ANAEROBIC DIGESTION OF FISH PROCESSING WASTEWATER WITH SPECIAL EMPHASIS ON HYDROLYSIS OF SUSPENDED SOLIDS

Anaerobic Digestion of Fish Processing Wastewater with Special Emphasis on Hydrolysis of Suspended Solids

DISSERTATION
Submitted in fulfilment of the requirements of
the Board of Deans of Wageningen Agricultural University
and the Academic Board of the International Institute for Infrastructural,
Hydraulic and Environmental Engineering for the Degree of DOCTOR
to be defended in public
on Tuesday, 6 April 1999 at 13:30 h in Wageningen

by

ANALIZA PALENZUELA ROLLÓN
born in Paco, Philippines

This dissertation has been approved by the promoters:

Prof. dr ir G. Lettinga	Professor in Anaerobic Treatment Technology and re-use (LUW)
Prof. dr ir G.J. Alaerts	Professor in Sanitary Engineering (IHE)

Co-promoters:

Dr G. Zeeman	Senior scientist, sub-department of Environmental Technology (LUW)
Dr H.J. Lubberding	Senior lecturer, sub-department of Environmental Science (IHE)

Published by
A.A. Balkema, P.O. Box 1675, 3000 BR Rotterdam, Netherlands
Fax: +31.10.4135947; E-mail: balkema@balkema.nl; Internet site: http://www.balkema.nl

A.A. Balkema Publishers, Old Post Road, Brookfield, VT 05036-9704, USA
Fax: 802.276.3837; E-mail: info@ashgate.com

ISBN 90 5410 417 1

Printed in the Netherlands

THESES

belonging to the dissertation:

Anaerobic Digestion of Fish Processing Wastewater with special emphasis on hydrolysis of suspended solids

Analiza Palenzuela Rollon

April 6, 1999
Wageningen / Delft,
The Netherlands

1. A cyclic cause-and-effect relation exists between methanogenesis and lipid hydrolysis: At high lipid levels, lipids inhibit methanogenesis. In the absence of methanogenesis, complete lipid degradation does not occur. Hence, lipids will inhibit their own hydrolysis (this dissertation).

2. The necessary low pH for an effective co-precipitation of fish proteins and lipids cannot be achieved in the first step of a two-step UASB system treating fish wastewater (this dissertation).

3. Based on the nature of LCFAs and the adsorption theory on LCFA inhibition it can be stated that a LCFA only becomes inhibitory when their concentration exceeds their aqueous solubility and the inhibitory effect is greater at higher LCFA levels.
Demeyer D. I. and Henderickx H. K. (1967). The effect of C_{18} unsaturated fatty acids on methane production *in vitro* by mixed rumen bacteria. *Biochem. Biophys. Acta.*, **137**, 484-497.

4. The use of a biodegradability constant for animal manure based on m^3 CH_4 per g VS will result in an overestimation of the predicted methane emission when less pre-acidified manure is involved as compared to that used for the determination of the constant.
Derikx P. J. L., Willers H. C. and ten Have P. J. W. (1994) Effect of pH on the behaviour of volatile compounds in organic manures during dry matter determination. *Bioresource Technology*, **49**, 41-45.

5. It is highly recommendable to consider the application of de-centralised instead of centralised systems for the treatment of industrial wastewater's, although combined industrial wastewater's might become better treatable.
Bozinis N. A., Alexio I. E. and Pistikopoulos E. N. (1996) A mathematical model for the optimal design and operation of anaerobic co-digestion plant. *Wat. Sci. Technol.*, **34**, 383-392.

6. There is so much the anaerobic microbial ecosystem can teach the stubborn human society: e.g., the ability to sustain each other's race.

7. Constant threat makes one an expert on it, else one perils.

8. Living in another country is like opening one's windows. We discover many interesting things about the country's culture and ours.

9. A modern-time expression of disgust is: "The network is down!". On the other hand, "Oh what a relief!".

10. In order to accomplish more human dignity in our society, women instead of men should predominate in ruling positions.

Contents

Acknowledgements

This Ph.D. research was financially supported by the Environmental Ecotechnology Program, a collaboration between the International Institute for Infrastructural, Hydraulic and Environmental Engineering (IHE) and Wageningen Agricultural University (WAU). This program, aiming at developing integrated approaches in water quality management, was designed specifically for conditions and problems of the participating developing countries, Philippines and Uganda. Prof. Dr. Wim van Vierssen played a major role in the conceptualization and initial operation of the program. Dr. Erik de Ruyter van Steveninck coordinated its execution. The research components of this program deal with different aspects of water pollution, for example, the physical and biological effects of pollutants in coastal waters. This dissertation deals with a technique to treat wastewater prior to discharge.

This dissertation would not have been realized without the support of many brains: I would like to express my gratitude to Prof. Dr. Ir. Gatze Lettinga, Prof. Dr. Ir. Guy Alaerts, Dr. Ir. Grietje Zeeman, and Dr. Henk J. Lubberding for their critical and enlightening ideas, encouragement, and constant guidance; and Dr. Wilfredo I. Jose of the Univeristy of the Philippines (UP) for suggesting to work on this topic, assisting me in setting up the laboratory, and for the immediate solutions to my logistic and intellectual queries.

