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5-Fluorouracil Accumulation in Green Microalgae and its Biogenetic Transfer into Ciliate Protozoan

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Abstract The study has demonstrated that anticancer drug 5-fluorouracil causes acute toxicity and interferes with the growth of green microalgae, *Scenedesmus vacuolatus*. It accumulates in microalgae biomass with bioaccumulation factor of 1.84×10^4 and further integrates into the DNA and RNA of microalgae. In addition, the labelled microalgae genome is transferred into protozoan *Tetrahymena pyriformis* on feeding and is retained in the food vacuoles of predator organisms. This biotransfer of labelled 5-fluorouracil via genomic material was evaluated using radioactivity in *Tetrahymena* cell pellets though radioactivity did not detect anticancer drug in the genome of the predator organism.

Keywords Bioaccumulation · Acute toxicity of 5-fluorouracil · Genetic transfer of 5-FU · Microalgae

The chemotherapeutic agent 5-fluorouracil (5-FU) is commonly used in the treatment of cancer and is usually discharged into the hospital effluent and consequently to the surface water with adverse effects on aquatic microcosms. However, the fate of this drug in the environment is largely unknown though it has the potential to contaminate wastewater treatment effluents and consequently affect aquatic ecosystems. In the year 2002, 5-FU was administered to over 2 million patients worldwide, and its overall production was estimated to 5,000 kg/year (Rich et al. 2004). Approximately 2%–35% of the administered 5-FU is excreted via urine within 24 h (Diasio and Harris 1989; Schalhorn and Kuhl 1992), which seemingly ends up into the hospital effluents. Antineoplastic drugs such as cyclophosphamide, infosfamide, 5-FU, doxorubicin, epirubicin and daunorubicin were identified in hospital waste water from hospital's oncogenic department where 5-FU was detected and measured at high concentration (Mahnik et al. 2007; Kümmerer 2000; Kümmerer and Al-Ahmad 1997; Susanne et al. 2004). In addition, 5-FU in the surface water and waste water treatment plants (STPs) effluents was found at concentrations of up to 1 μg/L (Kümmerer 2001). This substance has also been detected in some big rivers such as the Rhime, Elbe, Neckar, Danube, Po and others (Ternes 1998; Klinger and Brauch 2000; Zuccato et al. 2001) as well as lakes and ground water (Heberer et al. 1995).

Although 5-FU is not readily biodegradable, it is generally biotransformed into toxic fluoroacetate (Arellano et al. 1998) and affects the growth of bacteria, algae and daphnia (Zurita et al. 2007). For instance, biodegradation and the degradation rate of 5-FU was found to be up to 92% in the OECD confirmatory test. The degradation rate was directly proportional to the initial concentration (Kiffmeyer et al. 1998). In contrast, degradation was not observed in the closed bottle test (OECD 301 D) and in the Zahn-Wellens test (OECD 302 B) (Kümmerer 1998; Kümmerer and Al-Ahmad 1997). Notably, its degradation again decreased in hospital sewage probably as a result of synergistic effects

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with antibiotics present in hospital sewage (Omura 2003). The undegraded 5-FU affects microbial ecology in the surface water and enters into ground water via soil (UBAFB 1996). Thus, the aim of this study was to analyse the effect of 5-FU on green microalgae in large vessels. In this context, we evaluated the toxicity and bioaccumulation of 5-FU in *Scenedesmus vacuolatus* to determine the quantitative distribution of 5-FU between microalgae DNA and RNA using radioactive [2-^{14}C] 5-FU. Additionally, we investigated the genetic transfer of 5-FU integrated within the microalgae genome to the ciliated protozoan *Tetrahymena pyriformis* as predator.

Materials and Methods

Radioactive [2-^{14}C] 5-fluorouracil (50 µCi/500 µl at a concentration of 245.5 µg/mL or 1.88 mM) and non-labelled 5-fluorouracil were purchased from Hartmann Analytic GmbH, 38124 Braunschweig, Germany and Sigma-Aldrich Chemie GmbH Laboratory, Germany, respectively. Permafluor E, CARBO-SORB and Ultima Gold^{TM} XR were received from PerkinElmer LAS GmbH, 63110 Rodgau-Jügesheim, Germany. The 5-FU stock solution was prepared in Millipore water to a concentration of 10 mg/mL.

