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Sensitivity of Different Endpoints in *Euglena gracilis* to Wastewater Toxicity

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Mini Review

ABSTRACT

Chemical analysis of potential pollutants in wastewater is hampered by the large number of toxins, time constrains and high costs. Therefore bioassays are being employed to evaluate toxicity and monitor pollution levels. In addition to protozoa, invertebrates and vertebrates as well as algae and higher plants, flagellates are being used for this purpose. *Euglena gracilis*, a unicellular photosynthetic flagellate, is an excellent candidate because of its ease of cultivation, fast growth and rapid responses to environmental stress parameters. The cells are motile and respond to light and gravity and are capable of morphological changes of their cell form. The precision of phototaxis and gravitaxis is affected by a variety of organic and inorganic toxins. Swimming velocity and percentage of motile cells is a sensitive endpoint for pollution monitoring. Photosynthetic quantum yield and pigments are likewise suitable parameters in testing environmental stress parameters.

INTRODUCTION

The rapidly increasing human population on our planet is a major burden for the available resources. In addition to natural sources, industry, households and agriculture produce numerous pollutants such as SO_2, methane and soot, to name only a few, which are discharged into the atmosphere [1]. Especially in developing countries this is often done in an uncontrolled way; but these substances are globally distributed by wind affecting the global air quality [2]. Many of these substances find their way into freshwater aquatic ecosystems such as natural habitats as lakes, ponds and rivers when washed out of the atmosphere by rain.

In addition, many pollutants enter the aquatic ecosystems directly from industry, mining, municipalities and agriculture [3-5]. The degree of pollution has increased with increasing industrialization and growing populations. Furthermore, the problem is aggravated by global climate change altering wind and precipitation patterns [6].

Water pollutants and their sources

Heavy metals are among the most toxic pollutants affecting the biota [7]. On the one hand these substances are not biodegradable and concentrate in the water and sediment [8]. At low concentrations they may even be essential for life as compounds in biomolecules, but at higher concentrations heavy metals are toxic. They are taken up by aquatic organisms and concentrated in the food web reaching toxic concentrations affecting invertebrates, fish, birds and finally humans who consume contaminated aquatic animals [9,10].

As an example, heavy metal concentrations in Lake Naivasha (Kenia) and its contributing rivers have been analyzed to be 100-180 (Pb), 10 (Cd) and 30-32 (Cu) µg/L [11]. By bioaccumulation in the food web these chemicals reach high concentrations in higher trophic levels: In carp (*Cyprinus carpio*) caught in this lake the measured concentrations were 5–58 (Pb), 1–1.7 (Cd), and <0.03–2.3 (Cu) mg in each kg of fresh muscle tissue. Cd is especially notorious to accumulate in the food web and reach concentrations of 10^2 to 10^5 times higher than the water concentrations depending on the species [12]. Similar concentrations have been found in rivers in Pakistan and India [13,14].

At higher concentrations in the water some heavy metals, such as zinc and iron, induce irritation of the skin and mucous membranes and are responsible for gastric disorders and affect ventilation and heart physiology [15]. Nickel, chromium, lead, cadmium and copper also cause heart problems and are associated with leukemia and cancer [16,17].

Indiscriminate discharge of sewage is another source of pollution which is also a major problem in developing countries. It contains a wide range of organic and inorganic toxins, among which also heavy metals have a large share [18]. Untreated sewage may enter drinking water reservoirs and pipelines due to broken sewers or constructions where both sewage and drinking water tubes are laid down in close vicinity. In Pakistan about 1100 cubic meters per day of waste effluents are discharged by various industries containing untreated toxicants [19]. Since the polluted water is being used for irrigation heavy metals are taken up by crop plants leading to phytotoxic effects and accumulation in the soil and cereal crops [20,21].