This dissertation also benefitted from the cooperation of many other people: I would like to thank Dr. Miguel D. Fortes of the Marine Science Institute of the UP for the general coordination of the program in the Philippines; Fred Kruis, Ilse Bennehey, Johannes van der Laan, Jo Ackermans Jacobs, Sjoerd Hobma, and Rob Roersma for the chromatographic analyses assisting me in the laboratories, and preparing the needed equipment in the Philippines; Dave van Bergen and Wendy Sanders for helping me with ome of the analytical procedures; and the M.Sc. fellows involved in this project: Wilbert Menkveld, Mark Groeneveld, Agnes Goze, Santos Ome Jr., and Marilou Nabatilan. I also acknowledge Asia Brewery, Inc. for providing me with seed sludge. A number of fish canneries allowed me to sample their wastewaters on conditions of anonimity; many thanks to all of you.

This research benefitted from the facilities of many other institutions: the Department of Chemical Engineering, UP Diliman, where the bulk of the experimental work was done; the Department of Environmental Technology at WAU, IHE Analytical Laboratory, and the Environmental Management Bureau Laboratory (Philippines) for some of the chemical analyses; and the Netherlands Institute of Ecology Center for Limnology for providing my family with a warm home and a working place during the writing of this dissertation. My special thanks to Erik de Ruyter van Steveninck van family: the snow coats, child toys, dinner visits, and many other nice little things all together made our stay in Holland a very pleasant one.

The completion of this dissertation will nt be possible without the invaluable moral support of my parents, Perfecto and Myrna, and my sisters. I (and Rene) specially thank my mother for taking charge of our parental responsibilities during our absence and hardest times. To Rene and Elise, thank you for the inspiration all throughout.

Analiza Mar Palenzuela Rollón
April 1999

Chapter 1

General Introduction: Fish Processing Waste and Anaerobic Treatment

1. Fishery Industry in the Philippines

2. Fish and Aquatic Invertebrate Processing and Waste Generation
    - 2.1. Fish and Aquatic Invertebrate Processing
    - 2.2. Fish Balance
    - 2.3. By-Products and Solid Waste Utilization
    - 2.4. Fish Processing Wastewater Characteristics

3. Fish Processing Wastewater Treatment
    - Fish Processing Wastewater Treatment in the Philippines

4. Lipids and Proteins in Fish
    - 4.1. Fish Lipids
    - 4.2. Fish Proteins and Non-Protein Nitrogenous (NPN) Compounds

5. Anaerobic Digestion
    - 5.1. Biochemical Processes in Anaerobic Treatment of Wastes
    - 5.2. Degradation of Hydrolysis Products from Proteins and Lipids
        - Degradation of Long-Chain Fatty Acids (LCFAs)
        - Degradation of Amino Acids
    - 5.3. Inhibition and Other Problems Caused by Lipids and LCFAs
    - 5.4. Two-phase Anaerobic Treatment Systems
    - 5.5. Inhibition by NH_4^+ and NaCl

6. Hydrolysis of Lipids and Proteins
    - 6.1. Factors Affecting Hydrolysis
        - pH
        - Temperature
        - Oxygen Concentration
        - Seeding and Methanogenesis
    - 6.2. Aspects of Hydrolysis
        - Acidogenic Bacteria
        - Enzyme Production
        - Enzymes and Enzyme Activity
        - Nature of Complex Waste

7. Scope of this Dissertation

1. Fishery Industry in the Philippines

Fishery industry is an important component of the Philippine economy. It provides livelihood to about 5% of the national labor force (Aprieto 1995), contributes about 5% to the gross national products (BFAR Reports 1985-1993, BAS 1992), and provides a major protein source in the Filipino diet (Palomares *et al.* 1986). The annual volume of fish exports increased from 65,000 MT in 1979 to 164,000 MT in 1993, worth 500 million US$ (BAS 1988, BFAR Reports 1985-1993). The Philippines is among the top 15 fish producers in the world (Aprieto 1995). It produces ca. 1.9 million MT finfishes and 0.4 million MT aquatic invertebrates annually (BAS 1993).

Fish processing, which deals with not only finfishes but also aquatic invertebrates, is a major food processing industry in the Philippines. There are about 140 companies engaged in both fish processing and export (DTI 1994). Some of these companies are involved in fish canning, which is a rather new technology in the country. The first fish cannery in the Philippines was established in 1977 (Aprieto 1995). At present, there are about 30 fish canning factories, mostly located in Metro Manila, 15 of which (4 in Metro Manila and Luzon provinces, 4 in the Visayas islands, and 7 in Mindanao) are producers and exporters of canned tuna. These tuna canners, combined, can process at least 100,000 MT raw fish annually (DTI 1989, DTI 1994, Aprieto 1995).

2. Fish and Aquatic Invertebrate Processing and Waste Generation

2.1. Fish and Aquatic Invertebrate Processing

Processing schemes for fishes and aquatic invertebrates, raw materials, source of utility water, and unit processes vary between plants. The processes in other countries for the most commonly canned fishery products, e.g. tuna, salmon, sardines, blue crab, clam, oyster, shrimp, squid, lobster, and catfish are discussed in Edwards *et al.* (1981), Nair (1990), Veiga *et al.* (1994), and Wheaton and Lawson (1985).

