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# History and sensitivity comparison of two standard whole-sediment toxicity tests with crustaceans: the amphipod *Hyalella azteca* and the ostracod *Heterocypris incongruens* microbiotest

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## ABSTRACT

**Key-words:** whole-sediment toxicity tests, sensitivity comparison, *Hyalella azteca*, *Heterocypris incongruens*, ostracod microbiotest

The review first details the development of the test procedures with *Hyalella azteca* which historically emerged as one of the recommended test species for whole-sediment assays and its gradual standardization and endorsement by national and international organizations. The sensitivity and precision of the *H. azteca* test for application on chemicals and on real world sediments is discussed. The review subsequently addresses the development of the whole sediment microbiotest with the ostracod crustacean *Heterocypris incongruens* with larvae of this test species hatched from dormant eggs (cysts), rendering this assay stock culture/maintenance free. The application of the 6-day ostracod microbiotest on sediments in Canada and in Belgium is discussed, as well as its endorsement by the ISO subsequent to an extensive international inter-laboratory ring test. The sensitivity of the amphipod and ostracod tests is compared by data from studies in which both assays were applied in parallel. A comparison of more than 1000 ostracod/amphipod data pairs of a 12-year river sediment monitoring study in Flanders/Belgium confirmed that both whole-sediment assays have a similar sensitivity and that the 6-day ostracod microbiotest is a valuable and cost-effective alternative to the 10–14 day amphipod test for evaluation of the toxic hazard of polluted sediments.

## RÉSUMÉ

Historique et comparaison de la sensibilité de deux tests standard de toxicité sur crustacés de sédiments entiers : *Hyalella azteca* et *Heterocypris incongruens*

**Mots-clés :** tests de toxicité « de contact

Historique et comparaison de deux tests de toxicité « de contact direct » sur des sédiments avec le crustacé amphipode *Hyalella azteca* et le microbiotest ostracode avec *Heterocypris incongruens*. Cet article synthèse rappelle tout d'abord le développement des méthodes test faisant appel à l'amphipode *Hyalella azteca* – une espèce qui historiquement a été une des premières à être recommandée

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direct »  
sur sédiments,  
comparaison  
de la sensibilité,  
*Hyalella azteca*,  
*Heterocypris*  
*incongruens*,  
microbiotest  
ostracode

comme espèce test pour l'évaluation de la toxicité des sédiments et qui a ensuite graduellement fait l'objet de normalisation et d'acceptation par des instances nationales et internationales. L'article traite ensuite de la sensibilité et la précision de *H. azteca* quant à l'utilité de son application pour apprécier la dangerosité de contaminants chimiques et de sédiments naturels d'intérêt. La publication détaille ensuite le développement plus récent d'un microbiotest (essai biologique entrepris à petite échelle) dit « de contact direct », dans lequel des larves du micro-crustacé ostracode *Heterocypris incongruens* sont exposées à un sédiment. Comme ce microbiotest utilise des larves écloses de « stades de dormance » (cystes cryptobiotiques), il a l'avantage remarquable d'être totalement émancipé d'un besoin de culture en continu de stocks des organismes test. Sont décrites des applications pour ce microbiotest-ostracode de 6 jours d'exposition, conduites au Canada et en Belgique, lesquelles ont contribué à sa reconnaissance par l'ISO comme test standard, après la tenue réussie d'un vaste exercice international d'inter-calibration. Un regard intéressé est porté sur la comparaison de la sensibilité des tests amphipode et ostracode à partir de données d'études menées sur des échantillons semblables. Des sensibilités concordantes pour ces deux tests ont été trouvées au regard de plus de 1000 paires de données comparatives générées dans le cadre d'une étude de surveillance de sédiments de rivières réalisée en Flandres, Belgique, et qui s'est échelonnée sur une période de 12 ans. Celle-ci confirme l'utilité du test ostracode de 6 jours, lequel offre une alternative pratique au test amphipode de 10–14 jours pour l'évaluation de l'écotoxicité de sédiments contaminés.

## HISTORY OF THE *HYALELLA AZTECA* WHOLE-SEDIMENT TEST

When it gradually became clear during the past century that contaminated sediments constituted a serious environmental hazard for aquatic ecosystems, early sediment toxicity tests were performed with “pelagic” species on pore waters or elutriates. After some time, it was gradually acknowledged that “direct contact tests” with benthic invertebrates would be needed for an ecologically meaningful assessment of hazard from pollutants present and/or accumulating in sediments. Investigations were therefore initiated in several countries to select and culture benthic invertebrates which could be used for whole-sediment toxicity tests, and to develop reliable test methodologies. One of the first articles in this regard was published by Nebeker *et al.* from the USEPA, describing “Biological methods for determining toxicity of contaminated freshwater sediments to invertebrates”, and in which the amphipod crustacean *Hyalella azteca* (Saussure, 1858) and the midge larva *Chironomus tentans* (Fabricius, 1805) were the recommended test species (Nebeker *et al.*, 1984). Reasons for this selection were justified on the basis that both of these benthic invertebrates “are easy to rear and test, that they remain in intimate contact with the sediment, that they exhibit high control survival and that they are very sensitive to toxic organic chemicals”.

