Are Bacteria The Main Producers of Hydrolytic Enzymes in Aquatic Environment?

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ABSTRACT

Bacteria play an important role in the decomposition of organic matter, which is a key process in aquatic microbial food webs as well as its application in water bioremediation processes. DOM (Dissolved Organic Matter) is the major fraction of organic matter in most aquatic environments. Most of DOM is present as high molecular weight compounds that cannot be taken up by bacteria directly. Therefore, they must be hydrolysed enzymatically to be transported across microbial cell membranes. Molecular fluorosensors have been used as artificial substrates to study hydrolytic enzymes in situ. The present study wants to investigate the following hypothesis i.e. bacteria are the main producers of hydrolytic enzyme in aquatic systems. There were not any significant correlations between bacteria and the investigated hydrolases (esterase, peptidase, and β-glucosidase) in the meso- to eutrophic aquatic systems near Rostock city – North East Germany, although a wide range of bacterial abundances were covered. Enzyme saturations were not detected in all samples which may be explained by a high KM indicating a low affinity of enzymes (1) and or the contribution of many enzymes with different kinetics to the respective substrate degradation (2). There is also increasing evidence that bacteria may not be the sole or dominant source of esterases, peptidases and β-glucosidases in aquatic ecosystems. Invertebrate, fungi and other eukaryotes (diatoms, protozoa etc.) must be considered as possible and even important producers of hydrolytic enzymes. Several other factors may influence the correlations of bacteria to hydrolytic enzyme activities, i.e. variations in the species composition (1), a wide ranged variability of hydrolytic activities influenced directly by other substrates (low enzyme affinity), element availability (N and P), temperature and other abiotic factors (2), the existence and persistence of enzymes caused by other (passive) processes (e.g. cell lysis) (3).

Keywords: Hydrolases, esterase, aminopeptidase, glucosidase, fluorosensor, bacteria

INTRODUCTION

Microorganisms occur almost everywhere in nature and in/on artificial material. They can contribute to material corrosion, as they hydrolyze polymers, oxidize metals or enhance water retention by their mucous envelopes. This biocorrosion is responsible for serious maintenance and repair costs summing up to milliards of Euro per year. Additionally they potentially endanger men, e.g. by the allergenic potential of fungal spores. Hoppe *et al*. (2002) and Bidle & Azam (1999), for example, reported the important role of microorganism also for aquatic ecosystems. Microorganisms are responsible for the decomposition of organic matter which is a very important key process in (microbial) food chains as well as in water bioremediation processes.

Modern microbial diagnostics are very important to recognize the existence of the invisibly small bacteria and the transparent biofilms on material surfaces as early as possible. Classical microbiological detection methods often include an isolation and cultivation step, that is not only time consuming (up to several days), but also misses most individuals or even species. In water samples only up to 6% of all bacteria were found to grow colonies on agar plates, most often they don't exceed 1% of total number (Rheinheimer 1985). Molecular genetic methods, esp. genomic profiling protocols, were introduced into environmental research, esp. into marine microbiology, several years ago with great success (Muyzer *et al*. 1993). Fluorescent gene probes identify bacterial species without isolation (Amann *et al*. 1990), however, if they detect DNA they find also dead cells and if they are designed to hybridize with RNA they don't distinguish between active and inactive individuals. Since many "wild" cells are rather small compared to cultured ones, the detection of small marine bacteria with a low ribosome content using CARD-FISH (Catalyzed Reporter

Decomposition – Fluorescence in Situ Hybridization) was of great advantage (Pernthaler *et al*. 2002).

The novel microbial diagnostic strategy based on molecular fluorosensors is especially advantageous in applied environmental microbiology, because microorganisms do not need to be isolated and can be investigated on non-transparent surfaces with minor to none material damage. The specificity and universal applicability of fluorosensors for heterotrophic and autotrophic microorganism is a good advantage to develop it as new microbial diagnostic kit in applied environmental microbiology.

