

## *Tetrahymena* spp. (Protista, Ciliophora) As Test Species in Rapid Multi-level Ecotoxicity Tests

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**Summary.** This review summarizes the application of *Tetrahymena* spp. in ecotoxicology, in order to promote a more integrated, multi-level ecotoxicological assessment approach regarding the effects of chemical stressors on several biological levels (from molecule to ecosystem). Such a multi-level testing approach in one species facilitates the establishment of missing causal relationships between biochemical responses and ecological effects. The review illustrates that *Tetrahymena* spp. represent excellent ecotoxicological test species due to their important role in the microbial foodweb, wide distribution and abundance, sequenced genome in *T. thermophila*, large background knowledge and scientific publications in cellular biology, ecology and ecotoxicology. Several bioassays have already been developed on different biological organisation levels, such as enzyme assays (biochemical level), behavioral tests (individual level), population growth tests (population level) and microcosms (community level). Moreover, specific mode-of-action based assays are available (e.g. genotoxicity), or are in development (e.g. endocrine disruption and neurotoxicity). *Tetrahymena* spp. combine traits of (1) a single cell, thus might replace or complement specific cell-line testing approaches, with traits of (2) a whole organism and population, thus allowing to study complete metabolic pathways and its consequences on population growth and genetic adaptation. Assays involving *Tetrahymena* spp. might easily be adapted for a rapid multi-level *in situ* or *ex situ* toxicity biosensor test system for ecologically relevant risk assessment.

**Key words:** Biotest, biomarker, biosensor, population growth, toxicity test, risk assessment.

### WHY USING *TETRAHYMENA* SPP. FOR ECOTOXICOLOGICAL TESTS?

*Tetrahymena* spp. (Protozoa, Ciliata, Oligohymenophorea) are non-pathogenic, free-living eukaryotes and

ubiquitously distributed in nature (Sauvant *et al.* 1999). Their abundance may indicate healthy aquatic environments, and they represent an important trophic level where bio-accumulation or bio-concentration are important processes (Cooley *et al.* 1972, Carter and Cameron 1973).

Several other reviews previously stated the importance of this genus for biological, ecological and toxicological studies (Sauvant *et al.* 1999, Lukacinova *et al.* 2007). *Tetrahymena* spp. feed on organic matter and bacteria, i.e. they are at the base of microbial

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and the detritivore food webs. They play an important role as grazers of microbes in aquatic and soil environments, controlling bacterio-plankton production. Use of these organisms in toxicity testing could help reduce the number of animals used in ecotoxicity testing (Pauli *et al.* 1993, 2001). Because this genus can easily be cultured at high densities, bioassays with this genus enable the use of a large number of test organisms and replicates. Moreover, because they are unicellular organisms, *Tetrahymena* spp. possess features of both single eukaryotic cells and whole organisms. *Tetrahymena* spp. can easily be cultivated in a variety of media and represent well-known model organisms in microbiology and cell biology, esp. *T. pyriformis* and *T. thermophila*. Compared to other protozoans, they are quite large (50–60  $\mu\text{m}$  vs. 10  $\mu\text{m}$ ), they have a fast generation time (3–7 hours under optimal conditions in the exponential growth phase), and they show a high level of complexity, similar to that of metazoans and human epithelial tissue (Hausmann *et al.* 1996).

*Tetrahymena* spp. possess nuclear dimorphism; two types of cell nuclei exist in a single cell and carry out different functions with distinct cytological and biochemical properties (Collins and Gorovsky 2005). *T. pyriformis* is incapable of sexual reproduction as it does not possess a micronucleus. *T. thermophila* possesses a genetically fully sequenced macronucleus, thus facilitating the study of changes in gene expression patterns under pollution stress (toxicogenomics). Moreover, it possesses many core processes conserved across a wide diversity of eukaryotes (including humans) that are not found in other single-cell model systems (Brunk *et al.* 1990, Eisen *et al.* 2006, Stover *et al.* 2006). There is also a statistically-significant correlation ( $r = 0.928$ ;  $n = 52$  substances) between the sensitivity of *T. pyriformis* and *T. thermophila* to various toxicants (Pauli *et al.* 2000).