*H. azteca* is a benthic amphipod crustacean which undergoes a minimum of nine instars during its development, to reach an adult size of 6–8 mm. This species is found across Central America, the Caribbean and North America and is reported to be the most abundant amphipod crustacean in North American lakes (Mason, 2002). However, this species is not included in the European limnofauna and this can pose a problem in some countries where the use of non indigenous species is regulated or prohibited.

The solid-phase sediment tests described in the publication of Nebeker *et al.* (1984) are assays in which young amphipods and midge larvae are brought in contact with the sediment for 10 days after which survival is evaluated as the criterion for toxicity. A 28-day partial life cycle test is also mentioned for *H. azteca* with analysis of number of adults and number of young surviving. Two years later, a report developed by the Sediment Subcommittee and its Assessment Work Group to the Water Quality Board of the International Joint Commission (an independent binational organization established by the USA and Canada) recommended

a battery of laboratory bioassays for sediment bio-assessment which also included *H. azteca* (International Joint Commission, 1988). The timely remark in this report that “currently no standardized methodology is available to conduct such assays” must have triggered research in the Great Lakes Laboratory for Fisheries and Aquatic Sciences of the Department of Fisheries and Oceans in Burlington, Ontario in Canada, which led to a publication entitled “A new standardized sediment bioassay protocol using the amphipod *H. azteca*”. The test procedure is a chronic test in which young amphipods are exposed to sediments for 4 to 8 weeks with measurement of survival and growth (Borgmann and Munawar, 1989). In the same year, this laboratory published a paper on the chronic toxicity of chemicals to three test species, also including *H. azteca*, for even longer exposure times up to 12–14 weeks (Borgmann et al., 1989).

In 1990, the ASTM published a “Standard test method for measuring the toxicity of sediment-associated contaminants with freshwater invertebrates”, which also describes the test procedure for a 10-day acute assay with *H. azteca* and *C. tentans* (ASTM, 1990). An updated version of the standard test method which also includes approaches for evaluating sublethal effects in longer-term exposures was published in 2010 (ASTM, 2010). A detailed review was written by Burton (1991) on the toxicity of freshwater sediments, which includes an extensive list of publications on toxicity tests performed with *H. azteca*. This review mentions that sediment testing with this amphipod test species is performed in static renewal systems for exposure periods of 7, 10, 14, 28 and 29 days in acute and chronic tests. In 1994 the USEPA published its own standardized method for conducting toxicity tests with *H. azteca* in a 10-day test with growth and survival as primary endpoints (USEPA, 1994). A second edition was published 6 years later, which described methods for evaluating survival after 28, 35 and 42 days, growth after 28 and 42 days, and reproduction during a 28- to 42-day period (USEPA, 2000). Burton et al. (1996) reported and discussed in detail two ringtests organized by the US EPA in 1993 on the *H. azteca* assay, namely a 96-h test on a “water-only” reference chemical and a 10-day “whole-sediment” test, both of which involved 10 participants. In 1996/1997 the USEPA coordinated a second series of round-robin tests and reported the results of 10-day assays with survival as effect criterion, and of “long term” (28-day) tests based on survival and growth (USEPA, 2000). Eighteen laboratories participated in the 10-day ringtest and 12 laboratories in the long term ringtest. The long term tests could even be prolonged to 42 days, with measurement of reproduction.

With the objective of validating their own 14-day *H. azteca* standard test procedure, Environment Canada in 1996 carried out two round robin exercises, the first with copper-spiked formulated sediment and the second with field-collected contaminated sediment. The results of these two ringtests in which five Canadian testing laboratories participated, were published in a report by Milani et al. (1996). The data and findings of these exercises led to a publication by Environment Canada (1997) of its 14-day standard survival and growth sediment test with *H. azteca*, of which a revised and second edition appeared in 2013 (Environment Canada, 2013). The latter publication includes detailed information and references on “the historical use” of *H. azteca* in sediment toxicity tests.

In 2003, the AFNOR in France published its own method for toxicity testing with *H. azteca* on “natural sediments” with 10-day and 28-day test procedures (AFNOR, 2003).

In December 2013, ISO published ISO standard 16303 dealing with the determination of the toxicity of freshwater sediments using *Hyalella azteca* (ISO, 2013). This ISO standard closely follows the methodology of Environment Canada for a whole sediment assay based on survival and growth inhibition after 14 days and/or 28 days of exposure.

## TEST PROCEDURES, PRECISION AND SENSITIVITY OF THE *HYALELLA AZTECA* WHOLE-SEDIMENT TOXICITY TEST

During the course of research and subsequent standardization of the *H. azteca* sediment toxicity test, a variety of methodologies were developed and published in the scientific literature.

In time specific procedures for the standard tests as reported in the previous section were worked out and are prescribed to date by national and international organizations, with specifications on (a) culturing of the organisms, (b) type and volume of the test containers, (c) volume of sediment and water, (d) type of reference sediment and water, (e) ratio of volume of sediment to volume of overlying water, (f) age and number of organisms placed in each test container, (g) type and concentration of food, (h) number of replicates, (i) renewal of water, (j) incubation conditions and (k) handling of animals at start, during and at end of test. As mentioned above, test duration of the standard assays ranges from 10 to 14 days for the “short term” assays, with measurement of survival and growth of the organisms. In long term assays exposure is prolonged to 28 days, also with measurement of survival and growth, and assays can even be prolonged to 42 days for determination of effects on reproduction.