There are some different molecular based fluorosensors applied already to environmental samples. With the redox-sensitive dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) respiratory activity of (marine) bacteria can be visualized by epifluorescence microscopy as well as flow cytometry (Sieracki *et al*. 1999). So called live/dead kits distinguish between intact and dead bacteria to evaluate antibiotic treatments (Jacobsen *et al*. 1997). Schumann *et al*. (2003) investigated the viability of aquatic bacteria using different types of fluorescent markers and discussed the potential of intracellular hydrolytic enzymes as viability markers. However, there are very few and limited attempts to use them in applied environmental research, e.g. in biofilm or biodeterioration analysis (see a discussion in Surman *et al*. 1996). Different types of common fluorescent markers, e.g. redox sensitive, nucleic acid stains with different membrane permeability have been applied to natural bacterial assemblages of meso to eutrophied waters and biofilm samples from technical surfaces in this research. However, the detection of (cellular) hydrolytic enzyme activity will be of special interest. Bacterial hydrolytic enzymes play a key role in the cycling of organic matter in aquatic as well as terrestric ecosystems. They are also involved in biodeterioration of material and food going stale. Since cellular enzyme activity is one of the most important microbial activities, it can also be used to characterise viable cells in environmental samples. This position as "mineralizers" or "degraders" breakdown organic matter and liberate valuable inorganic nutrients for primary producers.

DOM (Dissolved Organic Matter) is the major fraction of organic matter in most aquatic environments (e. g. Münster & Chróst 1990). In aquatic systems, most of DOM consists of high molecular weight compounds (>95%) (Chróst 1993). These polymers are mostly unknown and very diverse structures, such as proteins, polysaccharides, nucleic acids and humic material (Münster 1985). Therefore, only a small fraction of total DOM (<5%) can be directly taken up by bacteria (Ammerman *et al*. 1984) because only low molecular weight organic compounds (mono- or small oligomers) can pass the cell walls and get transported across microbial cell membranes. The hydrolytic enzymes help to hydrolyze macromolecules outside into monomers, which are taken up by the cells. Through an active process mediated by membrane-located carriers, sometimes termed as permeases and after subsequent hydrolysis to mono- or oligomers, most of DOM can be incorporated by bacteria. The hydrolysis products serve as substrates for the hydrolase producers themselves as well as an input of substrates to the pool of uDOM (utilisable DOM). In the last decade, many studies on the enzymatic decomposition of organic matter (as microbial substrates) have been carried out to elucidate transformation rates of organic carbon in aquatic ecosystems (e.g. Sherr & Sherr, 1999). Most of ectoenzymes and extracellular enzymes in aquatic environments are hydrolases (Chróst 1990). This study focused on three hydrolytic enzymes i.e. esterase, peptidase and glucosidase, representing enzymes capable hydrolysing three main compounds in DOM pool i.e. lipids, proteins and carbohydrates.

The major step of the microbial loop concept is the conversion of uDOM into bacterial biomass and production (Azam *et al*. 1983). This process is strongly dependent on enzymatic capacities of the microbial community. Considering the importance of hydrolytic enzymes within the microbial loop, they operate at the molecular level in aquatic environments and affect the function of the whole aquatic ecosystem.

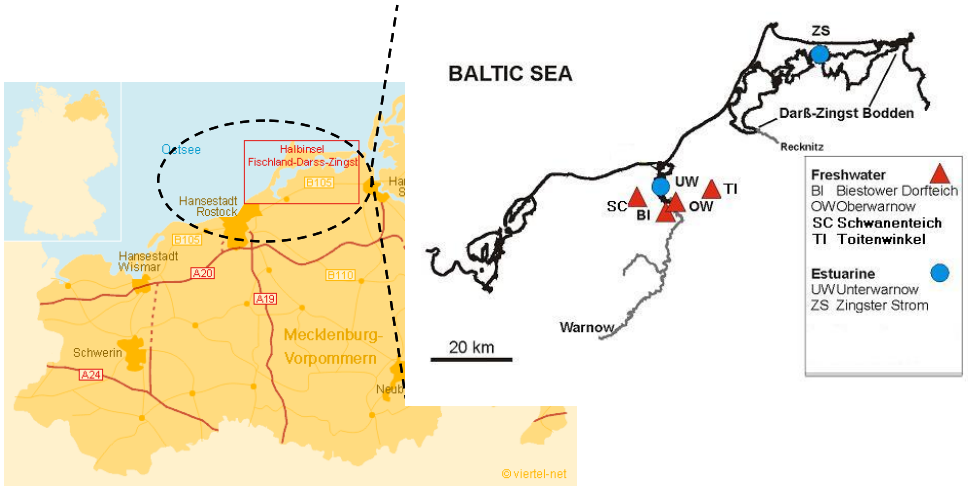


Figure 1. Map of sampling location.

