipment will be required:
– oxygen meter (with microelectrode or other suitable equipment for measuring dissolved oxygen in low volume samples); – adequate apparatus for temperature control; – pH-meter; – equipment for the determination of the hardness of water;	– oxygen meter (with microelectrode or other suitable equipment for measuring dissolved oxygen in low volume samples); – adequate apparatus for temperature control; – pH-meter; – equipment for the determination of the hardness of water;

2008	2012
– equipment for the determination of the total organic carbon concentration (TOC) of water or equipment for the determination of the chemical oxygen demand (COD); – adequate apparatus for the control of the lighting regime and measurement of light intensity.	– equipment for the determination of the total organic carbon concentration (TOC) of water or equipment for the determination of the chemical oxygen demand (COD); – adequate apparatus for the control of the lighting regime and measurement of light intensity.
Test Organism	**Test Organism**
11. The species to be used in the test is *Daphnia magna* Straus [1].	11. The species to be used in the test is *Daphnia magna* Straus [1].
[1]Other *Daphnia* species may be used provided they meet the validity criteria as appropriate (the validity criterion relating to the reproductive output in the controls should be relevant for the *Daphnia* species). If other ~~species of~~ *Daphnia* are used they must be clearly identified and their use justified.	[1]Other daphnids may be used provided they meet the validity criteria as appropriate (the validity criterion relating to the reproductive output in the controls should be relevant for *all* species). If other *daphnid* are used they should be clearly identified and their use justified.
12. Preferably, the clone should have been identified by genotyping. Research (1) has shown that the reproductive performance of Clone A (which originated from IRCHA in France) (3) consistently meets the validity criterion of a mean of ≥ 60 offspring per parent animal surviving when cultured under the conditions described in this Guideline. However, other clones are acceptable provided that the *Daphnia* culture is shown to meet the validity criteria for a test.	12. Preferably, the clone should have been identified by genotyping. Research (1) has shown that the reproductive performance of Clone A (which originated from IRCHA in France) (5) consistently meets the validity criterion of a mean of ≥ 60 living offspring per parent animal surviving when cultured under the conditions described in this Guideline. However, other clones are acceptable provided that the *Daphnia* culture is shown to meet the validity criteria for the test.
13. At the start of the test, the animals should be less than 24 hours old and must not be first brood progeny. They should be derived from a healthy stock (i.e. showing no signs of stress such as high mortality, presence of males and ephippia, delay in the production of the first brood, discoloured animals, etc). The stock animals must be maintained in culture conditions (light, temperature, medium, feeding and animals per unit volume) similar to those to be used in the test. If the *Daphnia* culture medium to be used in the test is different from that used for routine *Daphnia* culture, it is good practice to include a pre-test acclimation period of normally about 3 weeks (i.e. one generation) to avoid stressing the parent animals.	13. At the start of the test, the animals should be less than 24 hours old and should not be first brood progeny. They should be derived from a healthy stock (i.e. showing no signs of stress such as high mortality, presence of males and ephippia, delay in the production of the first brood, discoloured animals, etc). The stock animals should be maintained in culture conditions (light, temperature, medium, feeding and animals per unit volume) similar to those to be used in the test. If the *Daphnia* culture medium to be used in the test is different from that used for routine *Daphnia* culture, it is good practice to include a pre-test acclimation period of normally about 3 weeks (i.e. one generation) to avoid stressing the parent animals.
Test medium	**Test medium**
14. It is recommended that a fully defined medium be used in this test. This can avoid the use of additives (e.g. seaweed, soil extract ~~etc~~), which are difficult to characterise, and therefore improves the opportunities for standardisation between laboratories. Elendt M4 (4) and M7 media (see Annex 2) have been found to be suitable for this purpose. However, other media (e.g. (5) (6)) are acceptable provided the performance of the *Daphnia* culture is shown to meet the validity criteria for the test.	14. It is recommended that a fully defined medium be used in this test. This can avoid the use of additives (e.g. seaweed, soil extract), which are difficult to characterise, and therefore improves the opportunities for standardisation between laboratories. Elendt M4 (6) and M7 media (see Annex 2) have been found to be suitable for this purpose. However, other media (e.g. (7) (8)) are acceptable provided the performance of the *Daphnia* culture is shown to meet the validity criteria for the test.
15. If media are used which include undefined additives, these additives should be specified clearly and information should be provided in the test report on composition, particularly with regard to carbon content as this may contribute to the diet provided.	15. If media are used which include undefined additives, these additives should be specified clearly and information should be provided in the test report on composition, particularly with regard to carbon content as this may contribute to the diet provided.