With regard to the precision of methods and applications for sediment toxicity tests, the review of Ingersoll *et al.* (1995) reports on several studies performed with *H. azteca* on a variety of field collected or spiked sediment samples, as performed according to the USEPA standard method (1994). The authors concluded that the case studies “demonstrated the robustness of the methods for successfully conducting toxicity tests with *H. azteca*”. [Burton \*et al.\* \(1996\)](#) gave a table on the inter-laboratory precision of the survival values for the 10-day *H. azteca* toxicity tests performed in 1993 by the USEPA (USEPA, 1994) with four sediments. The mean percentage survival was quite different in the four sediments, ranging from 3.3% to 94.5%. Variation coefficients for the assays for which the 80% survival performance criterion was obtained ranged from 5.8% up to 114%.

ISO standard 16303 on the *H. azteca* assay (2013) reports performance data obtained in the 1996 Canadian round-robin exercise (Milani *et al.*, 1996) for the 14-day assay. The between-lab CV's for survival in this ringtest ranged from 2.5% to 11% and from 26% to 35.7% for growth.

The second volume of the USEPA document on methods for measuring toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates (USEPA, 2000) provides detailed information and tables on the inter-laboratory precision of the 10-day whole-sediment round robin testing with *H. azteca* performed in 1996/1997. Four types of sediment were involved in this exercise: a field controlled sediment, a sediment formulated with alpha-cellulose and two contaminated sediments spiked with a chemical. The between-lab CV's for percentage survival in the 10-day tests with acceptable survival values in the control (*i.e.* at least 80%) ranged from 5% to 171.3%.

The sensitivity of *H. azteca* for inorganic and organic chemicals was assessed in a substantial number of studies. Many of the investigations were, however, performed on a “water-only” basis, and were hence not “whole-sediment contact tests”. In the very first inter-laboratory study performed by the USEPA in 1992 (USEPA, 1994), the LC50 and the inter-laboratory precision of a “water-only” 96h *H. azteca* test with potassium chloride (KCl) was evaluated, based on 10 participating laboratories. The mean 96h LC50 of the eight labs which matched the mortality acceptability criterion in the controls was 306 mg·L<sup>-1</sup> with a variation coefficient of 15.8%. In a publication on the relative sensitivity of three freshwater benthic macro-invertebrates, [Phipps \*et al.\* \(1995\)](#) report 10-day LC50 values for 10 chemicals (metals and pesticides) for *H. azteca*, *C. riparius* and *Lumbriculus variegatus*. The data indicated that “none of the three species was most (or least) sensitive to the toxicants”.

[Borgmann \*et al.\* \(2005\)](#) report the toxicity of 63 metals and metalloids to *H. azteca*, as determined at two levels of water hardness in static (non renewal) one week exposures. The most toxic metals on a molar basis were cadmium, silver, lead, mercury and chromium. The authors also indicated that 4-day to 14-day LC50s for metal toxicity to *H. azteca* measured in other studies compared favorably with those determined in their investigations. They also report on a comparison of metal toxicity to *Hyalella* and *Daphnia*, which revealed an overall similarity in the metal toxicity response of the one week *H. azteca* assays and the three week *Daphnia* tests. [Becker \*et al.\* \(1995\)](#) had made a comparative study on the sensitivity of *H. azteca* and *Chironomus tentans* in 10-day whole-sediment assays collected from eight stations in a lake near New York. The biological endpoints appraised were survival, biomass and body length.



This study revealed that a “significant concordance” among all endpoints was found with respect to the relative toxicity of the sediments from the eight stations.

## HISTORY OF THE *HETEROCYPRIS INCONGRUENS* WHOLE-SEDIMENT MICROBIOTEST

Virtually all toxicity test methods are dependent on the culturing/maintenance of stocks of test organisms and this is for many reasons (e.g. biological, technical and/or not the least costs) an inherent burden in ecotoxicology. In order to try to eliminate this serious handicap, research endeavors were conducted as of the 1980s in the Laboratory for Biological Research in Aquatic Pollution (presently the Laboratory for Environmental Toxicology and Aquatic Ecology) at Ghent University in Belgium to develop toxicity tests which would be independent of the year-round culturing and maintenance of stocks of live test species needed for performing toxicity tests. This research was first devoted to select species known to produce “dormant (cryptobiotic) stages” during the course of their life cycle, to culture them in the laboratory, and to trigger these species to produce dormant stages under controlled laboratory conditions. Once a successful production of the cryptobiotic stages was achieved, research was then oriented towards storage conditions and on triggers required for “turning on” the biological clock in the dormant eggs to steer them to successful hatching. After these “prerequisites” were satisfactorily resolved, research focused on the development of a toxicity test method for the selected test species (Persoone, 1991). Specific efforts in this regard were paid to “simplicity and practicality” and sought to miniaturize the assays into “microbiotests”, owing to their attractive features as described by Blaise (1991), because they enable reducing materials, bench and incubation space, as well as sample volumes.

Over the last decades, more than a dozen “culture/maintenance free acute and short-chronic microbiotests” with micro-algae, ciliates, rotifers and several crustaceans were gradually developed. These assays are now commercialized as TOXKIT microbiotests and used worldwide for research and toxicity monitoring as shown by the more than 500 publications listed on the website [www.microbiotests.be](http://www.microbiotests.be).