In the Philippines, most of the fish processing industries are generally small-scale and situated in coastal areas. The methods of processing at small-scale industries vary between different regions. The common processes are drying, fermenting, canning, and smoking. About 46% of the total fish catch is dried in about 660 plants in the country (Palomares *et al.* 1986). The canning of fish and invertebrates is done by both small- and large-scale industries. The canned fishes are tunas (*Thunnus albacares, Thunnus obesus, Euthynnus pelamis*), sardines (*Sardinella* sp.), mackerel (*Scomber* sp.), milkfish (*Chanos chanos*), and roundscad (*Decapterus macrosoma*)[Aprieto 1995; Palomares *et al.* 1986; survey, this study], while the canned invertebrates include shrimps (*Penaeus* spp.) and squids (*Loligo* spp.). Canned tuna is mainly exported (55,000 MT export in 1993, NSO 1993) while canned sardines and mackerels are mainly for the local market.

The canning operations for tuna in Metro Manila, Philippines, in sequential order (Fig. 1.1-A), are thawing, eviscerating, pre-cooking, cooling, cleaning (removing dark meat, skins, bones, fins, and tails), slicing, manual or mechanical packing in cans, addition of ingredients

such as salt, oil, or sauce, can seaming, cooking by exhausting, washing, labelling and packing. For canning sardines, mackerels, or roundscads, the process scheme (Fig. 1.1-B) is similar to that of tuna except that the thawed fish is cleaned, cut to remove head and tail, eviscerated, washed with brine, and arranged in cans before pre-cooking. These process schemes are similar to those reported by Edwards *et al.* 1981, Nair 1990, and Wheaton and Lawson 1985.

2.2. **Fish Balance**

The finished-product: raw-material ratio in the processing of fishes or invertebrates varies according to species. Hence, the amount of generated solid wastes also varies accordingly. The total waste percentage (i.e. the weight percent of raw fish or invertebrate that is removed during the processing) is 15 for herring; 30 for mackerels, e.g. jack and Pacific mackerels; 30-33 for salmons, e.g. chinook, chum, pink, sockeye, and silver salmons; 40 for tuna species; 45 for sardines; 65 for clams; 75 for mussels or oysters; 80 for shrimps or lobsters; and 73-86 for crabs, e.g. king, blue, or Dungenese crabs (Wheaton and Lawson 1985). In tuna canning, the waste percentages are 10-15% in evisceration, 26-27% in pre-cooking, 18-19% in the removal of dark meat, head and bone, and 1-6% to wastewater stream (Edwards *et al.* 1981). For sardine canning, the waste percentages are about 33% due to fish cutting and evisceration, 18% in pre-cooking, and 4% to wastewater stream (Edwards *et al.* 1981). From these fish balances, the fish weight lost to the wastewater in a factory having a capacity of 100 MT raw fish per day is 1-6 MT per day. This amount is equivalent to a pollution load of 600-4000 kg $COD.d^{-1}$, considering the lipid and protein contents of fish particles.

2.3. **By-Products and Solid Waste Utilization**

Solid wastes in fish processing can be used as raw material for the production of the following:

- protease (Reece 1988),
- oil for pharmaceutical purposes (Pigott 1996),
- lubricants (Pigott 1996),
- protein hydrolysate as animal feed supplement (Vega and Brennan 1988),
- protein extract for culture media preparation (Brett Borup and Fenhaus 1990),
- carotenoid astaxanthin for diet formulation (Chen and Meyers 1982, Raa and Hansen 1983),
- chitin and chitosan (Johnson and Peniston 1982) as coagulants, and
- fish meal as animal feed (Johnson and Gallanger 1984, Milazzo 1983).

In the Philippines, some of these solids are processed into fish meal and fermented fish sauce or paste in either the same fish processing plant or another factory (surveys in fish canneries, this study). The research in the country on fish waste utilization includes

- production of chitin from crustacean shells, cuttlefish, and squid (Aquaculture Watch 1987);
- purification of fish oil for pharmaceutical use (surveys in fish canneries, this study; ITDI-DOST 1993);
- production of fish protein concentrate from the pre-cooking step in tuna canning (ITDI-DOST 1993); and
- production of food flavors from waste shrimp heads and shells (DOST 1990).