2008	2012
It is recommended that the total organic carbon (TOC) and/or chemical oxygen demand (COD) of the stock preparation of the organic additive be determined and an estimate of the resulting contribution to the TOC/COD in the test medium made. It is further recommended that TOC levels in the medium (i.e. before addition of the algae) be below 2 mg/l (7).	It is recommended that the total organic carbon (TOC) and/or chemical oxygen demand (COD) of the stock preparation of the organic additive be determined and an estimate of the resulting contribution to the TOC/COD in the test medium made. It is further recommended that TOC levels in the medium (i.e. before addition of the algae) be below 2 mg/l (9).
16. When testing substances containing metals, it is important to recognise that the properties of the test medium (e.g. hardness, chelating capacity) may have a bearing on the toxicity of the test substance. For this reason, a fully defined medium is desirable. However, at present, the only fully defined media which are known to be suitable for long-term culture of *Daphnia magna* are Elendt M4 and M7. Both media contain the chelating agent EDTA. Work has shown (2) that the 'apparent toxicity' of cadmium is generally lower when the reproduction test is performed in M4 and M7 media than in media containing no EDTA. M4 and M7 are not, therefore, recommended for testing substances containing metals, and other media containing known chelating agents should also be avoided. For metal-containing substances it may be advisable to use an alternative medium such as, for example, ASTM reconstituted hard fresh water (7), which contains no EDTA, ~~with added seaweed extract (8)~~. This combination of ASTM reconstituted hard fresh water and seaweed extract is ~~also~~ suitable for long-term culture ~~and testing~~ of *Daphnia magna* (2)~~, although it still exerts a mild chelating action due to the organic component in the added seaweed extract.~~	16. When testing substances containing metals, it is important to recognise that the properties of the test medium (e.g. hardness, chelating capacity) may have a bearing on the toxicity of the test substance. For this reason, a fully defined medium is desirable. However, at present, the only fully defined media which are known to be suitable for long-term culture of Daphnia magna are Elendt M4 and M7. Both media contain the chelating agent EDTA. Work has shown (2) that the 'apparent toxicity' of cadmium is generally lower when the reproduction test is performed in M4 and M7 media than in media containing no EDTA. M4 and M7 are not, therefore, recommended for testing substances containing metals, and other media containing known chelating agents should also be avoided. For metal-containing substances it may be advisable to use an alternative medium such as, for example, ASTM reconstituted hard fresh water (9), which contains no EDTA . This combination of ASTM reconstituted hard fresh water and seaweed extract (10) is suitable for long-term culturing of *Daphnia magna* (2).
17. The dissolved oxygen concentration should be above 3 mg/l at the beginning and during the test. The pH should be within the range 6 – 9, and normally it should not vary by more than 1.5 units in any one test. Hardness above 140 mg/l (as $CaCO_3$) is recommended. Tests at this level and above have demonstrated reproductive performance in compliance with the validity criteria (9) (10).	17. The dissolved oxygen concentration should be above 3 mg/l at the beginning and during the test. The pH should be within the range 6 – 9, and normally it should not vary by more than 1.5 units in any one test. Hardness above 140 mg/l (as $CaCO_3$) is recommended. Tests at this level and above have demonstrated reproductive performance in compliance with the validity criteria (11) (12).
Test solutions	**Test solutions**
18. Test solutions of the chosen concentrations are usually prepared by dilution of a stock solution. Stock solutions should preferably be prepared by dissolving the substance in test medium.	18. Test solutions of the chosen concentrations are usually prepared by dilution of a stock solution. Stock solutions should preferably be prepared, without using any solvents or dispersants if possible, by mixing or agitating the test substance in test medium using mechanical means such as agitating, stirring or ultrasonication, or other appropriate methods. It is preferable to expose test systems to concentrations of the test substance to be used in the study for as long as is required to demonstrate the maintenance of stable exposure concentrations prior to the introduction of test organisms. If the test substance is difficult to dissolve in water, procedures described in the OECD Guidance for handling difficult substances should be followed (13). The

2008	2012
	use of solvents or dispersants should be avoided, but may be necessary in some cases in order to produce a suitably concentrated stock solution for dosing.
~~19. The use of organic solvents or dispersants may be required in some cases in order to produce a suitably concentrated stock solution, but every effort should be made to avoid the use of such materials. Examples of suitable solvents are acetone, ethanol, methanol, dimethylformamide and triethylene glycol. Examples of suitable dispersants are Cremophor RH40, methylcellulose 0.01% and HCO 40. In any case, the test substance in the test solutions should not exceed the limit of solubility in the test medium.~~	
~~Solvents are used to produce a stock solution which can be dosed accurately into water. At the recommended solvent concentration in the final test medium (i.e. ≤ 0.1 ml/l), the solvents listed above will not be toxic and will not increase the water solubility of a substance.~~	
~~Dispersants may assist in accurate dosing and dispersion. At the recommended concentration in the final test medium (≤ 0.1 ml/l), the dispersants listed above will not be toxic and will not increase the water solubility of a substance.~~	
	19. A dilution water control with adequate replicates and, if unavoidable, a solvent control with adequate replicates should be run in addition to the test concentrations. Only solvents or dispersants that have been investigated to have no significant or only minimal effects on the response variable should be used in the test. Examples of suitable solvents (e.g. acetone, ethanol, methanol, dimethylformamide and triethylene glycol) and dispersants (e.g. Cremophor RH40, methylcellulose 0.01% and HCO-40) are given in (13). Where a solvent or dispersant is used, its final concentration should not be greater than 0.1 mL/L (13) and it should be the same concentration in all test vessels, except the dilution water control. However, every effort should be made to keep the solvent concentration to a minimum.
PROCEDURE	**PROCEDURE**
Conditions of Exposure	**Conditions of Exposure**
Duration	**Duration**
20. The test duration is 21 days.	20. The test duration is 21 days.
Loading	**Loading**
21. Parent animals are maintained individually, one per test vessel, with 50 – 100 ml of medium in each vessel.	21. Parent animals are maintained individually, one per test vessel, usually with 50 – 100 mL (for *Daphnia magna*, smaller volumes may be possible especially for smaller daphnids e.g. *Ceriodaphnia dubia*) of medium in each vessel, unless a flow-through test design is necessary for testing.