At the turn of the century attention was paid on a stock-culture free “whole-sediment” microbiotest. Eventually the choice for a suitable test species fell on the ostracod crustacean *Heterocypris incongruens* (Ramdohr, 1808) of which the biological cycle is known to shift to the production of dormant eggs (cysts) under particular environmental circumstances. *H. incongruens* whose biology and ecology is described in detail by Fryer (1997) is a cosmopolitan benthic bivalved micro-crustacean which lives in small water bodies of various kinds in temperate climates. Since this species is indigenous in Europe, it is actually a preferable test species for European countries than *H. azteca*, whose distribution is limited to North America. This ostracod can swim as well as crawl and reaches an adult size of about 1.8 mm. It reproduces mainly parthenogenetically with simultaneous formation of subitaneous eggs which develop into live offspring and dormant eggs (cysts) that resist desiccation. The cysts hatch as minute nauplii, of a size of 150  $\mu\text{m}$  to 200  $\mu\text{m}$  (see Figure 1), and pass through eight larval and pre-adult stages before reaching adulthood within a few weeks. Attempts in the laboratory to culture this potential test species and to make it produce cysts that could be stored and hatched were quite rapidly successful, and attention was then paid to the development of a simple and practical “whole-sediment” microbiotest.

## TEST PROCEDURES, PRECISION AND SENSITIVITY OF THE *HETEROCYPRIS INCONGRUENS* WHOLE-SEDIMENT MICROBIOTEST

The very first test protocol developed for the ostracod microbiotest was a 6-day assay, performed in 12 cup multiwell plates with 10 organisms per cup and 3 replicates.



**Figure 1**

Freshly hatched larva (150–200  $\mu\text{m}$ ) of the ostracod crustacean *Heterocypris incongruens*.

Organisms are collected 52 h after the start of the incubation of the cysts in standard freshwater at 25 °C under continuous illumination. Neonates are pre-fed with *Spirulina* micro-algae for 4 h prior to their transfer to the cups which are filled with 300  $\mu\text{L}$  sediment and 2 mL overlying (standard) freshwater containing a suspension of live algae ( $3 \times 10^7$  cells).

Standard sand is used as reference sediment for the controls. Surviving organisms are retrieved from the cups after 6 days of incubation at 25 °C in darkness. Ostracod mortality was selected as the “lethal” effect criterion and growth as a second “sublethal” effect criterion. This initial test procedure was published in 2002 (Chial and Persoone, 2002a), followed by a second publication the same year describing methodology and assay precision (Chial and Persoone, 2002b). The latter publication already recommended that 6 replicates (instead of only 3) are preferable to reach an acceptable precision, *i.e.* a variation coefficient of less than 20% in the controls. The test procedure proposes two validity criteria for the assay, namely that the percentage mortality of the ostracods in the reference sediment should not be higher than 20% and that the mean length of the organisms in the reference sediment after 6 days of exposure should be 600  $\mu\text{m}$ .

The very first attempt to evaluate the initial ostracod microbiotest method was a study on 26 sediments collected from various rivers of the Flemish hydrographic basin in Belgium (Chial and Persoone, 2002c). Subsequently the method was applied on 33 sediment samples from Peninsula Harbor located in Lake Superior of the Great Lakes water basin in Ontario, Canada (Chial *et al.*, 2003a). A third research project was performed in the framework of a study on a crude-oil contaminated freshwater shoreline of the St Lawrence River in Quebec, Canada, during which 53 sediment samples were analyzed (Blaise *et al.*, 2004). All three studies revealed mortality scores ranging from very low to very high, in relation to the degree of pollution of the concerned sediments, as also shown by assays with other test species. Based on these findings, an “Ostracodtoxkit” procedure was elaborated by the spin-off company MicroBioTests Inc., containing all materials to perform three mortality and growth whole-sediment assays with *H. incongruens*.

In view of the application of the ostracod microbiotest in the TRIADE evaluation of sediment pollution of the rivers in Flanders, Belgium, the Flemish Environmental Organization VMM undertook a study in 2002 with the objective of improving the original methodology of this microbiotest (Kwan, 2004). These investigations eventually led to the following changes and refinement of the original test procedure: execution of the assay in 6 replicates in 6 cups multiwells, with 1 mL sediment per test cup, 2 mL overlying standard freshwater and 2 mL algal suspension ( $1.5 \times 10^7$  cells·mL<sup>-1</sup>) and 10 freshly hatched neonates per test cup. Similarly to the original test procedure, the exposure lasted 6 days at 25 °C and in complete darkness. This adapted test is now the recommended method described in the standard Operational Procedure of the Ostracodtoxkit (Ostracodtoxkit-Standard Operational Procedure, 2004).