Other toxic effluents which reach wastewaters often untreated include persistent organic pollutants (POP) [22]. These pollutants contain chemicals such as organochlorine pesticides (OCPs), polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans (PCDD/Fs), perfluorooctane sulfonate (PFOS) and perfluorocotanoate (PFOA) which are often found in drinking water sources and also coastal waters, e.g., in China [22]. Even though the concentration levels may be below the regulatory limits of a country they may pose health risks for humans. Likewise the levels of DDTs in some Chinese rivers can exceed those considered a potential threat for human health. But as a whole data on the complexity of POP distribution in natural ecosystems as well as drinking water reservoirs are scarce. Even in remote areas as the Tibetan Plateau various POPs have been detected which probably arrived by airborne transport [23]. Other sources for POPs arriving in freshwater ecosystems are from land filling and dumping, threatening drinking water and food resources. These landfills are a potential risk for decades or even centuries to come [24].

Pollution by POPs is associated with obesogenic effects [25]. Especially in elderly populations there is a strong association between obesity and mortality with the serum concentrations of POPs [26]. Another wide range of adverse health effects is related with the placenta and the risk of neural tube defects which have been detected in a Chinese population [27]. Similar observations were made for o,p'-DDT and its metabolites, α-HCH, γ-HCH and α-endosulfan.

Finally, another major source for growing environmental pollution of aquatic habitats is agriculture. The increasing use of pesticides and fertilizers result in growing concentrations of these substances in natural ecosystems and drinking water reservoirs [28,29]. Modern agriculture heavily relies on the use of pesticides such as herbicides, insecticides, molluscicides, nematicides, algicides and fungicides [30,31]. Often these substances are applied in excess and therefore reach both the groundwater and open water reservoirs as runoff. When entering the drinking water supply they can induce health problems in livestock and pose an acute or delayed risk for humans [32]. These problems range from skin and eye irritation to more serious effects on the nervous and reproductive system and may induce cancer [33]. They may even cause epigenetic changes in the gene expression without altering the DNA sequence itself, e.g. by DNA methylation, histone modification and microRNA expression, the severity of which depends on the environmental exposure conditions and the individual susceptibility [34].

Also fertilizers and manure are often applied in excessive quantities resulting in a runoff which washes the excess chemicals into nearby open water reservoirs, rivers and into the ground water [35]. Nitrogen is one of the culprits which leach from farm land in the water-soluble form of NO_3^- when applied in large quantities. The overload induces algal growth in rivers, lakes and coastal waters where it harms drinking water quality as well as fishing and tourism and may even result in dead zones devoid of oxygen. Nitrogen can also reach aquatic ecosystems from polluted atmosphere as N_2O and NO_x gases. Excessive use of nitrogen augments climate change and has an impact on human health [36].

Needs for Bioassessment

Traditionally, water quality was determined by chemical analysis. However, the sheer number of potentially toxic organic and inorganic molecules prevents a stringent analysis, so that chemists are limited to monitoring for whole classes or groups of chemicals. Even then a systematic analysis is beyond the reach in routine monitoring due to time and financial constrains even with modern equipment such as coupled chromatographic and mass-spectrometric techniques [37]. A typical example is the chemical catastrophe in Seveso in 1976 where the chemical company ICMESA (Industrie Chimiche Meda Societá Azionaria) inadvertently released the toxin TCCD (tetrachloridibenzodioxine) into the atmosphere [38]. The main problem was that the toxicity of the substance was grossly underestimated and secondly routine monitoring failed to detect the chemical. Another example is the Minamata accident where residents suffered long-term exposure to methylmercury from gold mining leading to psychiatric symptoms [39].

Therefore environmental scientists started looking for alternatives to chemical analysis to determine pollution and exposure to toxins in aquatic ecosystems. One obvious option is to employ organisms to signal potential toxicity. The inherent drawback of this method is that it does not result in the identification of the toxic chemical, but they indicate a potential pollution problem which may pose a hazard to the environment and human health [40]. Therefore, when a potential problem is signaled by the biomonitoring system, a chemist needs to identify the culprit, but anyway this approach is much less time consuming and costly than exclusive chemical analysis.

An early example of biomonitoring was to observe the swimming behavior and mortality of fish when exposed to potentially toxic water [41]. In addition to being time consuming, this method for biomonitoring is prone to a subjective bias of a human interpreter. Consequently, methods were developed to determine the swimming behavior by computer-controlled image analysis [42]. Time constrains and bioethical questions led to the search for other organisms for biomonitoring of aquatic ecosystems yielding oligochaetes, microcrustaceans and protozoa as potential organisms [43,44]. *Daphnia* has been established as a recognized organism for biomonitoring of pollutants and a broad range of toxicants, using swimming behavior, reproduction and mortality as endpoints [45].