2008	2012
22. Larger volumes may sometimes be necessary to meet requirements of the analytical procedure used for determination of the test substance concentration, although pooling of replicates for chemical analysis is also allowable. If volumes greater than 100 ml are used, the ration given to the *Daphnia* may need to be increased to ensure adequate food availability and compliance with the validity criteria. ~~For flow-through tests, alternative designs may, for technical reasons, be considered (e.g. four groups of 10 animals in a larger test volume), but any changes to the test design should be reported.~~	22. Larger volumes may sometimes be necessary to meet requirements of the analytical procedure used for determination of the test substance concentration, although pooling of replicates for chemical analysis is also allowable. If volumes greater than 100 mL are used, the ration given to the *Daphnia* may need to be increased to ensure adequate food availability and compliance with the validity criteria.
Test animals	**Test animals**
23. For semi-static tests, at least 10 animals individually held at each test concentration and at least 10 animals individually held in the control series.	23. For semi-static tests, at least 10 animals individually held at each test concentration and at least 10 animals individually held in the control series.
24. For flow-through tests, 40 animals divided into four groups of 10 animals at each test concentration has been shown to be suitable (1). A smaller number of test organisms may be used and a minimum of 20 animals per concentration divided into two or more replicates with an equal number of animals (e.g. four replicates each with five daphnids) is recommended. Note that for tests where animals are held in groups, it will not be possible to express the reproductive output as the total number of living offspring produced per parent animal alive at the end of the test, if parent animals die. In these cases reproductive output should be expressed as 'total number of living offspring produced per parent present at the beginning of the test'.	24. For flow-through tests, 40 animals divided into four groups of 10 animals at each test concentration has been shown to be suitable (1). A smaller number of test organisms may be used and a minimum of 20 animals per concentration divided into two or more replicates with an equal number of animals (e.g. four replicates each with five daphnids) is recommended. Note that for tests where animals are held in groups, it will not be possible to exclude any offspring from the statistical analysis if inadvertent/ accidental parental mortality occurs when the reproduction has begun, and hence in these cases the reproductive output should be expressed as 'total number of living offspring produced per parent present at the beginning of the test'.
25. Treatments should be allocated to the test vessels and all subsequent handling of the test vessels should be done in a random fashion. Failure to do this may result in bias that could be construed as being a concentration effect. In particular, if experimental units are handled in treatment or concentration order, then some time-related effect, such as operator fatigue or other error, could lead to greater effects at the higher concentrations. Furthermore, if the test results are likely to be affected by an initial or environmental condition of the test, such as position in the laboratory, then consideration should be given to blocking the test.	25. Treatments should be allocated to the test vessels and all subsequent handling of the test vessels should be done in a random fashion. Failure to do this may result in bias that could be construed as being a concentration effect. In particular, if experimental units are handled in treatment or concentration order, then some time-related effect, such as operator fatigue or other error, could lead to greater effects at the higher concentrations. Furthermore, if the test results are likely to be affected by an initial or environmental condition of the test, such as position in the laboratory, then consideration should be given to blocking the test.
Feeding	**Feeding**
26. For semi-static tests, feeding should preferably be done daily, but at least three times per week (i.e. corresponding to media changes). Deviations from this (e.g. for flow-through tests) should be reported.	26. For semi-static tests, feeding should preferably be done daily, but at least three times per week (i.e. corresponding to media changes). The possible dilution of the exposure concentrations by food addition should be taken into account and avoided as much as possible with well concentrated algae suspensions. Deviations from this (e.g. for flow-through tests) should be reported.
27. During the test the diet of the parent animals should preferably be living algal cells of one or more of the following: Chlorella sp, *Selenastrum capricornutum* [now	27. During the test, the diet of the parent animals should preferably be living algal cells of one or more of the following: *Chlorella* sp, (formerly *Selenastrum capricornutum*)

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Pseudokirchneriella subcapitata, (11)] and *Scenedesmus subspicatus*. The supplied diet should be based on the amount of organic carbon (C) provided to each parent animal. Research (12) has shown that, for *Daphnia magna*, ration levels of between 0.1 and 0.2 mg C/*Daphnia*/day are sufficient for achieving the required number of offspring to meet the test validity criteria. The ration can be supplied either at a constant rate throughout the period of the test, or, if desired, a lower rate can be used at the beginning and then increased during the test to take account of growth of the parent animals. In this case, the ration should still remain within the recommended range of 0.1 – 0.2 mg C/*Daphnia*/day at all times.	*Pseudokirchneriella subcapitata*, (11b) and *Desmodesmus subspicatus* (formerly *Scenedesmus subspicatus*). The supplied diet should be based on the amount of organic carbon (C) provided to each parent animal. Research (14) has shown that, for *Daphnia magna*, ration levels of between 0.1 and 0.2 mg C/*Daphnia*/day are sufficient for achieving the required number of living offspring to meet the test validity criteria. The ration can be supplied either at a constant rate throughout the period of the test, or, if desired, a lower rate can be used at the beginning and then increased during the test to take account of growth of the parent animals. In this case, the ration should still remain within the recommended range of 0.1 – 0.2 mg C/*Daphnia*/day at all times.
28. If surrogate measures, such as algal cell number or light absorbance, are to be used to feed the required ration level (i.e. for convenience since measurement of carbon content is time consuming), each laboratory must produce its own nomograph relating the surrogate measure to carbon content of the algal culture (see Annex 3 for advice on nomograph production). Nomographs should be checked at least annually and more frequently if algal culture conditions have changed. Light absorbance has been found to be a better surrogate for carbon content than cell number (13).	28. If surrogate measures, such as algal cell number or light absorbance, are to be used to feed the required ration level (i.e. for convenience since measurement of carbon content is time consuming), each laboratory should produce its own nomograph relating the surrogate measure to carbon content of the algal culture (see Annex 3 for advice on nomograph production). Nomographs should be checked at least annually and more frequently if algal culture conditions have changed. Light absorbance has been found to be a better surrogate for carbon content than cell number (15).
29. A concentrated algal suspension should be fed to the *Daphnia* to minimise the volume of algal culture medium transferred to the test vessels. Concentration of the algae can be achieved by centrifugation followed by resuspension in ~~distilled water, deionised water or~~ *Daphnia* culture medium.	29. A concentrated algal suspension should be fed to the *Daphnia* to minimise the volume of algal culture medium transferred to the test vessels. Concentration of the algae can be achieved by centrifugation followed by re-suspension in *Daphnia* culture medium.
Light	**Light**
30. 16 hours light at an intensity not exceeding 15–20 $\mu E \cdot m^{-2} \cdot s^{-1}$.	30. 16 hours light at an intensity not exceeding 15–20 $\mu E \cdot m^{-2} \cdot s^{-1}$ measured at the water surface of the vessel. For light-measuring instruments calibrated in lux, an equivalent range of 1000 _ 1500 lux for cool white light corresponds close to the recommended light intensity 15–20 μE・m−2・s−1.
Temperature	**Temperature**
31. The temperature of the test media should be within the range 18–22° C. However, for any one test, the temperature should not, if possible, vary by more than 2° C within these limits (e.g. 18–20, 19–21 or 20–22° C). It may be appropriate to use an additional test vessel for the purposes of temperature monitoring.	31. The temperature of the test media should be within the range 18–22° C. However, for any one test, the temperature should not, if possible, vary by more than 2° C within these limits (e.g. 18–20, 19–21 or 20–22° C) as daily range. It may be appropriate to use an additional test vessel for the purposes of temperature monitoring.
Aeration	**Aeration**
32. The test vessels must not be aerated during the test.	32. The test vessels should not be aerated during the test.
Test concentrations	**Test design**