The pure isolates of green microalgae *Scenedesmus vacuolatus* were obtained from the Institute of Plant Physiology, University of Göttingen, Germany. During cultivation of microalgae, the cell suspension was facilitated by continuous shaking, illumination and blowing of 3% CO_2 mixed with air through growth medium inlet, which was maintained at an approximate pH-8 (OECD 201). Excess CO_2 and O_2 produced by cell exited by outlet of cell culturing system. Prior to the experiment, the cells were acclimatized in fresh medium by reseedings twice. On the other hand, ciliated protozoan *T. pyriformis* strain GL was obtained from the Institute of Biology: Ecotoxicology and Biochemistry, Free University of Berlin, Germany. Protozoan cell was grown anexically at 28°C in a nutrient proteose peptone yeast (PPY) extract medium (Ud-Daula et al. 2008).

In the exposure experiment, different amount of stock solution of 5-FU were added on 100 mL microalgae inoculums in 250-mL Erlenmeyer flasks, which resulted in concentrations of 200, 100, 50, 25, 12.5 and 6.25 mg/L. Six replicates (n = 6) were prepared for each concentration of 5-FU. Initial cell density was adjusted to 1×10^4cells/mL (OECD 201). 5-FU is a stable compound and is not expected to significantly biodegrade until 40 h incubation in closed bottle test (Kümmerer and Al-Ahmad 1997; Mahnik et al. 2007). Therefore, in this study, the actual concentration of 5-FU in microalgae medium was assumed to be the same throughout the exposure time. Furthermore, in this experiment, the actual exposure concentration of 5-FU was recalculated based on the result of the bioaccumulation test where 93.85% 5-FU was found after immediate measurement in a 200 mL algal medium as explained in bioaccumulation section. Hence, the actual exposure concentration of 5-FU was maintained at 187.7, 93.5, 46.93, 23.46, 11.73 and 5.87 mg/L. The flasks containing microalgae and labelled 5-FU were then placed on cell culturing system with controlled continuous shaking and uniform illumination at 21–25°C. The entire experiment was conducted in a closed system where cell growth was monitored by the measurement of OD_{660} (Spectrophotometer Unicam 5675, England) after 24-h, 48-h and 72-h exposure. The percentage of cell growth inhibition was determined in comparison with the untreated control (Wang et al. 2009). And the effective concentration (EC_{20}) of 5-FU was calculated from standard logistic curve, which was normalized based on an iterative 4-parameter equation as follows:

$$4\,\text{parameter equation } Y = \frac{A - D}{1 + (X/C)^B} + D \qquad (1)$$

where A = Y value of upper asymptote, B = degree of curvature (relative slope of middle region), C = X value at 50% point of curve and D = Y value of lower asymptote.

In order to test bioaccumulation, the radio-labelled [2-^{14}C] 5-FU consisting of 50,000 disintegration per minute (DPM) equivalent (22.53 nCi, 55.29 ng) was added into 200-mL cell growth medium in 500-mL Erlenmeyer flasks with exponentially growing microalgae cells. Initial cell concentrations were adjusted to 1×10^4 cells/mL and incubated for 24 h at six replicates (n = 6). Twenty millilitre of microalgae culture was centrifuged at 4,500 rpm/min for 30 min and washed twice with phosphate buffer saline (PBS). The cell pellets were filtered on a membrane filter (Sartorius Typ SM II 302, pore size 3.0 µm). Subsequently, microalgae cells including filter paper were ignited in an oxidizer (model 307; Packard, Meriden, CT, USA). The oxidized samples were collected in a vial containing 15 mL PermafluorE. Then radioactivity was counted using a liquid scintillation counter (Liquid Scintillation Analyzer, Tri-carb 1500 TR, Packerd, Meriden, CT, USA) for 10 min. Five millilitre of cell supernatant was spiked into 10 mL Ultima Gold^{TM} XR, and the radioactivity of free labelled 5-FU in the medium was counted. The bioaccumulation factor of 5-FU was determined from the radioactivity of microalgae cell divided by the radioactivity of the culture medium according to formula 2. Here, the activity of labelled 5-FU incorporated into microalgae was calculated in a nominal weight. The nominal dry weight of 10 mL microalgae cell suspension was 1.0 mg. On the other hand, 50,000 DPM of 5-FU was added in 200 mL cell-free microalgae medium, and the radioactivity was measured immediately for the determination of actual amount of active 5-FU in the microalgae

culture. In this case, 5 mL of water sample was taken in 10 mL Ultima Gold™ XR, and consequently the radioactivity of exposed 5-FU was measured.

$$\text{Bioaccumulation factor (BAF)} = \frac{5-\text{FU accumulated per kg microalgae}}{\text{Free } 5-\text{FU per litre culture medium}} \quad (2)$$