Lower and higher plants were also employed as early warning biomonitors. Heavy metals are being monitored by aquatic macrophytes in the river Nile [46] and lichens and aquatic mosses are used to detect POPs in aquatic ecosystems [47]. Growth and reproduction, mortality and photosynthetic performance as well as pigment, DNA and protein composition are common endpoints for biomonitoring purposes.

The free-floating aquatic macrophyte *Lemna minor* L. (duckweed) has been recognized as a sensitive indicator for a wide range of toxic substances [48]. Root length, leaf growth and pigmentation are used as indicators for potential pollution. The test is defined by ISO, EPA and OECD standards [49]. However the standard test duration for the duckweed bioassay is 7 days, which is considered too long for immediate biomonitoring purposes. A related aquatic plant is *Spirodela* which has also been employed in duckweed microbiotests e.g. for the detection of cyanobacterial microcystin-LR in drinking water of rural water treatment plants. Germination of the turions in a test plate is quite simple and requires little bench space and the test is completed in 3 days. Computer analysis of the growth rate of the leaves provides independence from subjective analysis and high statistical significance [50].

Euglena gracilis Used for Bioassessment

Euglena gracilis is a motile freshwater flagellate of the phylum Euglenophyta and is found in many aquatic habitats, especially shallow eutrophic ponds **(Figure 1)**. It reproduces non-sexually, and the cell division normally takes about 2 to 4 h [51]. It can be cultivated in a variety of media. If light is available, *E. gracilis* grows autotrophically and exhibits plant-like metabolism, but in the absence of light it can live heterotrophically on a variety of carbon sources showing animal-like metabolism [52]. The cell of *Euglena* lacks a cell wall but is surrounded by a pellicle, a flexible outer shell, which gives mechanical support and stability [53]. A large ovoid nucleus (5-7 μm long and 3-5 μm wide) surrounded by a double layered nuclear envelope is present with a nucleolus inside [54]. Numerous mitochondria ranging in shape from spherical to rod-shaped are present. Mitochondria are scattered throughout the cell, but are most abundant in the region between the chloroplasts and pellicle [54]. Numerous chloroplasts are found throughout the cells. In contrast to the chloroplasts of higher plants and green algae, which are surrounded by two membranes, the chloroplasts of *Euglena* are surrounded by three membranes [55]. When grown in light, it develops both chloroplasts and mitochondria, but only mitochondria when grown in the dark [56].

Figure 1. Light microscopic image of *Euglena gracilis* with the flagellae clearly visible (400x).

The motility of *Euglena gracilis* is powered by a single flagellum inserted at the front end (a second one originates in the reservoir, but does not reach the surface). The organism responds to both light and gravity by active phototaxis and gravitaxis [57]. It orients itself by using light and gravity as environmental hints to reach a region in the water column optimal for growth and reproduction. This flagellate is capable of changing its cell shape from an elongated to a rounded form e.g. under external stress such as in the presence of chemicals. In addition to normal swimming movement it can move using a euglenoid gliding motility [58]. Under osmotic stress, like increased salinity, *Euglena* cells form temporary palmella stages and recover as the stress is removed [59].

Parameters of *Euglena* Used in Bioassessment

Because of their trophic level, fast growth and ubiquitous occurrence, microalgae are good indicators of environmental stress and the health of aquatic ecosystems [60]. For toxicity assessment in the aquatic environments, different algal tests are being used with cell number, fresh or dry weight, protein and nucleic acid contents, chlorophyll *a* fluorescence, photosynthetic CO_2 fixation, ATP production, morphology or vital stainability as endpoints [61]. Single algal tests have gained popularity because of their simplicity and sensitivity [62].