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~~33. Prior knowledge of the toxicity of the test substance (e.g. from an acute test and/or from range-finding studies) should help in selecting appropriate test concentrations.~~	
	Range finding test
	33. When necessary, a range-finding test is conducted with, for example five test substance concentrations and two replicates for each treatment and control. Additional information, from tests with similar compounds or from literature, on acute toxicity to *Daphnia* and/or other aquatic organisms may also be useful in deciding on the range of concentrations to be used in the range-finding test.
	34. The duration of the range-finding test is 21 days or of a sufficient duration to reliably predict effect levels. At the end of the test, reproduction of the *Daphnia* is assessed. The number of parents and the occurrence of offspring should be recorded.
	Definitive test
34. Normally there should be at least five test concentrations arranged in a geometric series with a separation factor preferably not exceeding 3.2, and the appropriate number of replicates for each test concentration should be used (see paragraphs 23-24). Justification should be provided if fewer than five concentrations are used. Substances should not be tested above their solubility limit in test medium.	35. Normally there should be at least five test concentrations, bracketing effective concentration (e.g. ECx), and arranged in a geometric series with a separation factor preferably not exceeding 3.2 An appropriate number of replicates for each test concentration should be used (see paragraphs 24-25). Justification should be provided if fewer than five concentrations are used. Substances should not be tested above their solubility limit in test medium. Before conducting the experiment it is advisable to consider the statistical power of the tests design and using appropriate statistical methods (4).
35. In setting the range of concentrations, the following should be borne in mind:	In setting the range of concentrations, the following should be borne in mind:
(i) If the aim is to obtain the LOEC/NOEC, the lowest test concentration must be low enough so that the fecundity at that concentration is not significantly lower than that in the control. If this is not the case, the test will have to be repeated with a reduced lowest concentration.	(ii) When estimating the LOEC and/or NOEC, the lowest test concentration should be low enough so that the reproductive output at that concentration is not significantly lower than that in the control. If this is not the case, the test should be repeated with a reduced lowest concentration.
(ii) If the aim is to obtain the LOEC/NOEC, the highest test concentration must be high enough so that the fecundity at that concentration is significantly lower than that in the control. If this is not the case, the test will have to be repeated with an increased highest concentration.	(iii) When estimating the LOEC and/or NOEC, the highest test concentration should be high enough so that the reproductive output at that concentration is significantly lower than that in the control. If this is not the case, the test should be repeated with an increased highest concentration unless the maximum required test concentration for chronic effects testing (i.e., 10 mg/L) was used as the highest test concentration in the initial test.
(iii) If ECx for effects on reproduction is estimated, it is advisable that sufficient concentrations are used to define the ECx with an appropriate level of confidence. If the EC50 for effects on reproduction is estimated, it is advisable that the highest test concentration is greater than this EC50. Otherwise,	(i) When ECx for effects on reproduction is estimated, it is advisable that sufficient concentrations are used to define the ECx with an appropriate level of confidence. Test concentrations used should preferably bracket the estimated ECx such that ECx is found by interpolation rather than extrapolation. It is an

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although it will still be possible to estimate the EC50, the confidence interval for the EC50 will be very wide and it may not be possible to satisfactorily assess the adequacy of the fitted model. ~~(iv) The range of test concentrations should preferably not include any concentrations that have a statistically significant effect on adult survival since this would change the nature of the test from simply a reproduction test to a combined reproduction and mortality test requiring much more complex statistical analysis.~~	advantage for the following statistical analysis to have more test concentrations (e.g. 10) and fewer replicates of each concentration (e.g. 5 thus holding the total number of vessels constant) and with 10 controls.
~~36. Where a solvent or dispersant is used to aid preparation of test solutions (see paragraph 19), its final concentration in the test vessels should not be greater than 0.1 ml/l and should be the same in all test vessels.~~	
	36. If no effects are observed at the highest concentration in the range-finding test (e.g. at 10 mg/l), or when the test substance is highly likely to be of low/ no toxicity based on lack of toxicity to other organisms and/or low/no uptake, the reproduction test may be performed as a limit test, using a test concentration of e.g.10 mg/l and the control. Ten replicates should be used for both the treatment and the control groups. When a limit test might need to be done in a flow-through system less replicates would be adequate. A limit test will provide the opportunity to demonstrate that there is no statistically significant effect at the limit concentration, but if effects are recorded a full test will normally be required.
Controls	**Controls**
37. One test-medium control series and also, if relevant, one control series containing the solvent or dispersant should be run in addition to the test series. When used, the solvent or dispersant concentration should be the same as that used in the vessels containing the test substance. The appropriate number of replicates should be used (see paragraphs 23-24).	37. One test-medium control series and also, if relevant, one control series containing the solvent or dispersant should be run in addition to the test series. When used, the solvent or dispersant concentration should be the same as that used in the vessels containing the test substance. The appropriate number of replicates should be used (see paragraphs 23-24).
38. Generally in a well-run test, the coefficient of variation around the mean number of living offspring produced per parent animal in the control(s) should be ≤ 25%, and this should be reported for test designs using individually held animals.	38. Generally in a well-run test, the coefficient of variation around the mean number of living offspring produced per parent animal in the control(s) should be ≤ 25%, and this should be reported for test designs using individually held animals.
Test medium renewal	**Test medium renewal**
39. The frequency of medium renewal will depend on the stability of the test substance, but should be at least three times per week. If, from preliminary stability tests (see paragraph 7), the test substance concentration is not stable (i.e. outside the range 80 - 120% of nominal or falling below 80% of the measured initial concentration) over the maximum renewal period (i.e. 3 days), consideration should be given to more frequent medium renewal, or to the use of a flow-through test.	39. The frequency of medium renewal will depend on the stability of the test substance, but should be at least three times per week. If, from preliminary stability tests (see paragraph 7), the test substance concentration is not stable (i.e. outside the range 80 - 120% of nominal or falling below 80% of the measured initial concentration) over the maximum renewal period (i.e. 3 days), consideration should be given to more frequent medium renewal, or to the use of a flow-through test.