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In another experiment, radio-labelled [2-^{14}C] 5-FU consisting of 222,000 DPM equivalents (100.01 nCi, 245.5 ng) was added to 100-mL microalgae culture in 250-mL Erlenmeyer flask and cultivated for 24 h. The actual exposure amount of radioactive 5-FU was readjusted based on the calculation of bioaccumulation test. The microalgae cells were centrifuged in a glass tube at 4,500 rpm/min for 30 min, and the cell pellets from 30 mL microalgae culture were than harvested in the described manner. DNA and total RNA were extracted using the DNeasy Plant Mini Kit and the RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's guidelines. The final extraction volume of both DNA and RNA was 200 µL. Fifty microlitre of DNA and RNA extracts was oxidized (n = 6), and then oxidized product was placed in 15 mL Permafluor E in a vial for counting radioactivity. In addition, the extraction wastes of DNA (collected 2.2 mL) and total RNA (collected 2.3 mL) and also the used mini spin columns were subjected to count radioactivity. Besides, 100 µl cell supernatant was spiked into 15 mL Ultima Gold™ XR, and radioactivity of free 5-FU present was counted in the culture medium.

The microalgae genomic materials (DNA and RNA) containing 469 DPM activity corresponding to 24×10^{11} molecules of 5-FU were fed to exponentially growing *Tetrahymena* and cultivated in 40-mL PPY medium in a 300-mL Erlenmeyer flask. The initial cell concentration was adjusted to 1×10^4cells/mL, and the culture was incubated for 24 and 48 h at 28°C. After incubation, 5 mL of cell suspension was centrifuged at 5,500 rpm for 10 min and subsequently washed twice with PBS. Afterwards, cell pellets were filtered by a membrane filter and washed again twice with 5 mL PBS. The cells pellets along with membrane filter were combusted and counted for radioactivity. In addition, the *Tetrahymena* DNA was extracted using the DNeasy tissue kit (Qiagen, Germany). This DNA was also used to investigate radioactivity for whether 5-FU reintegrated in the genome of predator organisms.

Results and Discussion

This study demonstrates that 5-FU induces cell growth inhibition and causes acute toxicity in microalgae. The effect of 5-FU was estimated from a regression curve derived from a plot of percentage of cell growth inhibition versus concentration of 5-FU as shown in Fig. 1. Similarly, iterative 4-parameter equation and consecutive model regression curve were used to obtain effective concentration of 5-FU in microalgae. All raw data were normalized by the 4-parameter equation. The effective concentration (EC), EC_{20}, of 5-FU to microalgae was found to be 40.12, 55.86 and 54.25 mg/L after 24-, 48- and 72-h exposure, respectively, as shown in Fig. 2. However, no observed effect concentration (NOEC) was determined to be 0.06, 2.32 and 0.08 mg/L after 24-, 48- and 72-h exposure, respectively, as given in Fig. 2. The low observed effect concentration (LOEC) was also calculated from the regression curve presented in the Fig. 2. 5-FU enters into microalgae probably via lipophilic interaction and accumulates in DNA and RNA because of greater repetitive insertion versus excision from genetic materials (Ingraham et al. 1982, Kufe et al. 1983). The DNA, which incorporates 5-FU, has a slow rate of elongation during its synthesis (Scheutz and Diasio 1985). For this reason, the incorporation of 5-FU in the DNA may contribute to the

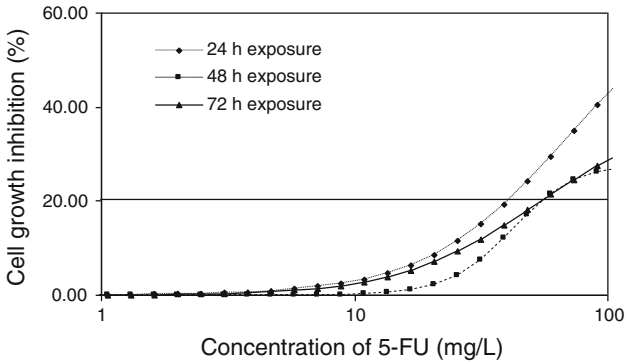

Fig. 1 Concentration–response regression curve of 5-FU in *Scenedesmus vacuolatus* after 24-h, 48-h and 72-h exposures, n = 6. The standard deviation of raw data for this experiment was found between ±0.47 and ±3.98