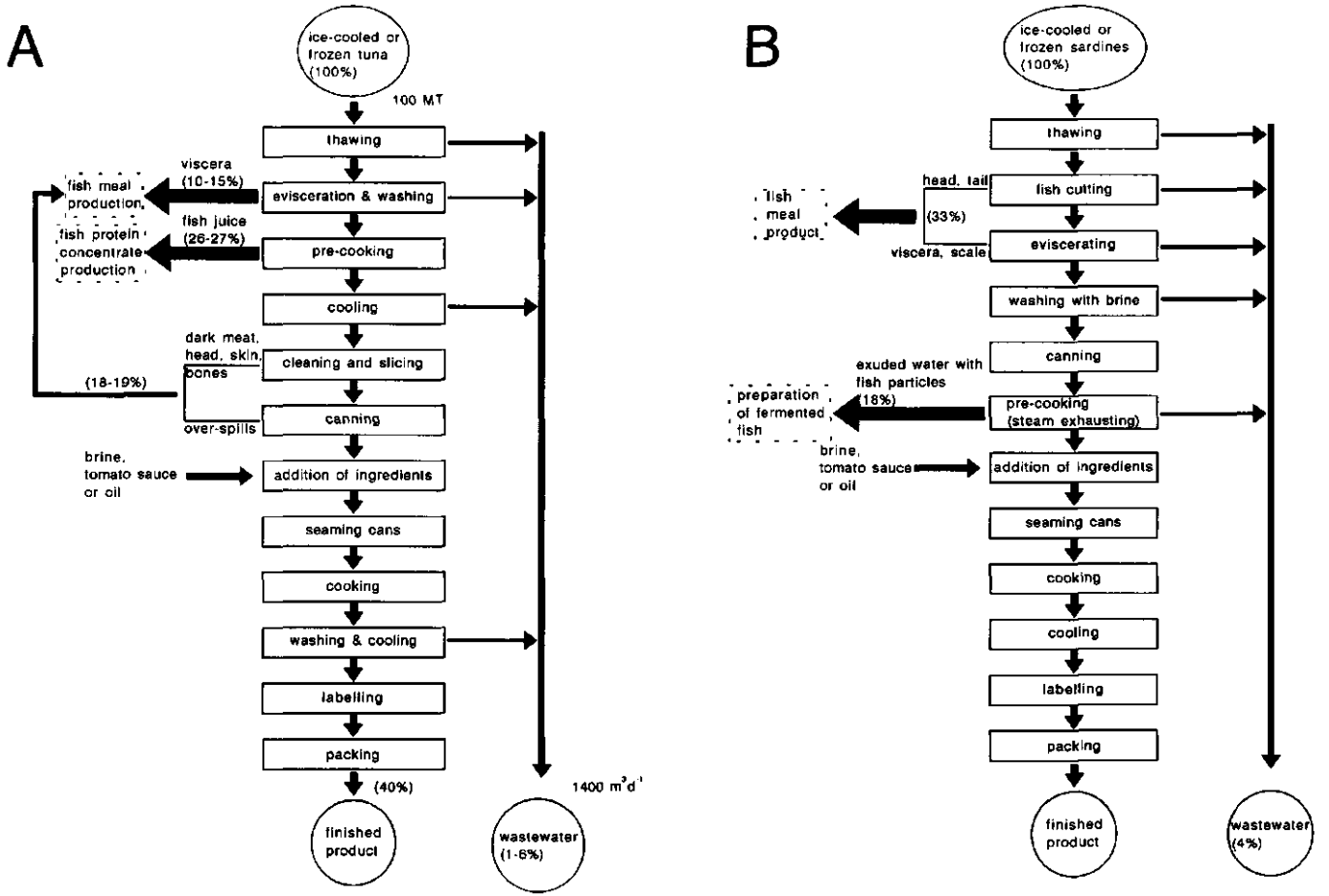

Figure 1.1: Process flow diagrams for tuna (A) and sardine (B) canning. Fish mass balances, shown as weight percent of raw fish accepted for canning.