The objective of this study is to investigate different hydrolytic enzyme activities in natural aquatic samples in relation to their producers. To achieve this goal, correlation analyses of hydrolytic activities with microorganism biomasses as well as organic substrates in waters of different organic loads were conducted. If bacteria are the main producer of hydrolytic enzymes, this study wants to evaluate following questions: are there any correlations between potential enzymatic activities and bacterial numbers in water samples of different organic loads? (1), what factors may influence these correlations? (2), is there any relationship between the portion of viable or active bacterial cell and hydrolytic activities? (3)

METHODS

Sampling location

Three different types of aquatic systems were investigated at or near the coast of the Baltic Sea in Mecklenburg-Vorpommern, Germany. A brackish site was sampled in the Baltic Sea together with different freshwater systems (River Warnow, Biestow Pond, Pond Schwanenteich, Toitenwinkel) and the estuaries (Unterwarnow and Zingster Strom (Figure 1). The Baltic Sea sampling site was situated in Warnemünde near Rostock city at a depth of 10 m. Water samples were collected from the jetties at the beach. The freshwater systems were all shallow including the slow running eutrophic River Warnow and several ponds. Inner coastal waters were oligohalob, tideless and shallow having an average depth of only 1 m and eutrophic (Figure 1). Overall, 41 water samples were collected from freshwater, estuarine waters and brackish waters for the correlation analysis.

Samples treatment

Water samples of 10 l volumes were collected at the upper 30-50 cm, transported to the laboratory within 1 h using polyethylene containers and then processed immediately upon arrival. At first, subsamples of unfiltered water and particle free filtrates (only water from the River Warnow) were prepared for measurements of total and free dissolved esterase, peptidase and β-glucosidase activity. Three 1 ml subsamples were incubated at *in situ* temperature for 3-5 h with the respective substrates to quantify respiring bacteria and those with intracellular esterase. Stored samples were prepared according to their sensitivity to handling. 10 ml of unfiltered water and 10 ml of filtrates were stored at -20°C, for TOC (Total Organic Matter) and DOC measurements respectively, and were measured within 3 months. Suspended particulate matter (SPM) was determined from dried filters after filtration of water samples, stored at 4 °C and measured within 2 months. Samples for chlorophyll *a* (chl *a*) were measured after filtration of 50-150 ml aliquots onto glass fibre filters and after storage at -20 °C for up to 3 months. Bacteria were counted in 20 ml fixed samples within 4 weeks.

Particulate Organic Carbon in Suspended Particulate Matter (POC in SPM)

Samples of 100-200 ml from mesotrophic sites and 50-100 ml from eutrophic waters were filtered onto precombused (450 °C for 4 h) and pre-weighed glass fibre filters (25 mm in diameter, GF/F Whatman). These filters were dried overnight at 60 °C. The dried filters were then weighed again on an electronic microbalance (M2P, Sartorius). The SPM

was calculated as the difference between the dried filter and the pre-weight filters.

Carbon contents of SPM were estimated according to Verardo *et al*. (1990) in an elementar analyser (vario EL Elementar). Triplicate of dry filters were packed in air-tight tin foils, stored at 4 °C until measured in the elementar analyser. POM in this research was an estimate for total microbial biomass in aquatic system.

Chlorophyll a (chl *a*) concentration as an estimate for phytoplankton biomass

Chlorophyll of previously filtered and frozen samples was extracted with N,NDimethylformamid (DMF, Fluka) and measured spectrophotometrically with a Shimadzu (UV-2401PC) spectrophotometer (Porra *et al*. 1989) for chlorophyll *a* corrected for chlorophyll *b*. A correction for chlorophyll *c* produced by diatoms was not possible by this procedure. Chl *a* was considered to be the general biomass parameter of primary producers, i.e. phytoplankton. Phytoplankton biomass was calculated using factor of 31 µg Carbon per µg phytoplankton (Schumann 1993).

Bacterial abundance as an estimate for bacterial biomass

Total bacterial cell numbers were counted in fixed samples (1 ml 25% Glutardialdehyde for 20 ml sample, final concentration 1.2%) after staining with 4',6 diamidino-2-phenylindole dihydrochloride (DAPI, Roth) following Porter & Feig (1980). Triplicate samples of 0.5 ml subsamples were filtered onto Irgalan Black stained 0.2 µm Isopore TM polycarbonate filters (SIGMA ALDRICH) with a maximum pressure of -400 mbar. Each filter was stained separately with 1 ml DAPI (1 mg DAPI in 100 ml phosphate buffer pH 7.6, final concentration 29 µM) for 5 min. Filters were embedded in immersion oil between slides and cover slips. Cells were counted with an epifluorescence microscope Olympus BX 51 (1000- fold magnification with an UPlan FL 100 NA objective, UV excitation by U-MWU2). At least 196 cells per filter were counted. Bacterial biomass was calculated according to Long (2004). 20 fg carbon per bacterium was used since sizing was not performed (biovolume).