Several studies have highlighted their potential as models in *in vitro* toxicological assessment of chemical pollutants using various endpoints (Carter and Cameron 1973, Larsen *et al.* 1997, Pauli and Berger 1997, Sauvant *et al.* 1999, Bogaerts *et al.* 2001, Schultz *et al.* 2005, Lukacinova *et al.* 2007) and their role in self-purification of natural aquatic ecosystems (Cooley *et al.* 1972, Martin-Cereceda *et al.* 1996). Moreover, their role in Waste Water Treatments Plants through biodegradation and grazing activities enhances the biological stability and performance in WWTPs (Curds 1982, Madoni *et al.* 1996, Cox and Deshusses 1997, Nicolau *et al.* 2001, Arregui *et al.* 2007). Additionally, *Tetrahyme-*

*na* spp. inactivated viruses in incubation media by the “killing the killer of the winner” principle, i.e. affecting indirectly virus levels by grazing on infected host bacteria and free phages (Kim and Unno 1996, Benyahya *et al.* 1997, Miki and Yamamura 2005, Pinheiro *et al.* 2007). *Tetrahymena* spp. have shown to affect horizontal gene transfer in a two-species microcosm consisting of *Euglena gracilis* and *T. thermophila* (Matsui *et al.* 2000), such gene transfer might be important for bacterial evolution and adaptation, e.g. resistance to antibiotics (Esteban and Tellez 1992, Young 1993, Lorenz and Wackernagel 1994, Davison 1999). Because they are protozoans, they do not have a cell wall compared to the frequently used yeast, bacteria or algae cells in *in vitro* toxicity testing, which might affect uptake and availability of chemicals (Matsui *et al.* 2003), and they show a higher degree of functional similarity with human genes than other microbial model eukaryotes (Gallego *et al.* 2007).

The sensitivity of *Tetrahymena* spp. to toxic substances compared to other standard test species is complex, e.g. the protozoan was less sensitive to herbicides than *Vibrio fischeri*, but more sensitive to metals (Madoni *et al.* 1996). *Tetrahymena* spp. is also moderately sensitive to perfluorinated compounds (Wang *et al.* 2010). Compared to *Daphnia magna*, *Tetrahymena* spp. was generally less sensitive (Fochtmann *et al.* 2000). Seward *et al.* (2002) assessed 127 chemicals using *Poecilia reticulata* and *T. pyriformis*, and found a good correlation in sensitivity for neutral narcotic chemicals (“baseline toxicity”). However, for polar narcotics and electrophilic substances exerting specific modes of actions, there were more differences in the interspecies comparison of sensitivity (Seward *et al.* 2002). Similar results have been found when comparing *Pimephales promelas* and *T. pyriformis* (Kahn *et al.* 2007). Hence, no single biotest can fully predict the ecological effects of substances, i.e. a biotest set consisting of complementary tests needs to be developed and applied for risk assessment. Fochtmann *et al.* (2000) reported that *T. thermophila* there was no correlation among biotests using Tetrahymina and other species using 30 pesticides, therefore *T. thermophila* provides complementary information regarding toxic action and sensitivity compared to algae and Microtox tests and should be part of a biotest set.

*Tetrahymena* spp. can easily be cultured and a large literature body of well-described methodology is available (De Corninck *et al.* 2004). Test conditions and media must consider the trade-offs between optimal growth

conditions for the species and potential artefacts due to, for example, binding of substances to organic matter (Jaworska and Wayne-Schultz 1994; Nacz-Jawecki and Sawicki 2002; Nilsson 1989, 2003; Zhu *et al.* 2006), pH effects on substance availability, and mortality of the test species (Carter and Cameron 1973, Schultz *et al.* 2003). However, *Tetrahymena* spp. has successfully been used in different types of environmental samples including spring water, i.e. even without media (Dayeh *et al.* 2004, Leitgib *et al.* 2007, Massolo *et al.* 2002, Chen *et al.* 2008).