Table 1.1: Characteristics of fish processing wastewaters

raw fish	Country	EF[a]	Temp. (°C)	pH	BOD $g.l^{-1}$	COD ($g.l^{-1}$)					VSS/TSS $g.l^{-1}$	NH_3-N/TKN $mg.l^{-1}$	PO_4^{3-}-P /P_{total} ($mg.l^{-1}$)	SO_4^{2-}-S $g.l^{-1}$	Cl $g.l^{-1}$
						total	soluble	protein	lipid	carbohydrate					
herring[1]	U.S.A.	nd	nd	nd	1.2-6.0	3-10	nd	nd	1.74-2.32	nd	nd/0.6-5	nd/nd	nd/nd	nd	nd
herring brine[b,2]	Denmark	nd	nd	3.8	78 (87)	90	nd	nd	11.6 (13)	nd	7/10	nd/3000	nd/1000	nd	65
herring/mac.[3]	Netherlands	nd	nd	6.5-7.4	nd	1.8-12.2	0.7-2.62	0.7-2.7	nd	nd	1.5/1.75	70-240/410	nd/nd	nd	0.12-0.31
salmon[1]	U.S.A.	nd	nd	nd	0.25-2.6	0.3-5.5	nd	nd	0.06-1.6	nd	nd/0.12-1.4	nd/nd	nd/nd	nd	nd
sardines[1]	U.S.A.	nd	nd	nd	1.3 (52)	2.5	nd	nd	0.72 (29)	nd	nd/0.92	nd/nd	nd/nd	nd	nd
sardines[a]	Philippines	22	25-26	6.9	7.0 (52)	13.5	1.25 (9)	2.12 (16)	10.1 (75)	nd	4.74/4.89	130/430	25/nd	0.23	0.45
sardines[a]	Philippines	nd	26	6.5	nd	5.8	1.52 (26)	nd	1.7 (30)	nd	nd/1.58	nd/390	nd/nd	nd	nd
tuna/sardine[a]	Philippines	14-20	ca.30	ca. 7	9.0 (64)	14.1	4.0 (28)	nd	5.37 (38)	nd	nd/3.85	nd/400	nd/nd	nd	1.5-2.0
tuna/sardine[a]	Philippines	nd	ca.30	nd	14.5 (57)	25.6	nd	nd	3.5 (14)	nd	nd/11.14	nd/nd	nd/nd	nd	nd
tuna/sardine[a]	Philippines	nd	nd	6.5	nd	6.2	1.8	nd	2.0 (32)	nd	nd/1.69	nd/350	nd/nd	nd	nd
tuna/sardine[4]	Thailand	14-20	nd	6.5-6.8	3.0-4.2 (66)	4.8-6.4	nd	nd	1.16-5.22	nd	nd/0.75-1.4	nd/250	nd/nd	nd	nd
tuna/sardine/mac.[5]	Brazil	nd	nd	6.3-6.4	4.1-6.4 (63)	6.5-10.2	nd	nd	0.35-7.5 (46)	nd	nd/1.7-2.7	44/390	nd/63-93	nd	0.2-1.5
tuna[6]	Spain	nd	45	nd	nd	34.5	nd	26.6 (77)	4.1 (12)	3.8 (11)	3.6/4.0	nd/nd	nd/nd	nd	14.0
tuna[7]	Spain	nd	nd	5.6-6.6	nd	20-53.6	19.7-52.1	18.5-37.5	nd	nd	1.9/2.0	200-6900/nd	600/nd	nd	2.0-9.2
tuna[8]	Thailand	nd	nd	6.4-6.6	0.8-7.5	1.5-10.6	nd	nd	1.92-8.22	nd	1.4-6.7/5-12.4	nd/126-703	nd/32-44	nd	3.3-4.1
tuna[1]	U.S.A.	nd	nd	6.2-7.2	0.71	1.9	nd	nd	0.93 (49)	nd	nd/0.55	6/82	nd/nd	nd	nd
fish meal[9]	Spain	18.3	nd	5.8	nd	55.2	25.8 (47)	23.0 (42)	21.6 (39)	1.87 (3)	16.1/16.6	nd/nd	220/nd	0.54	8.5
fish meal[10]	Spain	nd	nd	6.4	nd	55.4	nd	28.8 (52)	4.35 (8)	1.87 (3)	16.1/16.5	300/nd	200/nd	0.60	7.8
fish meal[c,11]	Denmark	nd	nd	9.3	nd	9.5	nd	nd	nd	nd	0.22/nd	nd/1150	nd	nd	nd
clam[12]	U.S.A.	nd	nd	7.2	1.1 (65)	1.7	1.25 (74)	nd	nd	nd	nd/0.7	62/220	34/40	nd	8.5
clam[1]	U.S.A.	nd	nd	nd	0.5-2.5	0.7-4.0	nd	nd	0.04-0.15	nd	nd/0.2-0.6	nd/nd	nd/nd	nd	nd
crab[1]	U.S.A.	nd	nd	nd	0.27-4.4	0.4-6.3	nd	nd	0.08-1.74	nd	nd/0.06-0.62	nd/nd	nd/nd	nd	nd
mussel[13]	Spain	nd	80-90	4.2-6.2	nd	11.5-26.6	10.0-25.0	2.31-4.65	nd	nd	1.1-1.4/1.1-1.5	120-312/nd	41-120/nd	nd	9.0-15.0
mussel[9]	Spain	nd	nd	6.0	nd	18.5	17.3 (93)	4.9 (26)	nd	12.2 (66)	0.83/1.1	nd/nd	60/nd	2.04	13.5
mussel[6]	Spain	nd	nd	nd	nd	18.5	nd	4.1 (22)	0.74 (4)	13.7 (74)	1.2/1.4	nd/nd	nd/nd	nd	13.0
mussel[14]	Spain	nd	nd	4.2-7.0	nd	1.2-40.0	11.0-38.1	2.25-7.05	nd	8.8-31.2	0.6-2.9/0.7-3.0	nd/nd	50-160/nd	0.7-2.3	8.0-16.0
octopus[9]	Spain	nd	nd	6.3	nd	22.0	19.12 (87)	16.0 (73)	nd	0.9 (4)	1.25/1.51	nd/nd	210/nd	2.5	15.8
shellfish[15]	Sweden	nd	nd	7.2-7.9	0.15-0.63	0.49-0.96	0.28-0.54	nd	nd	nd	1.4-1.8/1.6-2.1	4-11/24-45	4-59/nd	0.1	nd
shellfish[16]	U.S.A.	18.5	nd	7.0	0.31 (78)	0.4	0.36 (90)	nd	nd	nd	0.011/0.012	nd/nd	nd/nd	nd	nd

[1]Wheaton and Lawson 1985; [2]Balsev-Olesen *et al.* 1990; [3]Palenzuela 1993; [4]Nair 1990; [5]Aguiar and Sant'Anna 1988; [6]Mendez *et al.* 1992; [7]Veiga *et al.* 1992; [8]Prasertsan *et al.* 1994; [9]Soto *et al.* 1991; [10]Veiga *et al.* 1994; [11]Sandberg and Ahring 1992; [12]Boardman *et al.* 1995; [13]Lema *et al.* 1987; [14]Soto *et al.* 1993; [15]Pohland and Hudson 1976; [16]Hudson *et al.* 1978; [a]data from 1 sampling, this study; [b]data provided by factory (average), this study; [c]EF-wastewater emission factor (m^3/MT raw fish); [b]combined wastewater is approximately 6-7 times more dilute than the brine; [c]condensate from fish meal plant; mac = mackerel; nd = no data; values in parentheses are percentages of total COD; VSS and TSS = volatile and total SS

2.4. Fish Processing Wastewater Characteristics

The volume and concentration of the wastewater from fish canneries vary between different plants, depending on the composition of the raw fish and additives used, e.g. brine, oil, tomato sauce, the unit processes, and the source of processing water. The volume of wastewater produced in the canning of tuna and sardines is 14-22 m^3 per MT of raw fish (Table 1.1). In the canning of tuna, the wastewater is generated from fish thawing, washing, and eviscerating, cooling and washing of fish and cans after precooking and cooking, and clean-up of working areas (surveys in fish canneries, this study). In some factories, the BOD and COD (biological and chemical oxygen demand, respectively) loads mainly come from the pre-cooking step (Nair 1990). In the Philippines, the fish juice from this step is sent to other factories for the production of protein concentrates and fish sauce.