Since the commercial availability of the Ostracodtoxkit, a substantial number of studies were carried out with the standard ostracod toxicity test in different laboratories of several countries, on a variety of sediments. Findings of several of these studies have been published in

the scientific literature ([Latif and Licek, 2004](#); [Dirven-van Breemen et al., 2006](#); [Drobniowska et al., 2007](#); [Mankiewicz-Boczek et al., 2008](#); [Watanabe et al., 2008](#); [Wang et al., 2009](#); [García-Lorenzo et al., 2009](#); [Coccia et al., 2009](#); [Silva et al., 2011](#); [Kudlak et al., 2011](#); [Nalecz-Jawecki et al., 2011](#); [Steliga, 2011](#); [Sheahan and Fisher, 2012](#); [Huerta Buitrago et al., 2013](#); [Watanabe et al., 2013](#); [Ruiz et al., 2013](#); [Khanal et al., 2014](#); [Sevilla et al., 2014](#); [Palma et al., 2014](#)). Titles, abstracts, and posters of other studies presented at international symposia on ecotoxicology can be found on the website [www.microbiotests.be](http://www.microbiotests.be) ([Nalecz-Jawecki et al., 2009](#); [Hémart et al., 2012](#); [Gonçalves et al., 2012](#)). A few of these studies dealt with toxicity analysis of “pure chemicals” spiked in the reference sediment. Of particular interest in this regard is the publication by [Kudlak et al. \(2011\)](#) which reports LC50 values for the 6-day ostracod “mortality test” and EC50’s for the 6-day “growth inhibition” assay for a variety of metals (Cd, Hg, Cu, Cr, Ni, Mn, Zn, Pb, Li, Fe) spiked in reference sediment. The ostracod LC50 values were also compared with literature data on the whole- sediment test with *Chironomus riparius* and with “water-only” tests on *H. azteca*. The authors concluded that the sensitivity of *H. incongruens* to metal ions was similar to that of *H. azteca* and of *C. riparius*. In another investigation ([Coccia et al., 2009](#)) the toxicity of anthelmintic pharmaceuticals (ivermectins and benzimidazoles) was assessed on reference sediment spiked with these compounds. All the parasiticides were found to be very toxic to the ostracods (6-day LC50’s of a few µg/L). In several of the studies mentioned above, findings generated with the ostracod tests were compared with those obtained on pore waters with different types of test organisms, but only in two other investigations ([Hémart et al., 2012](#); [Gonçalves et al., 2012](#)) was another “whole-sediment” test carried out, namely the *Chironomus* assay.

Noteworthy to mention is an extensive monitoring study on the quality of river sediments, performed by the Scientific Institute of Public Service ISSEP in Wallonia, Belgium from 2010 to 2012 ([Hémart and Marneffe, 2013](#)). In the TRIAD approach applied, toxicity tests were performed on pore waters and on whole-sediments. Two whole-sediment tests were undertaken, namely the 7-day *C. riparius* test and the 6-day *H. incongruens* microbioassay. For both tests mortality as well as growth inhibition were used as effect criteria. Sediments were collected from 31 stations of the hydrographic basin in Wallonia and analyzed for their toxicity. They were ranked into five toxicity classes, in an arbitrary subdivision ranging from “not toxic” to “extremely toxic”. For all toxicity classes, it appeared that the ostracod microbioassay was equally sensitive and in most cases even more sensitive than the midge assay. For sediments where no mortality or a very low mortality in both the ostracod and the midge larvae assay occurred, no growth inhibition was found in the *C. riparius* test, whereas in many cases significant growth inhibition was noted in the ostracod assay.

Taking into account the increasing number of users of the stock culture-free ostracod assay, as well as the practicality and cost-effectiveness of this microbioassay, and following requests of many users, it was decided in 2009 to propose the standard ostracod microbioassay to the ISO for consideration as a standard toxicity test for the determination of fresh water sediment toxicity. A prerequisite favoring the acceptance of such a proposal demands that the precision and robustness of the submitted method must be demonstrated by inter-laboratory comparison data. An initiative was therefore taken in 2010 by the Laboratory of Environmental Toxicology and Aquatic Ecology of Ghent University in Belgium to organize an International Inter-laboratory Comparison with the testing procedure submitted to the ISO. The test was to be performed on reference sediment spiked with copper sulphate as the reference chemical. Twenty six laboratories, institutes, agencies and companies from 14 countries eventually participated in this round robin exercise and sent their results to the organizers. The Inter-laboratory Comparison was very successful and the results and discussion were detailed in an extensive report available on [www.microbiotests.be](http://www.microbiotests.be) ([Janssen and Persoone, 2011](#)). With regard to the precision of this international exercise, the mean 6-day LC50 for copper sulphate was 5.79 mg·L<sup>-1</sup> with a mean repeatability (within-laboratory variability) of 11.9% and a mean reproducibility (between-laboratory variability) of 30.9%. On the basis of the results submitted by the participating laboratories, the second validity criterion of the ostracod microbioassay was changed to “an increase by a factor of 1.5 from the initial length of the test



organisms". The major conclusion in the report on the extensive inter-laboratory comparison study states that "it can safely be concluded that the determination of the subchronic toxicity to *Heterocypris incongruens*, following the method outlined by the ISO/CD 14371 fulfils the requirements for a reliable and precise ecotoxicological test". Subsequent to the submission of precision data stemming from the International Interlaboratory Comparison, the standard ostracod microbioassay was accepted and officially published in 2012 by the ISO as standard 14371 (ISO 14371, 2012).