Intracellular esterase activity

Bacteria with intracellular esterase activity were detected using 5-chloromethylfluorescein diacetate (CellTrackerTM Green CMFDA, Molecular Probes Europe) as an artificial substrate for esterase. CMFDA starts to fluoresce after enzymatic hydrolysis and is bound to the intracellular protein pool with its chloromethyl-group (Haughland 2005). This reaction formed a covalently bound fluorescent molecule staining the entire cells yellow-green. Triplicate samples of unfixed 1 ml subsamples were incubated with a stock solution of CMFDA (25 µM final concentration) for at least 3 hours at *in situ* temperature. Filters were then embedded in immersion oil between slides and cover slips. Using 1000-fold magnification of a microscope (Olympus BX51, white green filter), more than 400 active bacteria were enumerated on each filter for most samples. At least 185 active bacteria were counted for several samples with very low cell numbers. These active bacteria could also be considered a viability parameter for the physiological state of bacteria (Schumann *et al*. 2003).

Cellular respiration

Respiring active bacteria were counted based on reduction of the fluorosensor 5-Cyano-2,3-ditolyltetrazoliumchlorid (CTC, 19292-100 Polysciences Inc.) according to Rodriguez *et al*. (1992). Active bacteria reduce monotetrazoliumredox (CTC) to the red fluorescing formazan derivative (CTF) given that there are sufficient amounts of NADPH+ available, e.g. via the respiratory chain. This fluorescence appeared intracellular by the formation and deposition of red fluorescing crystals (epifluorescence microscope Olympus BX51, blue-yellow-green excitation, UPlanFL 100× 1.3 Oil, U-MWB 2). Triplicate unfixed bacteria samples were stained with 100 µl CTC-solution (50 mM) resulting in a final concentration of 5 mM (Choi *et al*. 1999). Samples were incubated for 4-5 h at in situ temperature. Samples were counterstained with SYBR Gold (S-11949 Molecular Probes), immediately before counting.

Hydrolytic enzyme activity

Hoppe (1983) introduced a new method which linked substrates to the highly fluorescent compound 4-methylumbelliferon (MUF) and thus provided a very sensitive technique for the detection and quantification of specific and non-specific hydrolases of bacterioplankton in natural waters. The substrate-MUF complex does not fluoresce. Only after hydrolysis and cleavage of the complex which is catalysed by an enzymatic reaction (Figure 2) MUF will be activated to fluoresce blue under UV excitation. The slope of fluorescent signal was proportional to the amount of converted substrates and thus to enzyme activity. The hydrolytic activity of esterase, β-glucosidase and peptidase was measured as described above (Hoppe 1983) using the substrate-fluorescent compound complexes i.e. 4-methylumbelliferylacetate (MUF-acetate), 4-methylumbelliferyl-β-D-glucoside (MUFglucoside) and L-leucine-4-methyl-7-coumarinylamide hydrochloride (leucine-AMC) (Sigma Aldrich), respectively. Total enzymes activities were investigated in all water samples. The corresponding activities of dissolved enzymes were measured in the River Warnow only throughout the vegetation periods of 2004 and 2005 (data are not shown). Only minor adaptations were made to the procedure.

A kinetic approach was applied which allowed the calculation of the maximum reaction velocity (Vmax) and the Michaelis constant (KM) indicating

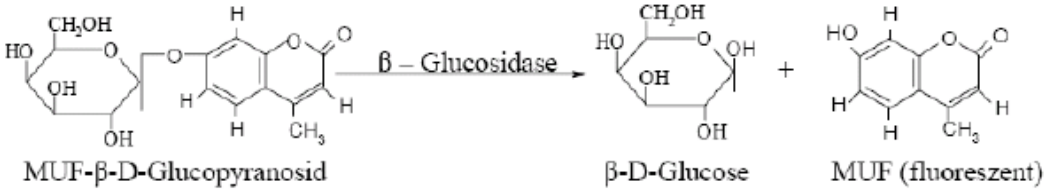


Figure 2. Cleavage of the bond between methylumbelliferyl marker (MUF) and the substrate through enzymatic reaction of β−glucosidase. The products were 4-methylumbelliferyl (MUF) and β-D-glucoside, the former fluorescing blue after excitation with UV-light.

enzyme affinity to the substrate. In most cases enzyme reaction follows the Michaelis Menten equation and displays a hyperbolic relationship.