To characterize (Table 1.1), the main components of fish processing wastewaters are lipids and proteins, except in mussel processing in which carbohydrates comprise a major fraction of the total COD. In most of the other species, carbohydrate COD is very low. The low carbohydrate levels in the wastewater can be expected considering that the raw fish constituents are mainly proteins and lipids. In comparison, the lipid COD in the wastewater from the processing of finfishes is higher than that of invertebrates. The levels of total, soluble, and suspended COD vary largely between factories and fish types. The BOD_5 level is 52-87% of the total COD. The temperature of fish processing wastewaters is hardly reported. Wastewaters from mussel processing plants are at extremely high temperatures, e.g. 80-90°C (Lema *et al.* 1987) because they mainly come from the steam-opening of mussel valves. In the fish processing plants visited in the Philippines, the wastewater is normally at ambient temperatures, 26-30°C. The pH values of the combined wastewater, i.e. the mixture of wastewater streams from different plant operations, are between 4.2 and 7.9 (Table 1.1). In some manufacturing plants, tuna is peeled using sodium hydroxide (Veiga *et al.* 1994). The pH of the effluent from this process is high, e.g. 10.4 (Veiga *et al.* 1994). Fish processing wastewaters also contain sea salts, e.g. Cl^-, Na^+, K^+, Ca^{2+}, and Mg^+, especially when sea water is used in the processing and/or when the raw material contains substantial amount of saline water as in the case of mussels. The ion levels are in the following ranges (in $g.l^{-1}$): 0.45-16.0 Cl^-, 0.087-2.51 SO_4^{2-}-S, 0.004-0.60 PO_4^{3-}-P, and 0.004-6.90 NH_4^+-N. The wastewater from the brining process has an extremely high Cl^- level, e.g. 65 $g.l^{-1}$.

3. Fish Processing Wastewater Treatment

Fish processing wastewaters are generally treated using either physical-chemical methods, biological methods, or a combination thereof. Physical-chemical methods include

- dissolved air flotation, DAF (Brett Borup and Fenhaus 1990, Iggleden and Van Staa 1984, Litchfield 1980, Litchfield 1983), in some cases with addition of polymers and either alum or $CaCl_2$;
- sedimentation with the aid of coagulants or flocculants such as $CaCl_2$, $FeCl_3$, $FeSO_4$, $Fe_2(SO_4)_3$ (Litchfield 1983), carrageenan, alginate (Kitabayashi 1983[a], Kitabayashi 1983[b]), chitosan (Milazzo 1983, Johnson and Gallanger 1984), polyacrylic acid (Hozumi 1988, Nagamine 1986), and alum (Hozumi 1988);

- adjustment of pH to 3-5.9 using hydrochloric acid, in some cases, with heating at 65-80°C to improve the recovery of soluble proteins (Civit *et al.* 1982, Fukuda and Nakatani 1979, Shimizu and Nishioka 1979);
- electrolysis (Litchfield 1980);
- hydrocyclones (Johnson and Lindley 1982); and
- membrane based methods such as ultrafiltration, reverse osmosis, and use of activated carbon filters (McComis and Litchfield 1985, McComis and Litchfield 1986, Miyata 1984, Watanabe 1985, Welsh and Zall 1984).

Sedimentation, DAF, and pH adjustment are usually employed as pre-treatment steps. Since electrolysis, ultrafiltration, reverse osmosis, and use of hydrocyclones are relatively expensive, these methods are used when the recovered fish proteins and oils can be recycled in fish processing or converted to valuable by-products.

In integrated fish wastewater treatment systems, the above physical and chemical methods are employed in combination with biological processes. In some cases, anaerobic and aerobic processes are applied in series (Frankel and Phongsphetraratana 1986, Okubo 1984[a], Okubo 1984[b]). The aerobic treatment methods for fish processing wastewater are activated sludge methods (Guida and Kugelman 1988, Kobayashi *et al.* 1985, Nakamura 1986, Okubo *et al.* 1980), aerated tanks (Aguiar and Sant'Anna 1988, Wheaton *et al.* 1984), and trickling filters (Battistoni *et al.* 1992, Verscharen 1989) while the anaerobic methods applied on laboratory and/or pilot plant scale to fish processing wastewaters are the following:

- Central activity digester (CAD, Mendez *et al.* 1992): a reactor made up of two co-centric cylinders: the influent goes to the inner cylinder which is equipped with a mixer while the effluent flows upward through the outer cylinder.

- Anaerobic filter: a reactor packed with filter media (clay, polystyrene, stone, or polyvinyl chloride) through which wastewater flows upward, e.g., upflow anaerobic filters (UAF or AF, Balsev-Olesen *et al.* 1990, Hudson *et al.* 1978, Lema *et al.* 1987, Prasertsan *et al.* 1994) or downward, e.g., downflow stationary fixed film (DSFF, Veiga *et al.* 1992). The microorganisms in the reactor exist both as attached to the filter media and partly as unattached flocs.

- Upflow anaerobic sludge blanket reactor (UASB, Boardman *et al.* 1995, Sandberg and Ahring 1992): a reactor composed of a digestion zone in which well-settling bacterial aggregates develop and a settling zone in which biogas is collected, sludge separates from the effluent and falls back to the digestion zone.

- Anaerobic fluidized bed (AFB, Balsev-Olesen *et al.* 1990): a reactor where biomass exist both as attached to carrier materials and as unattached flocs, in a bed fluidized by high upflow velocity so that mixing in the reactor is enhanced.