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40. When the medium is renewed in semi-static tests, a second series of test vessels are prepared and the parent animals transferred to them by, for example, a glass pipette of suitable diameter. The volume of medium transferred with the *Daphnia* should be minimised.	40. When the medium is renewed in semi-static tests, a second series of test vessels are prepared and the parent animals transferred to them by, for example, a glass pipette of suitable diameter. The volume of medium transferred with the *Daphnia* should be minimised.
Observations	**Observations**
41. The results of the observations made during the test should be recorded on data sheets (see examples in Annexes 4 and 5). If other measurements are required (see paragraphs ~~5 and~~ 44), additional observations may be required.	41. The results of the observations made during the test should be recorded on data sheets (see examples in Annexes 4 and 5). If other measurements are required (see paragraph44), additional observations may be required.
Offspring	**Offspring**
42. The offspring produced by each parent animal should preferably be removed and counted daily from the appearance of the first brood to prevent them consuming food intended for the adult. For the purpose of this guideline it is only the number of living offspring that needs to be counted, but the presence of aborted eggs or dead offspring should be recorded.	42. The offspring produced by each parent animal should preferably be removed and counted daily from the appearance of the first brood to prevent them consuming food intended for the parent. For the purpose of this guideline it is only the number of living offspring that needs to be counted, but the presence of aborted eggs or dead offspring should be recorded.
Mortality	**Mortality**
43. Mortality among the parent animals should be recorded preferably daily, at least at the same times as offspring are counted.	43. Mortality among the parent animals should be recorded preferably daily, or at least as frequently as offspring are counted.
Other parameters	**Other parameters**
44. Although this guideline is designed principally to assess effects on reproduction, it is possible that other effects may also be sufficiently quantified to allow statistical analysis. Growth measurements are highly desirable since they provide information on possible sublethal effects which may be more useful than reproduction measures alone; the measurement of the length of the parent animals (i.e. body length excluding the anal spine) at the end of the test is recommended. Other parameters that can be measured or calculated include time to production of first brood (and subsequent broods), number and size of broods per animal, number of aborted broods, presence of male neonates (OECD, 2008) or ephippia and possibly the intrinsic rate of population increase (see Annex 1 for definition and Annex 7 for the identification of the sex of neonates).	44. Although this guideline is designed principally to assess effects on reproductive output, it is possible that other effects may also be sufficiently quantified to allow statistical analysis. Reproductive output per surviving parent animal, i.e. number of living offspring produced during the test per surviving parent, may be recorded. This may be compared with the main response variable (reproductive output per parent animal in the start of the test which did not inadvertently or accidentally die during the test). If parental mortality occurs in exposed replicates it should be considered whether or not the mortality follows a concentration-response pattern, e.g. if there is a significant regression of the response versus concentration of the test substance with a positive slope (a statistical test like the Cochran-Armitage trend test may be used for this). If the mortality does not follow a concentration-response pattern, then those replicates with parental mortality should be excluded from the analysis of the test result. If the mortality follows a concentration-response pattern, the parental mortality should be assigned as an effect of the test substance and the replicates should not be excluded from the analysis of the test result.Growth measurements are highly desirable since they provide information on possible sublethal effects which may be useful in addition to reproduction measures alone; the measurement of the length of the parent animals (i.e. body length excluding the anal spine) at the end of

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	the test is recommended. Other parameters that can be measured or calculated include time to production of first brood (and subsequent broods), number and size of broods per animal, number of aborted broods, presence of male neonates (OECD, 2008) or ephippia and possibly the intrinsic rate of population increase (see Annex 1 for definition and Annex 7 for the identification of the sex of neonates).
Frequency of analytical determinations and measurements	**Frequency of analytical determinations and measurements**
45. Oxygen concentration, temperature, hardness and pH values should be measured at least once a week, in fresh and old media, in the control(s) and in the highest test substance concentration.	45. Oxygen concentration, temperature, hardness and pH values should be measured at least once a week, in fresh and old media, in the control(s) and in the highest test substance concentration.
46. During the test, the concentrations of test substance are determined at regular intervals.	46. During the test, the concentrations of test substance are determined at regular intervals.
47. In semi-static tests where the concentration of the test substance is expected to remain within ± 20 per cent of the nominal (i.e. within the range 80 – 120 per cent– see paragraphs 6, 7 and 39), it is recommended that, as a minimum, the highest and lowest test concentrations be analysed when freshly prepared and at the time of renewal on one occasion during the first week of the test (i.e. analyses should be made on a sample from the same solution – when freshly prepared and at renewal). These determinations should be repeated at least at weekly intervals thereafter.	47. In semi-static tests where the concentration of the test substance is expected to remain within ± 20 per cent of the nominal (i.e. within the range 80 – 120 per cent– see paragraphs 6, 7 and 39), it is recommended that, as a minimum, the highest and lowest test concentrations be analysed when freshly prepared and at the time of renewal on one occasion during the first week of the test (i.e. analyses should be made on a sample from the same solution – when freshly prepared and at renewal). These determinations should be repeated at least at weekly intervals thereafter.
48. For tests where the concentration of the test substance is not expected to remain within ± 20 per cent of the nominal, it is necessary to analyse all test concentrations, when freshly prepared and at renewal. However, for those tests where the measured initial concentration of the test substance is not within ± 20 per cent of nominal but where sufficient evidence can be provided to show that the initial concentrations are repeatable and stable (i.e. within the range 80 – 120 per cent of initial concentrations), chemical determinations could be reduced in weeks 2 and 3 of the test to the highest and lowest test concentrations. In all cases, determination of test substance concentrations prior to renewal need only be performed on one replicate vessel at each test concentration.	48. For tests where the concentration of the test substance is not expected to remain within ± 20 per cent of the nominal, it is necessary to analyse all test concentrations, when freshly prepared and at renewal. However, for those tests where the measured initial concentration of the test substance is not within ± 20 per cent of nominal but where sufficient evidence can be provided to show that the initial concentrations are repeatable and stable (i.e. within the range 80 – 120 per cent of initial concentrations), chemical determinations could be reduced in weeks 2 and 3 of the test to the highest and lowest test concentrations. In all cases, determination of test substance concentrations prior to renewal need only be performed on one replicate vessel at each test concentration.
49. If a flow-through test is used, a similar sampling regime to that described for semi-static tests is appropriate (but measurement of 'old' solutions is not applicable in this case). However, it may be advisable to increase the number of sampling occasions during the first week (e.g. three sets of measurements) to ensure that the test concentrations are remaining stable. In these types of test, the flow-rate of diluent and test substance should be checked daily.	49. If a flow-through test is used, a similar sampling regime to that described for semi-static tests is appropriate (but measurement of 'old' solutions is not applicable in this case). However, it may be advisable to increase the number of sampling occasions during the first week (e.g. three sets of measurements) to ensure that the test concentrations are remaining stable. In these types of test, the flow-rate of diluent and test substance should be checked daily.
50. If there is evidence that the concentration of the substance being tested has been satisfactorily maintained within ± 20 percent of the nominal or measured initial	50. If there is evidence that the concentration of the substance being tested has been satisfactorily maintained within ± 20 per cent of the nominal or measured initial