## SENSITIVITY COMPARISON OF THE *HYALELLA AZTECA* AND THE *HETEROCYPRIS INCONGRUENS* WHOLE-SEDIMENT TESTS

During the very first study on the application of the ostracod microbioassay to natural sediments reported above (Chial and Persoone, 2002c), this test was actually applied in parallel to whole-sediment *H. azteca* assays on 26 samples taken randomly from various watercourses in Flanders, Belgium. The amphipod tests were 10-day mortality assays performed according to the standard ASTM test procedure (ASTM, 2010). The results of this comparative study revealed that the intensity of the toxic effect varied from "nearly identical" to "substantially different" between the amphipod and the ostracod tests. Interesting in this regard is that some sediments were only slightly toxic to the ostracod and very toxic to the amphipod, whereas for other samples opposite trends were noted. The second application study of the ostracod microbioassay reported above (Chial et al., 2003a) and dealing with the analysis of freshwater sediments from Peninsula Harbor in Canada also comprised two other whole-sediment assays, namely with *H. azteca* and with *C. riparius*. The standard test used for *H. azteca* was the 14-day procedure, and the *C. riparius* test a 10 days assay, as prescribed by Environment Canada (Environment Canada, 1997). The majority of sediments were only slightly toxic to both crustacean test species, and those showing a high toxicity were also highly toxic to the amphipod and the ostracod. The pairwise correlation of toxicity data between the two crustacean assays revealed a significant correlation ( $r = 0.71$ ). However, the coefficient of determination  $R^2$  is equal to 0.5041 indicating that only 50% of the variability in the data set is accounted for by the correlation. This is not surprising considering that, as stated before, the two species present a similar sensitivity for many samples, albeit not for all. Results also showed that the midge larvae were the least sensitive of the three test species used in this study. In the third application of the ostracod microbioassay, namely the bioremediation project of oil-contaminated sediments on the shoreline of the St Lawrence River in Canada (Blaise et al., 2004), several toxicity tests on pore waters as well as the whole-sediment *H. azteca* assay were applied. The standard test used for the *H. azteca* assays in this study was again the 14-day test procedure prescribed by Environment Canada (Environment Canada, 1997). The calculation on the "toxicity response concordance percentage between pairs of bioassays" (which is the number of toxic or nontoxic responses of a set of two bioassays divided by the total number of sediment samples and multiplied by 100) revealed a 66% agreement for *H. azteca* with the ostracod microbioassay. A more detailed comparison of the two whole-sediment tests applied in this study (the amphipod and the ostracod assays) was made in a separate publication (Chial et al., 2003b) and revealed interesting findings with regard to the "evolution" of the degree of toxicity of treated sediments. The mean percentage mortalities for the two bioassays in samples collected immediately after the addition of oil were all extremely toxic to both crustaceans (75–100% mortality). After six weeks, the toxicity of sediments from some plots had decreased substantially for the amphipod (mortality around 30%) whereas for the ostracod mortality was still between 80% and 90%. After 21 weeks, sediment toxicity in the four oiled plots decreased overall, but in different proportions for the two test species. Similarly to the two former *H. azteca*/*H. incongruens* comparative studies, the degree of correspondence between data pairs ranged again from "very similar" to "quite different", as one can clearly not expect a total agreement from two phylogenetically different species. With regard to the "precision" aspect of the amphipod and the ostracod toxicity

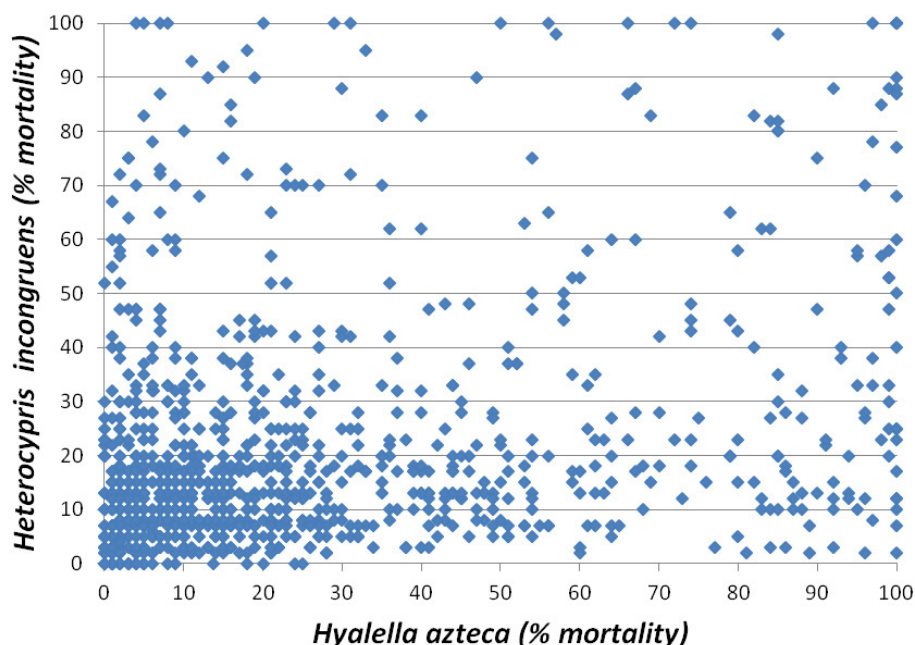
**Table 1**

Major characteristics of the *Hyalella azteca* test and the *Heterocypris incongruens* microbiotest used in the Flemish Environmental Agency VMM sediment monitoring study.