Table 1.2: Studies on anaerobic treatment of fish processing wastewaters

wastewater origin	reactor type	volume (l)	temperature (°C)	pH	total COD ($g.l^{-1}$)	CF ($g.l^{-1}$)	HRT (d)	OLR ($kg\ COD.m^{-3}.d^{-1}$)	% COD methane	% COD removal
shellfish[1]	UAF-shells[a]	18.5	18.5-24	nd	0.407	nd	3.1	0.15	43	81
shellfish[1]	UAF-shells	18.5	18.5-24	nd	0.407	nd	1.6	0.25	61	74
shellfish[1]	UAF-granite	11.9	18.5-24	nd	0.407	nd	2.51	0.18	20	33
shellfish[1]	UAF-granite	11.9	18.5-24	nd	0.407	nd	1.68	0.24	45	55
tuna[2]	DSFF-PVC	15,000	37	7.9-8.0	20-53.6 (27)	2.0-9.2	4	2	nd	75
herring brine[3]	AF-shells	365,000	34-36	6.8-7.3	13	9.3	1.7-2.6	3.3-10	76-78	74-85
herring brine[3]	AFB-quartz	360,000	34-36	6.9	13	9.3	1.5-2.6	3.3-10	80-88	80-88
mussel[4]	UAF-PVC	1	37	6.9-7.4	11.5-26.6	9-15	2.7-17.3	0.76-4.22	nd	85
mussel[4]	UAF-PVC	1	37	6.9-7.4	11.5-26.6	9-15	3.1-4.2	6.42-8.43	nd	79-81
mussel[4]	UAF-PVC	1	37	6.9-7.4	11.5-26.6	9-15	2.2	12	nd	74
mussel[4]	UAF-PVC	1	55	7.1-7.5	11.5-26.6	9-15	3-17.3	0.77-4.34	nd	70-79
mussel[4]	UAF-PVC	1	55	7.1-7.5	11.5-26.6	9-15	2.8-4.0	6.7-9.1	nd	77-84
mussel[4]	UAF-PVC	1	55	7.1-7.5	11.5-26.6	9-15	2.2	12	nd	74
mussel[5]	CAD	15,000	37	nd	16-18	14	5.0	4.2	48	75-85
tuna/mussel[5]	CAD	15,000	37	nd	18-22	14	5.6-7.5	3.2-3.8	64-67	90-95
tuna[5]	CAD	15,000	37	nd	20-25	14	5.0	4.5	67	80
tuna[6]	AF-PVC	3.2	30-35	7-7.3	1.6-50	0.5-4.1	6.6	1.3	nd	65
clam[7]	UASB	1	32	7.1	1.6	8.5	0.125	13.8	ca. 80	83*
fish meal[8]	UASB	4.7	34	7.3-7.9	9.5	nd	0.6	16	nd	92-97

[1]Hudson *et al.* 1978; [2]Veiga *et al.* 1992; [3]Balsev-Olesen 1990; [4]Lema *et al.* 1987; [5]Mendez *et al.* 1992; [6]Prasertsan *et al.* 1994; [7]Boardman *et al.* 1995; [8]Sandberg and Ahring 1992; UAF or AF = upflow anaerobic filter; DSFF = downflow stationary fixed film; AFB = anaerobic fluidized bed; CAD - central activity digester; UASB - upflow anaerobic sludge blanket; [a]filter packing material; HRT = hydraulic retention time; OLR = organic loading rate; *given as percentage removal of soluble COD

The performance of the different anaerobic methods used for fish processing wastewaters are shown in Table 1.2. Over 80% COD removal can be attained at organic loading rates exceeding 6 kg $COD.m^{-3}.d$ in UASB (Sandberg and Ahring 1992), AF, and AFB reactors (Balsev-Olesen *et al.* 1990, Lema *et al.* 1987). However, clogging problems are encountered in operating mesophilic filters at high loading rates (Soto *et al.* 1992). Toxicity effects caused by Na^+ (Soto *et al.* 1993), Cl^- (Soto *et al.* 1991), SO_4^{2-} (Soto *et al.* 1991), and NH_4^+ (Mendez *et al.* 1992) are potential problems in anaerobic treatment of fish processing wastewater. Nevertheless, there are possibilities of overcoming toxicity by sludge adaptation strategies. Ammonium, a potential inhibitor of methanogenesis, is produced during digestion of fish proteins and non-protein nitrogen containing (NPN) compounds.

Fish processing wastewater treatment in the Philippines

The results on wastewater treatment surveys in fish canning factories in Metro Manila are summarized as follows. The employed pre-treatment methods are screening and sedimentation. In some plants, chemical coagulants are used. The floating fats and oils in settling tanks are separated from the wastewater. The wastewater is then treated via trickling filters, aerated ponds, or methane upflow reactors (MUR), i.e. reactors in which sludge from an external settler is recirculated. The wastewater from trickling filters or MURs is post-treated in sedimentation tanks or aerated lagoons.