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concentration throughout the test, then results can be based on nominal or measured initial values. If the deviation from the nominal or measured initial concentration is greater than ± 20 per cent, results should be expressed in terms of the time-weighted mean (see guidance for calculation in Annex 6).	concentration throughout the test, then results can be based on nominal or measured initial values. If the deviation from the nominal or measured initial concentration is greater than ± 20 per cent, results should be expressed in terms of the time-weighted mean (see guidance for calculation in Annex 6).
DATA AND REPORTING	**DATA AND REPORTING**
Treatment of results	**Treatment of results**
51. The purpose of this test is to determine the effect of the test substance on the total number of living offspring produced per parent animal alive at the end of the test. The total number of offspring per parent animal should be calculated for each test vessel (i.e. replicate). ~~If, in any replicate the parent animal dies during the test or turns out to be male, then the replicate is excluded from the analysis. The analysis will then be based on a reduced number of replicates.~~	51. The purpose of this test is to determine the effect of the test substance on the reproductive output. The total number of living offspring per parent animal should be calculated for each test vessel (i.e. replicate). In addition, the reproduction can be calculated based on the production of living offspring by the surviving parent organism. However, the ecologically most relevant response variable is the total number of living offspring produced per parent animal which does not die accidentally2 or inadvertently3 during the test. If the parent animal dies accidentally or inadvertently during the test, or turns out to be male, then the replicate is excluded from the analysis. The analysis will then be based on a reduced number of replicates. If parental mortality occurs in exposed replicates it should be considered whether or not the mortality follows a concentration-response pattern, e.g. if there is a significant regression of the response versus concentration of the test substance with a positive slope (a statistical test like the Cochran-Armitage trend test may be used for this). If the mortality does not follow a concentration-response pattern, then those replicates with parental mortality should be excluded from the analysis of the test result. If the mortality follows a concentration-response pattern, the parental mortality should be assigned as an effect of the test substance and the replicates should not be excluded from the analysis of the test result.
	[2] Accidental mortality: non substance related mortality caused by an accidental incidence (i.e. known cause)
	[3] Inadvertent mortality: non substance related mortality with no known cause
~~52. For the estimation of the LOEC, and hence the NOEC, for effects of the chemical on reproductive output, it is necessary to calculate the mean reproductive output across replicates for each concentration and the pooled residual standard deviation, and this can be done using analysis of variance (ANOVA). The mean for each concentration must then be compared with the control mean using an appropriate multiple comparison method. Dunnett's or Williams' tests may be useful (14)(15)(16)(17). It is necessary to check whether the ANOVA assumption of homogeneity of variance holds. It is recommended that this be done graphically rather than via a formal significance test (18); a suitable alternative is to run a Bartlett's~~	

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~~test. If this assumption does not hold, then consideration should be given to transforming the data to homogenise variances prior to performing the ANOVA, or to carrying out a weighted ANOVA. The size of the effect detectable using ANOVA (i.e. the least significant difference) should be calculated and reported.~~	
~~53. For the estimation of the concentration which would cause a 50% reduction in reproductive output (i.e. the EC50), a suitable curve, such as the logistic curve, should be fitted to the data using a statistical method such as least squares. The curve could be parameterised so that the EC50 and its standard error can be estimated directly. This would greatly ease the calculation of the confidence limits about the EC50. Unless there are good reasons to prefer different confidence levels, two-sided 95% confidence limits should be quoted. The fitting procedure should preferably provide a means for assessing the significance of the lack of fit. This can be done graphically or by dividing the residual sum of squares into 'lack of fit' and 'pure error components' and performing a significance test for lack of fit. Since treatments giving high fecundity are likely to have greater variance in the number of juveniles produced than treatments giving low fecundity, consideration to weighting the observed values to reflect the different variances in the different treatment groups should be given. Useful background information can be found in (18).~~	
~~54. In the analysis of the data from the final ring test (2), a logistic curve was fitted using the following model, although other suitable models can be used:~~ ~~Y=c/[1+(x/x0)^b]~~ ~~where:~~ ~~Y is the total number of juveniles per parent animal alive at the end of the test (calculated for each vessel) and x is the concentration.~~ ~~c = the expected number of juveniles when x=0~~ ~~x0 = the EC50 in the population~~ ~~b = the slope parameter~~	
~~55. This model is likely to be adequate in a large number of situations, but there will be tests for which it is not appropriate. A check should be made on the validity of the model as suggested in paragraph 54. In some cases, a hormesis model in which low concentrations give enhanced effects may be appropriate (19).~~	
~~56. Other Effect Concentrations, such as the EC10 or EC20 can also be estimated, although it may be preferable to use a different parameterisation of the model from that used to estimate the EC50.~~	