Type of test	Whole-sediment test	Whole-sediment test
<b>Test species</b>	<i>Hyalella azteca</i>	<i>Heterocypris incongruens</i>
<b>Type of organism</b>	Amphipod crustacean	Ostracod crustacean
<b>Test procedure</b>	US EPA 2000	Ostracodtoxkit
<b>Origin of the test organism</b>	Live stock cultures	Dormant eggs (cysts)
<b>Age of test organisms</b>	7–14 day juveniles	Freshly hatched neonates
<b>Size of test organisms</b>	1800–2000 µm	150–200 µm
<b>Test containers</b>	300 mL beakers	6 well microplates (10 mL cups)
<b>Volume of test sediment</b>	50 mL per test container	1 mL per cup
<b>Type of overlying water</b>	Moderately hard EPA medium	Moderately hard EPA medium
<b>Volume of overlying water</b>	200 mL per test container	4 mL per cup
<b>Water renewal</b>	50% daily	No water renewal
<b>Control sediment</b>	Artificial formulated sediment	Commercial river sand
<b>Feeding</b>	3 times per week	At the start of the test
<b>Type of food</b>	Inert (YTC mixture)	Live algae
<b>Lighting</b>	16L/8D photoperiod	Darkness
<b>Test temperature</b>	23 °C	25 °C
<b>Number of replicates</b>	5	6
<b>Number of organisms per replicate</b>	20	10
<b>Test duration</b>	10 days	6 days
<b>Effect criteria</b>	Mortality	Mortality and growth inhibition

tests, the latter study showed a substantially higher uniformity of the effect levels found with the ostracod microbiotest than with the *H. azteca* bioassay. Whereas (barring one exception) all CV's for the percentage mortality in the four replicate samples analysed with the *H. incongruens* microbiotest did not surpass 30%; in turn they exceeded 50% (even up to 80%) for the amphipod assays in many cases. As mentioned by the authors this has, however, to be interpreted with caution since it is well known that low averages usually yield much higher CV's than higher ones.

The largest comparison study that ever took place on whole-sediment toxicity tests is undoubtedly that involving toxicity data generated in the framework of the sediment monitoring of rivers in Flanders, Belgium (de Deckere *et al.*, 2000). The Flemish Environmental Agency VMM indeed decided in 2000 to start a monitoring programme on the quality of river sediments in Flanders, in which sediments from 600 stations at different sites of Flemish rivers were collected and analyzed according to the TRIAD methodology, *i.e.* chemical, biological and ecotoxicological analyses. Ever since 2000, 150 stations are analyzed yearly, indicating that the same stations are sampled every four years to evaluate (possible) changes/improvements in sediment quality at these sites. Ecotoxicological analyses of this monitoring programme are performed on sediment pore waters with an algal test and a crustacean test, as well as on sediments with a whole-sediment test. At the start of this monitoring programme in 2000, the ostracod microbiotest was not yet available, and whole-sediment tests were performed with *H. azteca*, based on the 10-day ASTM standard test procedure with measurement of the percentage mortality (ASTM, 1990). Based on results of the investigations carried out by the VMM on improvement of the original ostracod microbiotest methodology (Kwan, 2004), this agency decided to include a second whole-sediment assay with the improved standard ostracod procedure (*i.e.* the Ostracodtoxkit) in their monitoring programme. Both mortality and growth inhibition were measured as effect criteria on the ostracod microbiotest. As of March 2002 and for the next 12 years, two whole-sediment assays were applied in parallel in the VMM river sediment monitoring programme on all collected sediments, *i.e.* the *H. azteca* test and the *H. incongruens* microbiotest. Table 1 shows



**Figure 2**

Data pairs for percentage mortality of the *H. azteca* and the *H. incongruens* tests.

**Table II**

Toxicity classes used by the Flemish Environmental Agency VMM for classification of sediments based on percentage mortality in the whole-sediment tests.

Toxicity class	Percentage mortality
1	0–20
2	20–40
3	40–60
4	60–80
5	80–100

the major characteristics of both whole-sediment assays as they were used in the VMM study. Results of the TRIAD analyses are used yearly by the VMM for their own reports on the quality of the rivers in Flanders, but data of the ecotoxicological tests were not published in the scientific literature. Permission was, however, given by the VMM to access their data bank on toxicity test results, and to use these data on whole-sediment tests for the present review. The considerations expressed hereafter specifically relate to the sensitivity comparison of the *H. azteca* assay and the ostracod microbioassay, for sediment samples from rivers in Flanders, collected during the 2002–2014 period. Figure 2 illustrates “the toxicity distribution” for the 1066 data pairs and clearly shows that, similarly to the studies mentioned above for the application of the *H. azteca* assay and the ostracod microbioassay, the mortality percentages range from “very low” to “very high”. The dots also reveal that a large percentage of sediments displayed a quite low toxicity for the two test species. In the TRIAD data treatment process, the VMM used “toxicity classes” for ranking toxicity data. Table II shows the five toxicity classes used by the VMM, subdivided according to a 20% increase in the percentage mortality (0–20%, 20–40%, 40–60%, 60–80%, 80–100%). Table III displays the number of *H. azteca* and *H. incongruens* mortality results for each toxicity class, for the 1066 sediment samples for which data on both whole-sediment tests were generated. This table confirms what Figure 3 shows visually, namely that more than 50% of sediment samples (more precisely 59% of the amphipod tests and 68% of the ostracod tests) are in class 1, and hence demonstrated a quite low toxicity (between 0% and 20%). For the highest toxicity class, 9% of the 1066 sediment samples (or 97 sediments) were highly toxic for *Hyalella* whereas only