However due to difficulties during the determination of Vmax and KM using this model, non-linear parameter estimation by Solver of Microsoft Excel program (Microsoft Office XP version 2003) was used to fit the hyperbolic relationship to estimate Vmax and KM. This study used termed KM to represent apparent KM obtained from this kinetic analysis of community samples. A kinetic experiment was designed at the beginning of this research project to determine the saturated concentrations of each artificial substrate. At least five different concentrations of artificial substrates, 0 to 600 µM (for example: 0, 50, 100, 200, 400, 600 µM) were applied to the enzyme reaction and plotted graphically. The saturated concentration is defined as the amount of substrate which does not influence the reaction velocity of the enzyme, thus velocity remains constant. Further increase in substrate concentrations does not affect reaction velocity.

Samples were buffered with Tris Buffer pH 8.2 (final concentration 5 mM in each of the 2 ml subsample). Artificial substrates were dissolved in pure Ethanol (98.99% p.a., Roth) and added to natural water at saturated concentration i.e. 400 µM for MUF-Acetate, 600 µM for MUF-glucoside, and 200 µM for leucine-AMC. Three replicates of each sample were incubated at room temperature (ca. 21°C). The fluorescent hydrolysis product was recorded in a Hitachi F4010 fluorometer (excitation 365 nm, emission 451 nm, bandpass 1.5 nm, response 2 s, average over 2 s) within 1 h for esterase, 2 h for peptidase and 2 to 8 h for β-glucosidase. Blanks of filtered, sterilised and distilled water were treated in the same way to correct the results for non enzymatic hydrolysis. The hydrolytic enzyme activity was calculated from the linear increase of fluorescence over time. At least four measurements were made throughout the entire incubation period. The fluorescence signal of triplicate samples was recorded until the initial slope showed no further increase.

Standard solutions of MUF and AMC were measured at each sampling day in order to calibrate for hydrolysis rates of fluorescence units (calibration factor). For this, geometric dilution series of MUF or/and AMC stock solution were used with concentrations ranging from 0.3 to 25 µM in Tris Buffer pH 8.2. The slope of the fluorescence-concentration curve was calculated and used as a calibration factor to correct the final enzyme activity calculations.

RESULTS AND DISCUSSION

It has been widely assumed that most extracellular hydrolytic activities in aquatic systems except phosphatase originate predominantly from bacteria (e.g. Sala & Güde 1996). However, there have been more and more reports on weak or missing relationships between bacterial numbers as well as biomass and hydrolytic activities (e.g. Hoppe *et al*. 1998, Vrba *et al*. 2004). There were also not any significant correlations between bacteria and the investigated hydrolases in the meso- to eutrophic aquatic systems investigated here (Table 1), although a wide range of bacterial abundances were covered. That should have provided enough steady-state conditions for an overall balanced cell number to activity ratio.

This can be explained by one or a combination of the following causes. Free dissolved enzymes, especially esterase, are abundant (Schumann *et al*. 2003b, this study in river Warnow) and may persist for days and are included in the total hydrolytic activity (1). Many bacteria counted were dead or inactive (e.g. Schumann *et al*. 2003a, Freese *et al*. 2006, this study in river Warnow) (2). Bacteria may not be the sole producers of hydrolytic enzymes, substantial hydrolytic activities may originate from other organisms, e.g. marine copepods (Bochdansky *et al*. 1995), diatoms (Smucker & Kim 1991), phagotropic flagellates (Karner *et al*. 1994), phototrophic dinoflagellates (Mulholand *et al*. 2002), or crustaceans (Vrba *et al*. 2004) (3).

Table 1. Spearman rank order correlation between bacterial abundance (10^6 ml^{-1}), chlorophyll a (chl *a*) (µg l^{-1}), DOC (mg C l^{-1}), POC (mg C l^{-1}), esterase, β-glucosidase and aminopeptidase (µmol l^{-1} h^{-1}). Data from all station, r_s: correlation coefficient; p: error probability; ***p***, significantly correlated with p<0.05.