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	52. In summary, when LOEC and NOEC or ECx are being used to express the effects, it is recommended to calculate the effect on reproduction by the use of both response variables mentioned above i.e. ● as the total number of living offspring produced per parent animal which does not die accidentally or inadvertently during the test and; ● as the number of living offspring produced per surviving parental animal; and then to use as the final result the lowest NOEC and LOEC or ECx value calculated by using either of these two response variables.
	53. Before employing the statistical analysis, e.g. ANOVA procedures, comparison of treatments to the control by Student t-test, Dunnett's test, Williams' test, or stepdown Jonckheere-Terpstra test, it is recommended to consider transformation of data if needed for meeting the requirements of the particular statistical test. As non-parametric alternatives one can consider Dunn's or Mann-Whitney's tests. 95% confidence intervals are calculated for individual treatment means.
	54. The number of surviving parents in the untreated controls is a validity criterion, and should be documented and reported. Also all other detrimental effects, e.g. abnormal behavior and toxicological significant findings, should be reported in the final report as well.
	ECx
	55. ECx-values, including their associated lower and upper confidence limits, are calculated using appropriate statistical methods (e.g. logistic or Weibull function, trimmed Spearman-Karber method, or simple interpolation). To compute the EC10, EC50 or any other ECx, the complete data set should be subjected to regression analysis.
	NOEC/LOEC
	56. If a statistical analysis is intended to determine the NOEC/LOEC appropriate statistical methods should be used according to OECD Document 54 on the Current Approaches in the Statistical Analysis of Ecotoxicity Data: a Guidance to Application (4). In general, adverse effects of the test substance compared to the control are investigated using one-tailed hypothesis testing at $p \leq 0.05$.
	57. Normal distribution and variance homogeneity can be tested using an appropriate statistical test, e.g. the Shapiro-Wilk test and Levene test, respectively ($p \leq 0.05$). One-way ANOVA and subsequent multi-comparison tests can be performed. Multiple comparisons (e.g. Dunnett's test) or step-down trend tests (e.g. Williams' test, or stepdown Jonckheere-Terpstra test) can be used to calculate whether there are significant differences ($p \leq 0.05$) between the controls and the various test substance

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	concentrations (selection of the recommended test according to OECD Guidance Document 54 (4)). Otherwise, non-parametric methods (e.g. Bonferroni-U-test according to Holm or Jonckheere-Terpstra trend test) could be used to determine the NOEC and the LOEC.
	Limit test
	58. If a limit test (comparison of control and one treatment only) has been performed and the prerequisites of parametric test procedures (normality, homogeneity) are fulfilled, metric responses can be evaluated by the Student test (t-test). An unequal-variance t-test (such as Welch test) or a non-parametric testsuch as the Mann-Whitney-U-test may be used, if these requirements are not fulfilled.
	59. To determine significant differences between the controls (control and solvent or dispersant control), the replicates of each control can be tested as described for the limit test. If these tests do not detect significant differences, all control and solvent control replicates may be pooled. Otherwise all treatments should be compared with the solvent control.
Test report	**Test report**
57. The test report ~~must~~ include the following:	60. The test report includes the following:
Test substance:	**Test substance:**
– physical nature and relevant physicochemical properties; – chemical identification data, including purity.	– physical nature and relevant physicochemical properties; – chemical identification data, including purity.
Test species:	**Test species:**
– the clone (whether it has been genetically typed), supplier or source (if known) and the culture conditions used. If a different species to *Daphnia magna* is used, this should be reported and justified.	– the clone (whether it has been genetically typed), supplier or source (if known) and the culture conditions used. If a different species to *Daphnia magna* is used, this should be reported and justified.
Test conditions:	**Test conditions:**
– test procedure used (e.g. semi-static or flow-through, volume, loading in number of *Daphnia* per litre); – photoperiod and light intensity; – test design (e.g. number of replicates, number of parents per replicate); – details of culture medium used; – if used, additions of organic material including the composition, source, method of preparation, TOC/COD of stock preparations, estimation of resulting TOC/COD in test medium; – detailed information on feeding, including amount (in mg C/*daphnia*/day) and schedule (e.g. type of food(s), including, for algae the specific name (species) and, if known, the strain, the culture conditions);	– test procedure used (e.g. semi-static or flow-through, volume, loading in number of *Daphnia* per litre); – photoperiod and light intensity; – test design (e.g. number of replicates, number of parents per replicate); – details of culture medium used; – if used, additions of organic material including the composition, source, method of preparation, TOC/COD of stock preparations, estimation of resulting TOC/COD in test medium; – detailed information on feeding, including amount (in mg C/*daphnia*/day) and schedule (e.g. type of food(s), including, for algae the specific name (species) and, if known, the strain, the culture conditions);