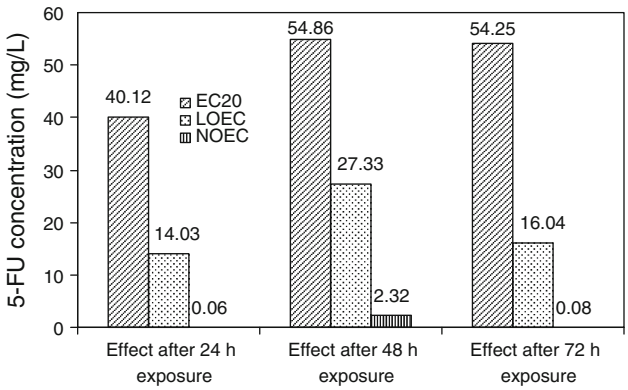

Fig. 2 Acute toxic effect of 5-FU on microalgae, *S. vacuolatus,* in terms of effective concentration (EC)

cytotoxicity of the compound and consequently causes the decline in cell growth rate. However, the effective concentration may be underestimated because of the depletion and potential low existing concentrations of 5-FU in this test due to its fast uptake during reproduction of cells. Thus, the frequent discharge of 5-FU into the aquatic environment may cause severe effects in algae populations.

Bioaccumulation of 5-FU in microalgae cell was evaluated using radio-labelled [2-^{14}C] 5-FU. The immediate measurement (0 h) of radioactivity of 5-FU in 200-mL microalgae medium was found to be 46,927 ± 268 DPM out of 50,000 DPM. Therefore, the radioactivity of 5-FU in the microalgae medium was found to be 93.85% in microalgae medium that was activated and exposed to the microalgae cell. The rest of 6.15% 5-FU may be have been inactivated or was not counted by liquid scintillation counter. The microalgae cell pellets (whole cell) of the entire cell culture after 24-h incubation showed 25,090 DPM radioactivity count while the supernatant of the culture contained 13,660 DPM as presented in Table 1. Therefore, the total radioactivity count in this experiment was found to be 38,750 DPM out of 46,927. The untreated control microalgae did not exhibit radioactivity count. Thus, the bioaccumulation factor (BAF) of 5-FU was calculated to be 1.84×10^4 according to the formula 2. This findings supported a claim that 5-FU actively bioaccumulated in the microalgae cells, perhaps partly through lipophilic interaction. As a result, it has the potential to affect aquatic biocenosis, especially where primary producers/microalgae are prominent. So far, no report exists that explains the mechanism of 5-FU penetration through cell membranes, and it possible that some 5-FU penetrates into *Tetrahymena* via the membrane rather than through feeding.

The exposure of radioactive 5-FU in microalgae causes slight incorporation into both DNA and RNA. But maximum 5-FU molecules are unaccumulated, which has resulted in the radioactivity count of 179,100 DPM in microalgae cell culture supernatant. DNA in the entire cell of the culture contains approximately 872.4 DPM of radioactivity counts out of 222,000 DPM. However, large amount of radioactive 5-FU passed through the DNeasy mini elute column during the DNA extraction and was retained on the column. The radioactivity obtained in the eluted waste fraction and the DNeasy mini columns were found to be 27593 DPM and 3165 DPM, respectively, as given in Table 2. Therefore, the sum of radioactivity counts for the three subsamples of 100 mL culture was found to be 31630.4 DPM, which demonstrates that 5-FU accumulates in the algae cells. The study has also revealed that 0.49% of 5-FU was integrated into the DNA while 18% was accumulated in the cell cytoplasm or elsewhere in the algae cell. On the other hand, the sum of radioactivity counts for the three subsamples of total RNA extraction from 100-mL cell culture (total RNA, eluted extraction waste and RNeasy mini column itself) together showed 27718 DPM radioactivity count as shown in Table 2. This finding shows that 0.68% of 5-FU was integrated into the

Table 1 Determination of bioaccumulation of 5-FU applying radio-labelled compound in *Scenedesmus vacuolatus* after 24-h exposure, where n=6

Name of experiment		Radioactivity (DPM) ± SD	Radioactivity in 200-mL culture	Amount of [2-^{14}C]5-FU	Bioaccumulation factor
Control	Cell pellet of 20 mL	0	0	0	0
Microalgae	Supernatant of 5 mL	0	0	0	
Microalgae	Cell pellet of 20 mL	2,509 ± 253	25,090	27.76 ng	1.84×10^4
+ 5-FU	Supernatant of 5 mL	341.5 ± 33	13,660	15.11 ng	