		Chl *a*	DOC	POC	Esterase	β-Glucosidase	Amino-Peptidase
Bacterial	r_s	0.52	-0.04	0.41	-0.43	-0.05	-0.22
abundance	p	***0.00**	0.83	***0.01**	***0.02**	0.77	0.25
Chl *a*	r_s		0.12	0.78	-0.22	0.43	0.13
	p		0.49	***0.00**	0.24	***0.01**	0.48
POC	r_s				0.10	0.61	0.42
	p				0.61	***0.00**	***0.02**
Esterase	r_s					0.34	0.61
	p					0.11	***0.00**
Glucosidase	r_s						0.71
	p						***0.00**

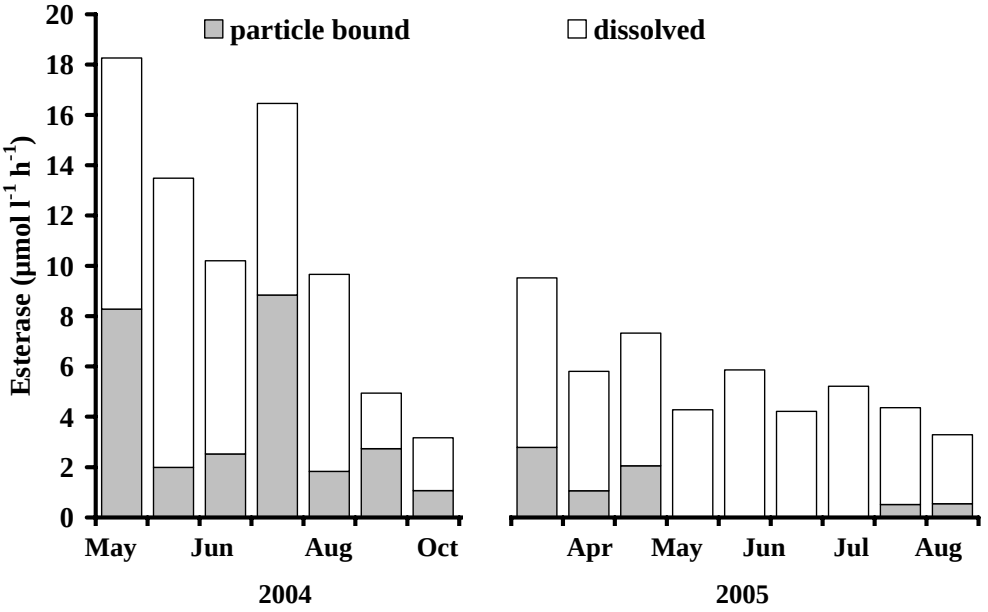


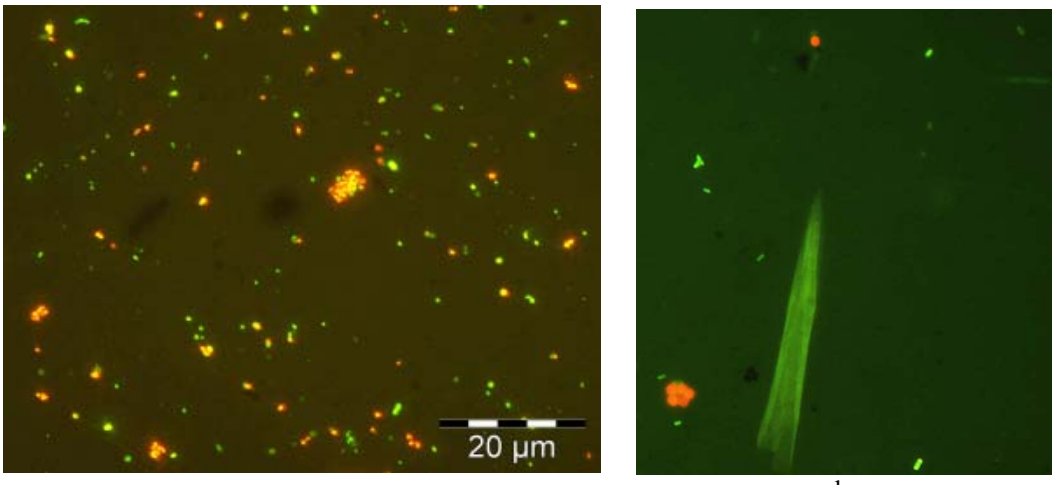
Figure 4. Seasonal development of particulate and free dissolved esterase activity (v_{max}, µmol l^{-1} h^{-1}) in river Warnow during the growth season of 2004 and 2005, 3 weekly sampling intervals, triplicate samples.