## SUBCELLULAR BIOCHEMICAL BIOMARKER TESTS

Biochemical biomarkers can provide valuable information about the potential mode of toxic action of chemicals. For example, the relative percent of the fatty acid methyl esters might be used to study the physical accommodation of compounds into cell membranes (Bearden *et al.* 1999a, b; Schultz *et al.* 2002). Whereas narcotic effects (“baseline toxicity”) are a result of an unregulated, passive process resulting from severe cell damage, including loss of membrane integrity, swelling of the cytoplasm and cell rupture, apoptosis is a highly regulated process of cell death by plasma membrane blebbing, aggregation of nuclear chromatin, shrinkage of cytoplasm. Bogaerts *et al.* (1998) determined the cytotoxic effects of several inorganic and organic compounds on *T. pyriformis* using the fluorescein diacetate assay (FDA) (Rotmann and Papermaster 1966; Bogaerts *et al.* 1998, 2001), which has also been shown to be a reliable indicator for cytotoxicity in marine microalgae (Gilbert *et al.* 1992). This assay revealed higher or at least similar sensitivity compared to Microtox and *Daphnia* spp. swimming inhibition assays. Other assays apply two or three dyes simultaneously, e.g. to stain nuclei of dead cells or mark additional cell functions, e.g. by fluorescence markers (Dias *et al.* 2003, Dayeh *et al.* 2004), hence allowing for multi-parameter cytotoxicity tests, which can be performed on multiwell filter plates (Dayeh *et al.* 2005) instead of in traditional microcentrifuge tubes.

Biomarkers for genotoxicity have traditionally been DNA-integrity and DNA-strand breakage (Gallo *et al.* 2008). *Tetrahymena* spp. might be an excellent test species for genotoxic effects as they have a large genome (Orias *et al.* 2000) and possess a complex eukaryotic

cellular structure, which is an advantage for testing substances that need metabolic activation before showing genotoxic effects. Damage of the macronuclei in *Tetrahymena* spp. has been studied with image analysis (Stefanidou *et al.* 2002, 2008) after staining the DNA with Feulgen reagent. Stefanidou *et al.* (1999, 2002) found that increase in DNA synthesis was correlated with suppression of phagocytosis under cocaine exposure. Increase in DNA content could also be recorded after exposure to tartazine, sodium nitrate, sodium benzoate and butylated hydroxytoluene (Stefanidou 2008). The comet assay, or single-cell gel electrophoresis (Cotelle and Ferar 1999, Kassie *et al.* 2000, Hartmann *et al.* 2001, Akcha *et al.* 2003) is a rapid and sensitive assay for genetic damage for different pro- and eukaryotic cells (Cotelle and Farard 1999), incl. *Tetrahymena* spp. (Lah *et al.* 2004). No difference was found when comparing the comet assay performance with different human cell lines and *Tetrahymena* spp. (Lah *et al.* 2005).

Enzyme biomarkers are used as indicators for the metabolic state of cells. Under toxicant exposure cells express defense mechanisms, which can be reflected by the acid phosphatase or dehydrogenase activity (MMT assay) or by antioxidant enzymes. The ATP content (Adenosin-Tri-Phosphate: as indicator of general energetic state) and the Acp activity (acid phosphatase: as indicator of intracellular digestive function) have both been valuable biomarkers of zinc and triton-X-100 toxicity in *T. pyriformis* (Nicolau *et al.* 2004). Müller *et al.* (2006) have suggested that a combination of ATP content and oxygen consumption can be used assess toxic effects of oxidative stress. Mountassif *et al.* (2007) suggested that determining D-β-hydroxybutyrate dehydrogenase (an inner mitochondrial membrane enzyme, well-studied in several species; Bergmeyer *et al.* 1967, Nielsen *et al.* 1973, Latruffe and Gaudemer 1974), in addition to antioxidant enzymes, may achieve a more complete estimation of cellular detoxification processes.