**Table III**Number of *H. azteca* and *H. incongruens* data in each toxicity class.

Toxicity class	<i>Hyalella azteca</i>	<i>Heterocypris incongruens</i>
1	633	727
2	181	187
3	102	69
4	53	38
5	97	45
<b>TOTAL</b>	1066	1066

**Table IV**

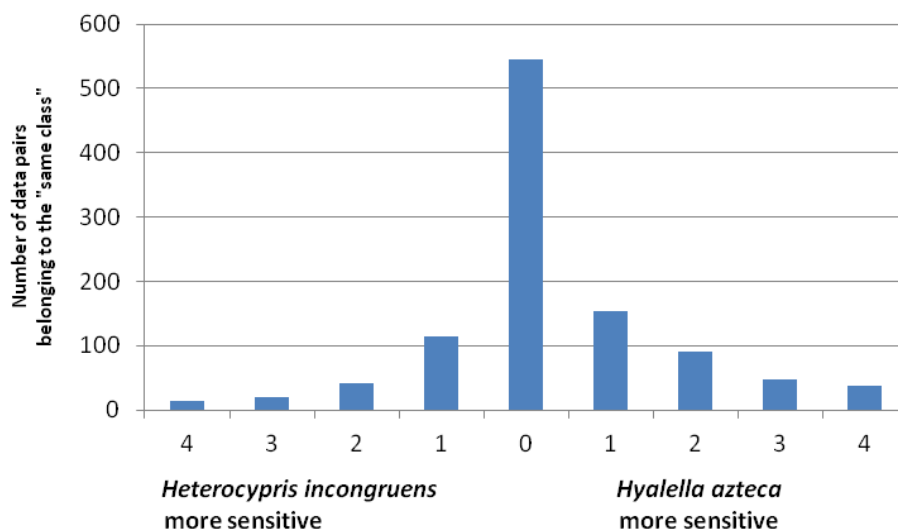
Difference in toxicity classes for toxicity data pairs, in absolute numbers and in percentage.

Class difference	Number of data pairs	Percentage
0	545	51
1	268	25
2	132	12
3	69	6
4	52	5
<b>TOTAL</b>	1066	100

4% (or 45 sediments) proved toxic for ostracods. Despite the substantial difference in toxic effects for the highest toxicity class which suggests that the amphipod is substantially more sensitive than the ostracod, this conclusion must be put in perspective with the findings for “the total number” of data pairs generated by the VMM during the 12-year monitoring study. When one considers “the similarity” or “dissimilarity” of the mortality results of both tests for the five toxicity classes, the outcome is shown in Table IV and visualized in the bar graph of Figure 3. This table and figure indicate the number of 1066 data pairs belonging to “the same class” (i.e. do not differ according to the ranking in the five toxicity classes), and how many differ by one (or more) class(es). The table and the bar graph show that 545 data pairs (or 51%) are “in the same class”. The bar graph further shows that more than 100 data pairs to the left and to the right of the 0 level bar only differ “by one class” i.e. a total of 268 (=114 + 154) as expressed in Table IV. The class differences “to the left” of the central 0 bar indicate that the ostracod was more sensitive than the amphipod, whereas those “to the right” of the central 0 bar indicate that the amphipod was more sensitive than the ostracod. From the graph and the table it thus appears that 832 of the 1066 data pairs (545 + 287), i.e. 78%, only differ “by one toxicity class”, indicating that both whole-sediment tests give roughly a similar signal on the intensity of sediment toxicity. The bar graph and Table IV also inform that out of 1066 sediment samples only 5% differ by four classes, i.e. 52 sediment samples analyzed with the two whole-sediment tests. As can be extrapolated from Figure 3 concerning these 52 sediments, 14 and 38 were highly toxic to the ostracod and amphipod, respectively. As indicated in Table I on the characteristics of the *H. azteca* and the *H. incongruens* assays performed in the VMM sediment monitoring study, percentage growth inhibition was also determined for the ostracods as the second (sublethal) effect criterion. Interestingly, out of 556 class 1 sediment samples for ostracod mortality (i.e. from 0% to 20% mortality), 75% virtually showed no growth inhibition for this organism. In turn, 19 % of these samples showed a 30% growth inhibition, and 6% even a 50% decrease in growth. As corroborated by other studies reported previously for the ostracod microbioassay, the (sublethal) growth inhibition criterion is clearly an informative rapid additional effects-based endpoint offered by this 6-day bioassay.

## CONCLUSIONS

This review on the development and application of whole-sediment testing with the amphipod crustacean *H. azteca* establishes that this toxicity test, presently used extensively especially in the USA, comprises a most valuable tool for detection and quantification of the toxic hazard of contaminated sediments. As shown by the growing number of studies performed since



**Figure 3**

Bar graph showing differences in toxicity classes for toxicity data pairs.

the development of the whole-sediment microbioassay with the ostracod crustacean *H. incongruens*, this assay, besides its attractive feature linked to independence of culturing and maintenance of live stocks, has also shown its value as a practical, robust and cost-effective assay for hazard analysis of polluted sediments. The sensitivity comparison studies of the *H. azteca* and *H. incongruens* tests clearly revealed that both assays have similar potential for evaluating the degree of toxic hazard of sediments. In addition, since the geographical distribution of *H. incongruens* is larger than that of *H. azteca*, the ostracod microbioassay is unquestionably a valuable, practical and cost-effective alternative to whole-sediment toxicity testing conducted with amphipod crustaceans.

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