Esterase activity was high and mostly free dissolved (Schumann *et al*. 2003b, Figure 4). They can be released by (dying) phytoplankton (Riegman *et al*. 2002, Berman & Wyne 2005) and/or due to resuspension of material from the sediment bacteria. Highest activities of esterase averaged at the Baltic Sea coast. The Baltic Sea coast is a mesotrophic system and had the lowest bacterial biomass. However, bacterial biomass had the highest contribution to POM (Particulate Organic Matter). Esterase activity was well coupled with phytoplankton in river Warnow, which was dominated by diatoms. In contrast to phytoplankton, non significant correlation between esterase and bacterial parameters was observed in river Warnow in 2004/05 (data are not shown) as well as by Hübener *et al*.(1996). The low portion of active bacteria in this system (Figure 5) may have caused an especially low contribution of bacteria to esterase activity, thus, leading to a pronounced phytoplankton impact. In 2004, only 1 to 5% of total bacteria showed intracellular esterase activity and 3 to 15% in

2005. Respiring bacteria varied from 1 to 19% of total number in 2004 and 2 to 9% in 2005. So far, esterase activity is not a good indicator for hydrolytic activity following a high microbial activity or biomass in pelagic systems.

The highest aminopeptidase activities was also observed at the Baltic Sea coast where bacteria had the highest contribution to POM. Aminopeptidase activity was associated with heterotrophic bacteria in most studies of aquatic environments (e.g. Hoppe *et al*. 1998). However, non significant correlations between bacterial abundance and aminopeptidase activitiy were also observed (Caruso & Zaccone 2000, Williams & Jochem 2006).tis study (Table 1)), indicated that aminopeptidase activity was not always associated with bacteria and/or other factors are contributed to the aminopeptidase pool. Variations in the species composition of bacterial assemblages or in the enzyme expression by a single bacterial species (Martinez *et al*. 1996) may explain the uncoupling of the bacterial assemblage from aminopeptidase activity. Many authors reported also an important role of phytoplankton on the aminopeptidase production (e.g. Taylor *et al*. 2003, Caruso *et al*. 2005). The impact of phytoplankton was not so high in the here investigated systems that phytoplankton correlated to aminopeptidase activity. However, the correlation with POC, which is dominated by organism biomass, suggests that phytoplankton as the main planktonic biomass component may still contribute to the total aminopeptidase pool (Table 1). Recently, aminopeptidase activities were also found in photo- and mixotrophic dinoflagellates (Stoecker & Gustafson 2003), heterotrophic nanoflagellates (Mohapatra & Fukami 2004). Under bloom conditions, photothropic dinoflagellates were associated with a significant fraction of peptide hydrolysis (Mulholland *et al*. 2002).

Heterotrophic microorganisms (predominantly bacteria and fungi) are assumed to be the producers of β-glucosidase in waters and sediments of both freshwater and marine environments (e.g. Hoppe 1983, Chróst *et al*. 1989). However, total bacteria were not significantly correlated to the β-glucosidase activity. Additionally, freshwater systems, which had the highest portion of phytoplankton, exhibited the highest activity of β-glucosidase in this study. A strong positive correlation between β-glucosidase and POC (Table 1) suggested the existence of other possible contributors to the β-glucosidase pool in aquatic systems.



a b

Figure 5. Water sample of river Warnow - Rostock stained by CTC for respiring bacteria (crystal orange) (a) and CMFDA for cells with intracellular esterase (green) which are very few (b). Samples were diluted 20x in aquadest steril, 10 min incubation T_{room}, Olympus BX70, blue-yellow-green excitation, camera: Minolta Dynax 505 Si, film material: Fuji Sensia II 200, software: Image Analysis.