Various substances from different chemical families may exert endocrine disruptive effects in animals (Kase *et al.* 2009). Whereas several bioassays already determine estrogenic effects based on cell-lines from mammals (e.g. CALUX tests), *Tetrahymena* spp. also possess endogenous steroids (Csaba *et al.* 1985), even though the equivalents to mammalian steroid receptors cells have so far not been found. However, estrogen/androgen receptor binding assays only cover a small percentage of endocrine disrupting effects. Steroidgenid assays based on Aromatase recording have recently

been developed and validated for both fish (Hinfray *et al.* 2006) and animal cell lines (e.g. H 295 R) (Kase *et al.* 2009). Aromatase alteration was correlated with reproductive effects in fish from contaminated sites (Lavado *et al.* 2004), and laboratory tests with many different substances proved effects on Aromatase activity (Sanderson *et al.* 2000, 2002; Hinfray *et al.* 2006; Cheshenko *et al.* 2008). Because transformation of testosterone to estradiol was found in *Tetrahymena* spp., it might possess cytochrome P 450 aromatase as well (Csaba *et al.* 1998).

Although *Tetrahymena pyriformis* does not possess a nervous system, they produce biogenic monoamines such as dopamine, adrenaline, noradrenaline and other catecholamines (Brizzi and Blum 1970, Goldman *et al.* 1981, Gundersen and Thompson 1983, Le Roith and Roth 1985, Takeda and Sugiyama 1993, Naokuni and Kanji 1993). Neurotoxicity can be assessed with biomarkers such as levels of dopamine and its derivatives Noradrenaline and Adrenaline in *Tetrahymena* spp. (Ud-Daula *et al.* 2008), thus allowing to use this species in pharmacological studies of drugs to treat/prevent dopaminergic cell disorders related to human neurological and psychiatric diseases. Although the recent research indicates the existence of both the aromatase and dopaminergic pathways in *Tetrahymena* spp., the relevant DNA sequences remain unknown, which complicates the extrapolation of results to other/higher test species.

Ecotoxicogenomics provides some potential for the development of new molecular biomarkers, because it provides insight in the effects of chemicals on gene expression patterns. The genome of the macronucleus in *Tetrahymena thermophila* has been sequenced in 2006 (Eisen *et al.* 2006) and a genome database has been created ([www.ciliate.org](http://www.ciliate.org)) (Eisen *et al.* 2006, Stover *et al.* 2006). *Tetrahymena* spp. have a fairly complete set of ancestral eukaryotic functions, and show a high degree of functional homology with human and mammalian genomes. Therefore, it has been suggested as a potential model organism in ecotoxicological monitoring (Fillingham *et al.* 2002, Turkewitz *et al.* 2002). For example, *T. thermophila* exposed to DDT or TBT showed changes in gene expression patterns using the suppression subtractive hybridization (SSH) library (Miao *et al.* 2006, Feng *et al.* 2007). However, extrapolation from ecotoxicogenomic results to physiological responses, which are usually a consequence of complex gene interactions, remains difficult unless other biomarkers on different biological levels are measured si-

multaneously. *Tetrahymena* can also be an alternative to several transfected reporter-gene cell-lines for e.g. toxic evaluation of endocrine disruptors (e.g. Yeast assays, CALUX systems). For example, La Terza *et al.* (2008) tested soil elutriates from three agricultural farms using *T. thermophila* transfected with a green fluorescent protein gene *i* under control of heat shock promoter elements derived from the *T. thermophila* hsp70 gene. This assay is being further developed into a real-time *in vivo* biosensor system, and other and more specific promoter genes than hsp70 gene expression have to be developed, too.