Table 2 Quantitative distribution (mean ± SD) of radio-labelled 5-fluorouracil into cellular DNA and total RNA of *Scenedesmus vacuolatus* after 24-h exposure, n = 6. In addition, radioactivity was also measured for other subsamples during their extraction, n = 6

Name of the experiment	Radioactivity (DPM) of 100-mL culture		Distribution of labelled 5-FU (%)		Distribution of radio-labelled 5-FU in cell
	DNA	Total RNA	DNA	Total RNA	
Control cell	0	0	0	0	
Nucleic acid	872.4 ± 132.7	1,211 ± 69.9	0.49	0.68	
Extraction waste	27,593 ± 668.4	17,513 ± 113.67	13.2	8.47	16–18 (%)
Extraction column	3,165 ± 420	8,994 ± 673.4	1.50	4.35	
Culture supernatant	179,100 ± 1,483.3	179,100 ± 1,483.3	84.9	86.6	

total RNA whereas 16% was accumulated either in the microalgae cell cytoplasm or elsewhere in the algae cell. However, the slight difference in the accumulation of 5-FU accumulation using two different extraction procedures (DNA and total RNA extraction) may be due to the differential capacities of the extraction columns. In general, these findings clearly demonstrate that 5-FU actively accumulates in the microalgae cell and also integrates into the genome.

5-FU is a pyrimidine analogue, which is transformed inside the cell into different cytotoxic metabolites that are then incorporated into DNA and RNA. It finally induces cell cycle arrest and apoptosis by inhibiting the cell's ability to synthesize DNA (Longley et al. 2003). 5-FU is also incorporated into the genetic materials and causes the heterogeneity of ribosomal RNA formation as well as production of improper proteins in yeast (Mayo et al. 1968). Other mechanisms suggest that 5-FU is involved in the activation of ribonucleoside, namely 5-fluorouridine, and its subsequent phosphorylation and incorporation into RNA (Morris 1993). Nevertheless, our study found slightly higher disparity in concentration of 5-FU in microalgae RNA than DNA. Despite the little variation of 5-FU in DNA and RNA extraction, it was estimated that 16–18% 5-FU was accumulated in fast-growing microalgae cell in the genome, cytoplasm or other locations in the cell. Hence, this experiment demonstrates the uptake of 5-FU by microalgae cells and its dispersal into the whole cell. In addition, it was exhibited that higher amount of radioactive 5-FU was incorporated in the total RNA than DNA despite extraction from the same volumes of cells culture. It may be caused by the fact that cell systems (uracil–DNA glycosylase) rapidly removed fluorinated pyrimidine from the genome but continuously incorporate into RNA (Morris 1993).

The genetic biotransfer of labelled 5-FU through microalgae genomes was identified in *Tetrahymena*. *Tetrahymena* cell was fed on radioactive genomic material of microalgae as nutrient during their growth. Cell growth was monitored by counting with a Neubauer cell counter, and cell concentration was found to be 1.6×10^5 cells/mL and 14×10^5 cells/mL after 24 and 48 h of incubation, respectively. The endocytosis of radioactive genomic material by *Tetrahymena* was confirmed by exhibition of radioactivity counts in cell pellets. Therefore, the cell pellet of entire culture (40 mL) was estimated to be 361 DPM radioactivities out of 469 DPM as illustrated in Fig. 3. Less radioactivity count (352 DPM) was found in the whole culture after 48 h of incubation, although the cell number had increased. This slight reduction in radioactivity might have been caused by the degradation of ingested genomic materials into nucleotide and decreasing concentration in the medium. Small unit of nucleotide that was not precipitated with *Tetrahymena* cell could also explain this

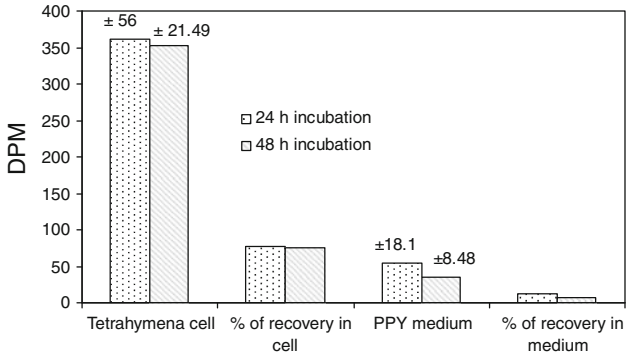

Fig. 3 Transfer of labelled 5-FU embodied in the genetic material of *Scenedesmus* to *Tetrahymena*, n = 6