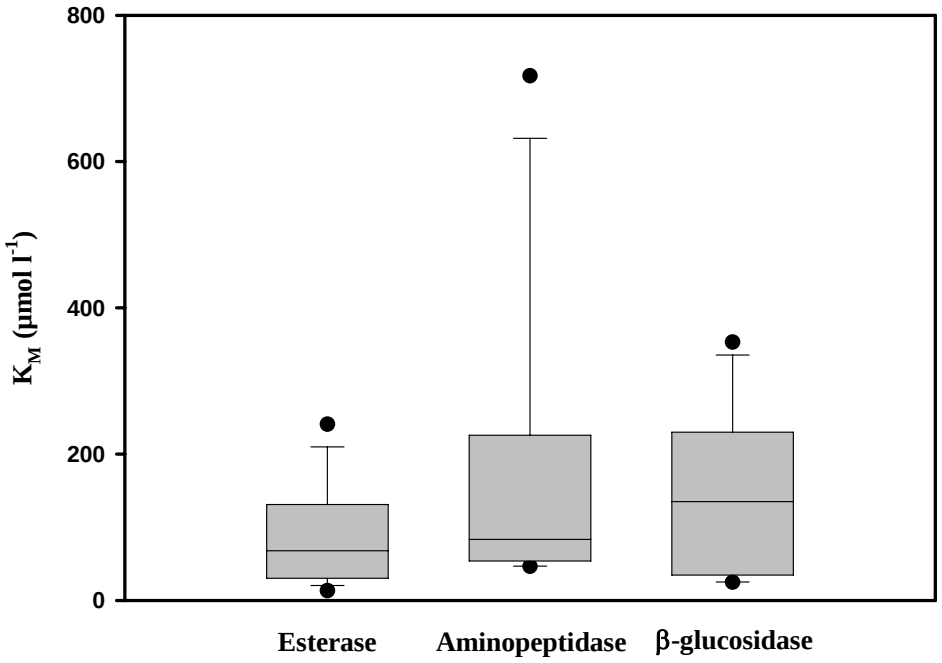


Figure 6. Average concentrations of K_M (µmol l^{-1}) of MUF-acetate for esterase, leucine-AMC for aminopeptidase and MUF-β-glucoside for β-glucosidase of aquatic systems Line in box: median, boxes: interquartile distance, error bars: 5% and 95% percentiles, dots: outliers. Irregularly sampling intervals during August 2003 – August 2005, number of observations: $n_{MUF\text{-}acetate}$=15, $n_{MUF\text{-}\beta\text{-}glucoside}$=18, $n_{leucine\text{-}AMC}$ =15.

Many other planktonic organisms contributed significantly to glycolytic enzymes in aquatic environments, such as phagotrophic flagellates (Vrba *et al*. 1992, Karner *et al*. 1994) and crustacean zooplankton (Bochdansky *et al*. 1995). In alpine lakes, glucosidase correlated weakly with bacteria and phytoplankton, but strongly to dry weight of *Daphnia longispina*. Freely released cladoceran digestive enzymes were believed to be an important part of the extracellular glycolytic activity (Vrba *et al*. 2004). Hydrolases affinities (K_M values) seem to be highly varied from each enzymes (Figure 6). Many high K_M values indicated low enzyme affinity. That is supported by the fact that aminopeptidase saturation was rarely observed (data are not shown), what could have been caused by the overlapping or joint measurement of enzymes with different K_M and V_{max} which may be originated from variety organisms (producers). Thus, apparent K_M is hard to calculate and overestimated.

There seems to be many producers of hydrolytic enzymes in aquatic environments. There is also increasing evidence that bacteria may not be the sole or dominant source of esterases, aminopeptidases and β-glucosidases in aquatic ecosystems. Invertebrate animals, fungi and other eukaryots (diatoms, protozoa etc.) must be considered as possible and even important producer of hydrolytic enzymes. If bacteria are indeed the major producers of hydrolytic enzymes in aquatic environments, several factors influence the correlations of bacteria with hydrolytic enzyme activities further: variations in the species composition of bacterial assemblages with different abilities to express enzymes (1), a wide range variability of hydrolytic enzymes activities influenced directly by substrates (enzyme induction), temperature and other abiotic factors (2), the existence of enzymes caused by other (passive) processes (e.g. cell lysis) because there is no evidence that extracellular enzymes in aquatic environments are actively secreted by intact living microorganisms (3). The low portion of active bacterial cells in this study may also explain the missing correlation between activity and total bacterial counts (4).

CONCLUSION

Investigation in the meso- to eutrophic aquatic systems showed that bacteria were not significantly correlated with the investigated hydrolases. This can be explained by one or a combination of the following causes. Bacteria may not be the sole producers of hydrolytic enzymes (1). It is very likely that substantial hydrolytic activities originate from other organisms (e.g. invertebrates, fungi and other eukaryots (diatoms, protozoa) and therefore, should be accounted as a potential decomposer (2) and other sources (cell lysis and high abundant of free dissolved enzymes) (3). Considering bacteria as major decomposers of organic matter, expanding the investigation to encompass the regulation of hydrolytic activities in species level will be very challenging (constitutive, inducible enzyme as well as their inhibitors and competitors). This will yield new insight into the factor controlling activities per cell. Model species used in the investigation should be representative for the community composition in natural communities. Thus, the development of experimental means to directly link specific measurements of microbial enzymatic activity with the identity of particular organisms is obviously needed. This responsible organism would be a very decided factor effecting not only natural community function but also as crucial decomposer for further application on bioremediation process by microorganisms.

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