## INDIVIDUAL LEVEL BASED TESTS

Behavioral endpoints have been developed as suitable indicators for both the individual fitness and ecosystem health, since they link biochemical processes on the suborganismal level to ecological consequences on the ecosystem level (Gerhardt 2007). Behavioral effects often occur at short response times, low toxicant concentrations and because they can be recorded in a non-destructive way just by observation in real-time and on repeated basis, allowing for time-series analysis (Gerhardt 2007). Behavioral studies with *Tetrahymena* spp. have concentrated on chemotaxis, phagocytosis and motility. The chemotactic response of *Tetrahymena* spp. has been proposed as indicator for the evaluation of contamination of water and soil (Koppelhus *et al.* 1994, Doi *et al.* 2005). Behavior has been studied in choice chambers (Leick and Helle 1983, Kohidai *et al.* 1999). For example, Gilron *et al.* (1999) developed a sublethal method to record the chemotactic response of *Tetrahymena* spp. Phagocytosis is an important defence mechanism, both in protozoans and in mammalian cells (Chiesa *et al.* 1993, Renaud *et al.* 1995). Additionally, filter-feeding activity of *Tetrahymena pyriformis* on fluorescent latex beads were studied under exposure to metals (copper, zinc) or Triton-X (Nicolau *et al.* 1999). This method was eventually automatized by advanced image analysis (Dias *et al.* 2003). In order to differentiate between dead and living ciliates, genetically-modified bacteria were used, which carry a green fluorescent protein that loses its fluorescence in food vacuoles of the ciliate (Parry *et al.* 2001). The use of fluorimetric tests systems offers high throughput methods such as fluorescence microtiter plates (Parry *et al.* 2001) or flow cytometry (Fu *et al.* 2003).

Several other studies with *Tetrahymena* spp. have been performed to examine the relationship between ciliary movement, locomotion under exposure to copper, morphine (Power *et al.* 2006) or single-walled carbon nanotubes (Ghafari *et al.* 2008). Naitoh and Eckert (1969) showed that the rate and direction of ciliary beat of *Tetrahymena* spp. is affected by changes in ion permeability, especially  $\text{Ca}^{2+}$  and  $\text{K}^{+}$  ions, which are the most important ion channels in *Tetrahymena* spp., thus changing membrane potential. Goto *et al.* (1982) confirmed this by showing a correlation between swimming speed and membrane fluidity. Toxicants which alter ion fluxes might thus have an inhibitory effect on swimming speed, which serves as easily accessible indicator for membrane stability (Cassidy *et al.* 1989; Wu *et al.* 1994, 1996, 1997; Al-Saadi *et al.* 1981; Darcy *et al.* 2002), however, the precise background mechanism in membrane perturbation has still to be elucidated.

## POPULATION LEVEL TEST METHODS

Under optimal culture conditions, *Tetrahymena* spp. grow first logarithmically, followed by a prestationary phase, ending in a stationary phase. In the log growth phase, the generation time can be as fast as 3–7 hours. Population growth can be recorded by different direct and indirect methods. Cell counts can be performed by an electronic particle counter or microscopically by a hemocytometer. According to the OECD ([www.oecd.org](http://www.oecd.org)) the 50% inhibitory growth concentration ( $\text{IGC}_{50}$ , mmol/l) is a widely applied toxicity endpoint. Indirect counting methods rely on spectrometric methods, such as the TETRATOX assay (Schultz 1997), measuring the turbidity of the medium caused by the population growth of the species, at 440 nm. Additionally to this fast, efficient and validated counting method (Larsen *et al.* 1997, Pauli and Berger 1997), toxicity tests with *Tetrahymena* spp. can be conducted in high throughput (e.g. microtiterplates, Sauvart *et al.* 1995) and in miniaturized microbioreactor systems (Ritzthaler 2006). A large database (TETRATOX, [www.vet.ulk.edu/TETRATOX](http://www.vet.ulk.edu/TETRATOX)) for ca. 2.400 industrial organic chemicals has been developed, and serves as basis for linear or non-linear QSAR development (e.g. Schultz *et al.* 2003, Aptula *et al.* 2006). However, Stewart *et al.* (2001) found that toxic potency values of chemicals acting via the electro(nucleo)philic mode of action could have lower reproducibility using the TETRATOX assay. In

addition to TETRATOX a miniaturized tox-kit (Prototoxkit) has been developed (Microbiotests Inc, Deinze, Belgium), which is frequently used in ecotoxicological assessment of waste water, river water and sediments and considers 5–6 generations within a test duration of 24 hours (Kristiansen *et al.* 1996, Fochtmann *et al.* 2000, Latif and Lieck 2004, Papadimitriou *et al.* 2008). For semi-volatile substances Wang *et al.* (2010) developed a closed test system with enough head space for the aerobic organisms, including a protocol to correct for estimation errors of the substance concentrations in the headspace.