finding. Most importantly, it was found that the radioactivity count in the *Tetrahymena* cell decreased with increasing incubation time. In contrast, much less radioactivity was found in PPY medium at 54 DPM and 35.5 DPM after 24- and 48-h exposure, respectively, as shown in Fig. 3. Therefore, the radioactivity measurement in PPY-medium and *Tetrahymena* cell culture has clearly demonstrated that the label 5-FU integrated microalgae genome is phagocytose or ingested by the predator cell. A steady occurrence of radioactive microalgae genome in the *Tetrahymena* cell would suggest that 5-FU naturally biotransfer from one aquatic species to another via genetic material, and may cause hazard in the aquatic microcosm due to continuous contamination.

The reintegration experiments of radio-labelled 5-FU via microalgae genome in the genome of *Tetrahymena* was conducted for 24 h. DNA extract of *Tetrahymena* was used to count radioactivity instead of the whole cells. No radioactivity count was detected at levels higher than the background (basal level), which suggest that 5-FU did not reintegrate in the genome of predator cell. Two points could explain this observation: first, labelled 5-FU never reintegrated into the DNA of *Tetrahymena* under the experimental conditions chosen; and secondly, the protocol to determine radioactivity was not sensitive enough. The background radioactivity in the DNA of *Tetrahymena* shows small amounts of radioactivity count, which corresponds to approximately 5.1×10^{11} molecules of 5-FU. However, the fate of 5-FU integrated in microalgae genome ingested by *Tetrahymena* remains a vital question. The ingested genetic materials by the predator organisms form food vacuoles in the cell cavity. These food vacuoles accumulate foreign genetic materials and later degrade nucleic acid polymers into oligonucleotides or single nucleotides by the action of host enzymatic systems. Hence, the fragmented DNA had probably not been recovered during DNA extraction of *Tetrahymena*. The fragmented algae genome was passed through the DNeasy tissue column because the lower limit of the extraction

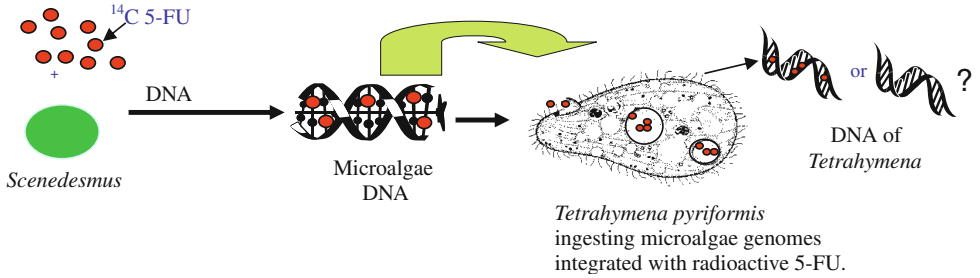

Fig. 4 A brief scheme of experiment and mechanism of biogenetic transfer of 5-FU from microalgae, *Scenedesmus,* into protozoan, *Tetrahymena.* Predator cell feed microalgae genome embodied with labelled 5-FU. This figure presented, 'Where is the final fate of 5-FU'? and 'Can the 5-FU reintegrate into the genome of *Tetrahymena* or not'?

column for capturing DNA is at 100 base pairs. Therefore, one can recover only DNA of *Tetrahymena* but not digested foreign genomes of microalgae, which show no radioactivity. However, the addition of high radioactivity genetic material of microalgae into the *Tetrahymena* culture might cause sufficient integration of 5-FU into the genome of *Tetrahymena* to allow for radioactive detection.

In brief, the results of exposure of microalgae to 5-FU and ciliated protozoan demonstrate the risk of 5-FU to aquatic microbial community. Since 5-FU interferes with cell growth, causes moderate acute toxicity in green microalgae, and also accumulates rapidly in cells and integrates into the DNA and RNA, there is a need to regulate its disposal to aquatic ecosystem. Reintegration of labelled 5-FU was not observed in the DNA of the predator organisms although the predator cell ingested labelled genetic materials of microalgae. The complete experiment and the mechanism of transfer of 5-FU into *Tetrahymena* is illustrated in Fig. 4. Indeed, it would be a convenient experiment to add whole microalgae to the predator organisms. Unfortunately, we could not carry out these experiments due to the difficulties of separation of *Tetrahymena* from microalgae. Nevertheless, this study demonstrates for the first time the adverse effect of 5-FU on microalgae in large test vessels and describes a probable mechanism of 5-FU biotransfer from one organism to another organism in an aquatic environment.

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