E. gracilis can easily be grown and handled; it is an ideal candidate for use in bioassays [63]. Due to its high sensitivity

to different environmental stresses, different organic and inorganic pollutants, heavy metals, UV radiations and salinity etc. *E. gracilis* has widely been used in bioassessment of pollution in aquatic environments [56,63-72]. Numerous physiological, behavioural, biochemical and morphological parameters of *Euglena* can be used as end points in biomonitoring. Cell growth in *Euglena* was monitored in many studies to evaluate the effects of different substances [65,67,68,73]. Motility, cell compactness and gravitactic orientation in *E. gracilis* have been studied for assessing the toxicity of water pollutants like organic substances, toxic metals and wastewaters [63,66,71,74]. Similarly, photosynthetic efficiency and concentration of light-harvesting pigments in *Euglena* were monitored as end points for toxicity assessment of different chemical pollutants [66,69-71,75]. As a conclusion, *E. gracilis* has been recommended as a sensitive organism in ecotoxicological studies.

Comparative Sensitivity of Different Parameters of *E. gracilis* to Wastewater

Euglena has widely been applied in ecotoxicity assessment of water pollutants of different nature both in short- and long-term tests routinely ranging from 24 hours to 7 days. The results of ecotoxicological studies are usually described by calculating EC_{50} values (concentration that induces 50% of the maximal effect) or NOEC values (the highest tested concentration at which no significant effect is observed) for a specific pollutant. In the case of wastewater, the NOEC value can be equivalent to the G value which represents the highest concentration (lowest dilution) without a significant effect on the test organism. A few examples of NOEC/G values and EC_{50} values for wastewater or water pollutants obtained for different parameters of *Euglena* in short- and long-term tests are shown in **Tables 1 and 2**.

Table 1. NOEC and EC_{50} values (G values instead of NOEC in the case of wastewater) for wastewater and water pollutants obtained for different parameters of *Euglena*. The values given are in %, g/L or mg/L as mentioned for each substance. The values were obtained immediately after exposure except for quantum yield of PS II (Fv/Fm) where the exposure time was 24 h. The values given before the slash (/) are NOEC while after the slash are EC_{50}. nd means the value is not determined. Asterisk (*) indicates that the effect was stimulatory instead of inhibitory.

	Motility (% motile cells)	Velocity	Cell shape	Gravitaxis	Orientation (r-value)	Fv/Fm	Reference
Glass industry wastewater (%)	34/nd	34/nd	6/nd	6/nd	3/nd	21/nd	[77]
Ceramics industry wastewater (%)	nd	nd	Nd	6/0	6/0	<6*/nd	[77]
Pharmaceutical industry wastewater (%)	nd	34/nd	21/nd	3/nd	2/nd	6*/nd	[77]
Match industry wastewater (%)	nd	nd	12/nd	3/ nd	3/ nd	6*/ nd	[77]
Textile industry wastewater (%)	nd	21/nd	6/nd	6/ nd	3/ nd	nd	[85]
Soap industry wastewater (%)	21/nd	nd	3/nd	6/ nd	3/ nd	nd	[85]
Ink chemicals industry wastewater (%)	nd	34/nd	12/nd	3/ nd	3/ nd	nd	[85]
Sugar industry wastewater (%)	nd	2*/nd	34/nd	2/ nd	2/ nd	nd	[78]
Municipal wastewater DI Khan, Pakistan (%)	nd	6*/ nd	6/ nd	12/ nd	12/ nd	21*/nd	[78]
Rubber industry wastewater (%)	34/ nd	nd	12/ nd	6/ nd	6/ nd	nd	[77]
Industrial effluents collecting drain, Peshawar (%)	6/ nd	3/ nd	1.26/ nd	1.26/ nd	0.36/ nd	nd	[77]
Wastewater treatment plant (%)	nd /174.3	nd /245.5	nd	nd /189	nd /80	nd	[66]
Cu (mg/L)	nd -/19.09	nd -/23.09	nd	nd -/50	nd /20.4	5/nd	[80]
Cd (mg/L)	/nd/202	nd	nd	nd	nd/183	nd	[82]
Ni (mg/L)	nd/235	nd	nd	nd /292	nd /221	nd	[82]
Ariel detergent (mg/L)	90/181	90/193	10.8/225	90/253	nd	250/nd	[72]
Green Care detergent (%)	0.04/0.227	0.04/0.14	nd/0.027	<0.02/0.6	nd	0.1/1.4	[75,86]
Urea (g/L)	6/14.973	3/13.361	6/18.514	6/16.517	6/16.327	nd	[60]
DAP (g/L)	1.8/3.46	0.63/1.91	3.6/6.896	1.8/5.486	1.5/7.547	nd	[60]
Pentachlorophenol (mg/L)	nd/26.8	nd	nd	nd/34.5	nd	1.56/nd	[87]
Dichlorophenol (mg/L)	nd/205	nd/125	nd	nd/205	nd/200	12/nd	[87]