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– method of preparation of stock solutions and frequency of renewal (the solvent or dispersant and its concentration must be given, when used).	– method of preparation of stock solutions and frequency of renewal (the solvent or dispersant and its concentration should be given, when used).
Results:	**Results:**
– results from any preliminary studies on the stability of the test substance;	– results from any preliminary studies on the stability of the test substance;
– the nominal test concentrations and the results of all analyses to determine the concentration of the test substance in the test vessels (see example data sheets in Annex 5); the recovery efficiency of the method and the limit of determination should also be reported;	– the nominal test concentrations and the results of all analyses to determine the concentration of the test substance in the test vessels (see example data sheets in Annex 5); the recovery efficiency of the method and the limit of determination should also be reported;
– water quality within the test vessels (i.e. pH, temperature and dissolved oxygen concentration, and TOC and/or COD and hardness where applicable) (see example data sheet in Annex 4);	– water quality within the test vessels (i.e. pH, temperature and dissolved oxygen concentration, and TOC and/or COD and hardness where applicable) (see example data sheet in Annex 4);
– the full record of living offspring by each parent animal (see example data sheet in Annex 4);	– the full record of the production of living offspring during the test by each parent animal (see example data sheet in Annex 4);
– the number of deaths among the parent animals and the day on which they occurred (see example data sheet in Annex 4);	– the number of deaths among the parent animals and the day on which they occurred (see example data sheet in Annex 4);
– the coefficient of variation for control fecundity (based on total number of living offspring per parent animal alive at the end of the test);	– the coefficient of variation for control reproductive output (based on total number of living offspring per parent animal alive at the end of the test);
– plot of total number of living offspring per parent animal (for each replicate) alive at the end of the test vs concentration of the test substance;	– plot of total number of living offspring produced per parent animal in each replicate excluding any parent animal which may have accidentally or inadvertently died during the test vs. concentration of the test substance;
	– as appropriate plot of total number of living offspring produced per surviving parent animal in each replicate vs. concentration of the test substance
– the Lowest Observed Effect Concentration (LOEC) for reproduction, including a description of the statistical procedures used and an indication of what size of effect could be detected and the No Observed Effect Concentration (NOEC) for reproduction; where appropriate, the LOEC/NOEC for mortality of the parent animals should also be reported;	– where appropriate the Lowest Observed Effect Concentration (LOEC) for reproduction, including a description of the statistical procedures used and an indication of what size of effect could be expected to be detected (a power analysis can be performed before the start of the experiment to provide this) and the No Observed Effect Concentration (NOEC) for reproduction; information on which response variable that has been used for calculating the LOEC and NOEC value (either as total living offspring per maternal organism which did not die accidentally or inadvertently during the test or as total number of living offspring per surviving maternal organism), where appropriate, the LOEC or NOEC for mortality of the parent animals should also be reported;
– where appropriate, the ECx for reproduction and confidence intervals and a graph of the fitted model used for its calculation, the slope of the dose–response curve and its standard error;	– where appropriate, the ECx for reproduction and confidence intervals (e.g. 90% or 95%) and a graph of the fitted model used for its calculation, the slope of the concentration–response curve and its standard error;

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– other observed biological effects or measurements: report any other biological effects which were observed or measured (e.g. growth of parent animals) including any appropriate justification; – an explanation for any deviation from the Test Guideline.	– other observed biological effects or measurements: report any other biological effects which were observed or measured (e.g. growth of parent animals) including any appropriate justification; – an explanation for any deviation from the Test Guideline.

ANNEX1 DEFINIIONS

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For the purposes of this Guideline the following definitions are used:	For the purposes of this Guideline the following definitions are used:
	Fecundity: number of living offspring produced per mother animal within the test period
	Reproductive output: number of living offspring produced by parental animals within the test period
Parent Animals are those female *Daphnia* present at the start of the test and of which the reproductive output is the object of study.	**Parent Animals** are those female *Daphnia* present at the start of the test and of which the reproductive output is the object of study.
Offspring are the young *Daphnia* produced by the parent animals in the course of the test.	**Offspring** are the young *Daphnia* produced by the parent animals in the course of the test.
	Accidental mortality: non substance related mortality caused by an accidental incidence (i.e. known cause)
	Inadvertent mortality: non substance related mortality with no known cause
Lowest Observed Effect Concentration (LOEC) is the lowest tested concentration at which the substance is observed to have a statistically significant effect on reproduction and parent mortality (at $p < 0.05$) when compared with the control, within a stated exposure period. However, all test concentrations above the LOEC must have a harmful effect equal to or greater than those observed at the LOEC. When these two conditions cannot be satisfied, a full explanation must be given for how the LOEC (and hence the NOEC) has been selected.	**Lowest Observed Effect Concentration (LOEC)** is the lowest tested concentration at which the substance is observed to have a statistically significant effect on reproduction and parent mortality (at $p < 0.05$) when compared with the control, within a stated exposure period. However, all test concentrations above the LOEC should have a harmful effect equal to or greater than those observed at the LOEC. When these two conditions cannot be satisfied, a full explanation should be given for how the LOEC (and hence the NOEC) has been selected.
No Observed Effect Concentration (NOEC) is the test concentration immediately below the LOEC, which when compared with the control, has no statistically significant effect ($p < 0.05$), within a stated exposure period.	**No Observed Effect Concentration (NOEC)** is the test concentration immediately below the LOEC, which when compared with the control, has no statistically significant effect ($p < 0.05$), within a stated exposure period.
ECx is the concentration of the test substance dissolved in water that results in a x per cent reduction in reproduction of *Daphnia* ~~*magna*~~ within a stated exposure period.	**ECx** is the concentration of the test substance dissolved in water that results in a x per cent reduction in reproduction of *Daphnia* within a stated exposure period.
Intrinsic rate of increase is a measure of population growth which integrates reproductive output and age-specific mortality (1) (2) (3). In steady state populations it will be zero. For growing populations it will be positive and for shrinking populations it will be negative. Clearly the latter is not sustainable and ultimately will lead to	**Intrinsic rate of population increase** is a measure of population growth which integrates reproductive output and age-specific mortality (1) (2) (3). In steady state populations it will be zero. For growing populations it will be positive and for shrinking populations it will be negative. Clearly the latter is not sustainable and ultimately will lead to

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extinction.	extinction.
Limit of detection is the lowest concentration that can be detected but not quantified.	**Limit of detection** is the lowest concentration that can be detected but not quantified.
Limit of determination is the lowest concentration that can be measured quantitatively.	**Limit of determination** is the lowest concentration that can be measured quantitatively.
~~**Mortality.** An animal is recorded as dead when it is immobile, i.e. when it is not able to swim, or if there is no observed movement of appendages or postabdomen, within 15 seconds after gentle agitation of the test container. (If another definition is used, this must be reported together with its reference).~~	

※ANNEX2 以降は省略