However, turbidity measurements might suffer from errors such as 1) lack of all dead organisms have precipitated, and 2) test substance or metabolism products might also affect turbidity. An alternative is microcalorimetry, i.e. recording the change in heat production, which allows for automatic and non-invasive recording of thermodynamic and kinetic data of aquatic animal species (Wegener and Moratzky 1995, Stangel and Wegener 1996) and *Tetrahymena pyriformis* (Beemann *et al.* 1999). Other studies that have successfully used microcalorimetry with *Tetrahymena* spp. in studying toxic effects on population growth are Wu *et al.* (2006), Chen *et al.* (2007) and Kong *et al.* (2009). *Tetrahymena* spp. can be assessed directly, but also be used as “vehicle”, e.g. in the test system BACTOX (Schlimme *et al.* 1999). In the BACTOX assay, bacteria are co-cultivated with *Tetrahymena* spp., and the alteration of *Tetrahymena* spp. population serves as indicator for the assessment of bacteria (Schlimme *et al.* 1999).

## MICROCOSM TESTS

Laboratory microcosms represent small model ecosystems and consist of several interacting species, usually a foodweb. Kawabata *et al.* (1995) constructed a microcosm simulating the microbial food web with the flagellate algae *Euglena gracilis* as primary producer, the ciliate *T. thermophila* as consumer and the bacterium *Escherichia coli* as decomposer. Matsui *et al.* (2000) found that the three species in this microcosm can co-exist for about one year, whereas in single cultures the species cultures were less stable. This microcosm has been used in several ecotoxicological studies of effects of  $\gamma$ - or UV-radiation (Fuma *et al.* 1998, Takeda *et al.* 1998), nickel (Fuma *et al.* 1998a, b), manganese, gadolinium (Fuma *et al.* 2001), acidification

(Miyamoto *et al.* 1998), copper, aluminium (Fuma *et al.* 2003) and dysprosium (Fuma *et al.* 2005). The effect index (EIM) is calculated as the difference in cell densities between exposed and control microcosms by Euclidean distances (Fuma *et al.* 2003). Doi *et al.* (2007) used a computer simulation model, where all constituents of the microcosm microbial food web are defined as factors of crucial functions for the sustainability of the system, e.g. by feeding on bacteria, *T. thermophila* prevents their extensive growth. Thus, this model allows to estimate functional ecosystem effects, too.

## CONCLUSIONS

Thanks to all the efforts made in the past decades, the application fields of *Tetrahymena* spp. in ecotoxicology have been widely extended. Their unique biological and ecological advantages allows them to be used as sensitive and easy-to-handle “toxic action indicators” at the biochemical, organismal and population levels, as an important part of a microcosm simulating a microbial food web, and to assess the direct/indirect effects of chemicals at the community level. Moreover, due to the advancements in cellular and molecular biology, more and more bioassays based on behavioral alteration or new molecular biomarkers have been introduced. These assays, not only to elucidate toxic mode of actions, but also to shed light on the mechanisms at the molecular and individual levels. Concurrently, some previous studies have further shown their potential being adapted for the future ecotoxicological research, which focus on complex samples and long-term effects, more closely reflecting ecological effects. Hence, *Tetrahymena* spp. have been, and will be, one of key representatives for integrated multi-level (eco) toxicological research. However, the recently developed biotests and biosensors still have to be standardized, validated and implemented for practical risk assessment and multi-level tests should be developed by simultaneous assessment of biomarkers from different biological organization levels. In this sense we agree with Lukacinova *et al.* (2007) that *Tetrahymena* spp. tests might replace mammal tests in toxicology and fish/fish cell line tests in ecotoxicology in the future, thus concordant with the European 3 R strategy in ecto/toxicity testing approaches.

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