Table 2. NOEC/EC$_{50}$ values for different synthetic pollutants obtained for different parameters of *Euglena* after 7 days growth in the toxicant. The values given are in % or mg/L as mentioned for each substance. The value before the slash (/) is NOEC while the value after the slash is EC$_{50}$. nd means value not determined.

	Motility (% motile cells)	Velocity	Cell shape	Gravitaxis	Orientation (r-value)	Fv/Fm	Reference
Glass industry wastewater (%)	34/nd	34/nd	6/nd	6/nd	3/nd	21/nd	[77]
Ceramics industry wastewater (%)	nd	nd	nd	6/0	6/0	<6*/nd	[77]
Pharmaceutical industry wastewater (%)	nd	34/nd	21/nd	3/nd	2/nd	6*/nd	[77]
Match industry wastewater (%)	nd	nd	12/nd	3/ nd	3/ nd	6*/ nd	[77]
Textile industry wastewater (%)	nd	21/ nd	6/ nd	6/ nd	3/ nd	nd	[85]
Soap industry wastewater (%)	21/nd	nd	3/ nd	6/ nd	3/ nd	nd	[85]
Ink chemicals industry wastewater (%)	nd	34/ nd	12/ nd	3/ nd	3/ nd	nd	[85]
Sugar industry wastewater (%)	nd	2*/ nd	34/ nd	2/ nd	2/ nd	nd	[78]
Municipal wastewater DI Khan, Pakistan (%)	nd	6*/ nd	6/ nd	12/ nd	12/ nd	21*/nd	[78]
Rubber industry wastewater (%)	34/ nd	nd	12/ nd	6/ nd	6/ nd	nd	[77]
Industrial effluents collecting drain, Peshawar (%)	6/ nd	3/ nd	1.26/ nd	1.26/ nd	0.36/ nd	nd	[77]
Wastewater treatment plant (%)	nd /174.3	nd /245.5	nd	nd /189	nd /80	nd	[66]
Cu (mg/L)	nd -/19.09	nd -/23.09	nd	nd -/50	nd /20.4	5/nd	[80]
Cd (mg/L)	/nd/202	nd	nd	nd	nd/183	nd	[82]
Ni (mg/L)	nd/235	nd	nd	nd /292	nd /221	nd	[82]
Ariel detergent (mg/L)	90/181	90/193	10.8/225	90/253	nd	250/nd	[72]
Green Care detergent (%)	0.04/0.227	0.04/0.14	nd/0.027	<0.02/0.6	nd	0.1/1.4	[75,86]
Urea (g/L)	6/14.973	3/13.361	6/18.514	6/16.517	6/16.327	nd	[60]
DAP (g/L)	1.8/3.46	0.63/1.91	3.6/6.896	1.8/5.486	1.5/7.547	nd	[60]
Pentachlorophenol (mg/L)	nd/26.8	nd	nd	nd/34.5	nd	1.56/nd	[87]
Dichlorophenol (mg/L)	nd/205	nd/125	nd	nd/205	nd/200	12/nd	[87]

The main objective of this review is to emphasize the suitability of *E. gracilis* in wastewater assessment. Several studies show its application for wastewater toxicity assessment in short-term tests (immediately after exposure) but very few studies exist for long-term assessment of wastewater with *Euglena*. Studies evaluating direct toxicity of wastewater (immediately after exposure) with *Euglena* usually involved motility, cell shape and orientation parameters of this flagellate as end points. These parameters showed immediate responses to wastewater exposure, but other parameters such as cell growth cannot be used for immediate tests as these parameters need a long time to respond. A literature survey reveals that a large number of municipal and industrial wastewater samples have been assessed applying motility and orientation parameters of *Euglena* as end points [66,74,76,77]. The authors of these studies concluded that gravitactic orientation (orientation with respect to the gravity field of the Earth) in *Euglena* was the most sensitive parameter toward wastewater toxicity. For example, Azizullah et al. [77] evaluated wastewater samples collected from a stream in Peshawar, Pakistan and reported that orientation parameters (gravitactic orientation and upward swimming) of *Euglena* had much lower G values (1.26 and 0.36% of wastewater, respectively) than motility and cell speed (6 and 3%, respectively). Similar results were reported for dozens of different wastewater samples [66,76-78]. These observations were supported by other studies reporting that in short-term tests gravitactic orientation of *Euglena* was more sensitive than other parameters to water pollutants like heavy metals and certain synthetic pollutants [63,72,79,80]. The use of gravitactic orientation of *Euglena* in bioassessment as a sensitive endpoint has recently been reviewed [81]. The higher sensitivity of orientation in *Euglena gracilis* has been attributed to the presence of mechano-sensitive ion channels in the cell membrane acting as gravireceptors [80]. It has also been reported that motility and orientation parameters of *Euglena* were more sensitive than photosynthesis toward wastewater measured by fluorescence techniques. For example, wastewater samples from different sources impaired motility and orientation in *Euglena* immediately after exposure but did not affect quantum yield of PSII after 24 h exposure [76,77]. Similarly,

short-term toxicity tests of heavy metals revealed that motility and gravitactic orientation of *Euglena* were more sensitive than photosynthetic parameters to heavy metals including Cd and Ni [82]. Second to gravitactic orientation, cell shape of *Euglena* was reported to be very sensitive to wastewater exposure as revealed by experiments with a large number of wastewater samples [76,77]. The change in cell shape may also be attributed to the presence of sensitive ion channels in the cell membrane which trigger a change in cell shape in response to external stress [82].

Wastewater toxicity tests with *Euglena* usually involved short-term tests except a very few studies which used photosynthetic performance of *Euglena* as an end point in long-term (7 days) toxicity assessment of wastewater [83]. In their short-term tests (24 h exposure), Azizullah et al. [76,77] reported that wastewater did not adversely affect photosynthesis in *Euglena* but rather had a stimulatory effect. Similarly, Danilov and Ekelund [84] reported stimulatory effects of wastewater on photosynthesis in *Euglena* in short-term tests. But, in a long-term study (7 days exposure), Azizullah [83] reported that 50% of the tested samples (5 out of the total 10) had negatively affected the quantum yield in *Euglena*. It shows that the length of exposure time is an important factor in toxicity assessment of industrial wastewater. However, no other parameters of *Euglena* were tested in this study for comparison purpose.

In addition to wastewater, several different pollutants of aquatic ecosystems have been evaluated for their ecotoxicity using different parameters of *Euglena* as endpoints (a few examples are given in **Tables 1 and 2**). In long-term tests, cell growth and photosynthetic pigments in *Euglena* have also been found to be very sensitive to different toxicants. Overall studies revealed different NOEC and EC_{50} values of a test substance for different endpoints which revealed that the same parameters may not always be the most sensitive to every pollutant. The sensitivity may depend on the nature of a pollutant, exposure time and other environmental conditions.

CONCLUSIONS

Numerous studies support the application of *Euglena* in wastewater toxicity assessment with different parameters as endpoints under different experimental conditions which gave vastly diverse results. It may not be easy to conclude a generalized statement which parameter of *Euglena* is more sensitive to pollution in aquatic ecosystems, but from an overall survey of literature it is evident that in immediate exposure experiments, gravitactic orientation of *Euglena* can be used as the most sensitive parameter in wastewater quality assessment. Second to gravitactic orientation, cell shape can be applied as a sensitive endpoint in wastewater quality assessment after immediate exposure. In long-term tests, cell growth and photosynthetic pigments can give promising results. However, the results can vary depending upon the nature of the pollutant tested and the prevailing conditions. Therefore, the use of multiple parameters as endpoints may be the best strategy in evaluating ecotoxicity of wastewater or a specific pollutant.

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