Table 1.3: Composition of raw fish used for canning (g per 100 g)

Fish	Proteins	Lipids	Carbohydrates	Ash	Water
Finfishes:					
Herrings	16 - 21 (18)[@]	5 - 14 (10)	0	1.3 - 2.4	67 - 72 (70)
Mackerels	19 - 21 (20)	5 - 14 (9)	0 - 5 (2.5)	1.2 -2.4	64 - 67 (66)
Salmons	20 - 29 (22)	3 - 10 (8)	0 - 3 (1.5)	1.2 - 1.7	60 - 72 (69)
Sardines	18 - 20 (19)	4 - 16 (9)	0	3	65 - 71 (69)
Tunas	23 - 25 (24)	1 - 7 (4)	0 - 4 (2)	1.2 - 1.7	67 - 71 (69)
Invertebrates:					
Clams	8 - 14 (11)	0.8 - 2.7 (1.3)	2 - 5.1 (2.8)	0.1 - 2.2	82 - 85 (83)
Crabs	15 - 17 (16)	0.8 - 1.3 (1.1)	0 - 0.6 (0.4)	0.5 - 1.9	80 - 83 (81)
Mussel[~]	12	1.7	4.5	2.2	80
Oysters	8 - 11 (10)	1.6 - 2.1 (1.8)	4.8 - 5.8 (5.2)	1.5 - 2.2	80 - 84 (82)
Shrimps	19 - 22 (20)	0.3 - 0.8 (0.6)	0	1.4 - 2.7	76 - 79 (77)
Scallops	15 - 17 (16)	0.4 - 0.5 (0.6)	2.6 - 2.9 (2.8)	1.5 - 2.6	78 - 80 (79)
Squids	13 - 17 (15)	0.7 - 1.4 (1.1)	0.6 - 6 (3.8)	1.0 - 2.6	78 - 80 (79)

[@]Values in parentheses are averages between different species: round, Atlantic, and Pacific herrings; Atlantic, chub, horse, and Japanese horse mackerels; Atlantic, chinook, chum, pink, sockeye, and silver salmons; Adriatic, Spanish, and Indian oil sardines; albacore, bluefin, skipjack, and yellowfin tunas; bean, surf, razor, and softshell clams; king, blue, and Dunganese crabs; [~]blue mussel; common, Eastern, and Pacific oysters; brown, pink, and white shrimps; bay and sea scallops; longfinned and Pacific squids. Source: Kinsella 1987, Krvaric-Skare 1955, Spotte 1992, and Suzuki 1981

4. Lipids and Proteins in Fish

Lipid and protein contents in fishes and invertebrates depend on species, size, age, their food conditions, their reproductive stage, i.e. whether they are harvested at pre- or post- spawning season, and part of the organism (Sikorski 1994[a], Suzuki 1981). The composition of the wastewater generated in the processing of fishes and invertebrates is expected to vary due to these factors as well. The finfishes usually canned are composed of 16-25% proteins, 1-16% lipids, 0-5% carbohydrates, 1.2-3% inorganic substances, and 60-72% water (Table 1.3). Sardines, herrings, salmons, and mackerels, are among the fish groups that contain relatively high percentage lipids. The invertebrates contain less lipids (0.3-2.7%) and more water (76-85%).

Lipids

Lipids are normally extracted from biological tissues with non-polar solvents. They comprise a wide range of macromolecules of different chemical structures, from the simplest fats and oils to the more complex phospholipids. Fixed oils and fats are esters of glycerol and fatty acids, e.g. mono-, di- and triglycerides. Phospholipids are esters of glycerol in combination with fatty acids, phosphoric acid, and nitrogenous compounds, usually choline or ethanolamine. The more complex structures known as lipopolysaccharides and lipoproteins are lipids covalently bound to polysaccharides and proteins, respectively.

The most commonly occurring lipids in nature are the triglycerides, also known as triacylglycerols or neutral fats. A triglyceride is composed of three fatty acids linked by ester bonds to the hydroxyl groups of glycerol (Fig. 1.2). Fatty acids are composed of long, mostly even-numbered chains of 12 to 24 carbon atoms, ending in a carboxyl group. They vary in both chain length and degree of saturation of the alkyl chain. Saturated fatty acids contain only single bonds within the alkyl chain, while unsaturated fatty acids contain at least one double bond. The more unsaturated fatty acids are those that contain more double bonds. The fatty acid components of a triglyceride render the lipid its characteristics. The fluidity of a lipid, hence its melting point, is determined largely by its degree of unsaturation, i.e. the number of double bonds per unit mass. Fish lipids are more unsaturated than those of mammalian fats (Spotte 1992).

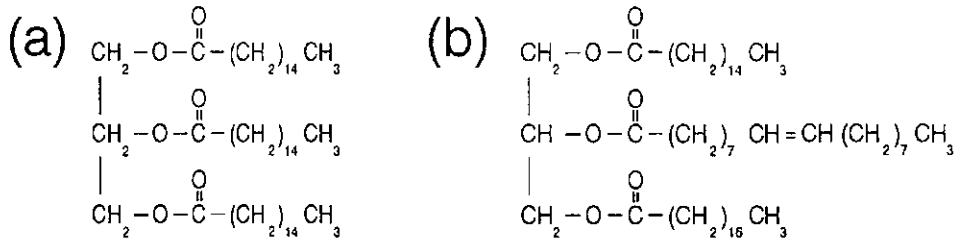

Figure 1.2: Examples of triglycerides: (a) tripalmityl glyceride, (b) 1-palmityl,2-oleyl,3-stearyl glyceride

Proteins

Proteins are biopolymers of amino acids that are linked by peptide bonds, the amino group of one interacting with the carboxyl group of another (Fig. 1.3). They have an enormous variety of structure since they have four orders of structure: primary - the amino acid sequence; secondary - the regular arrangement maintained by H-bonding; tertiary - overall folding of the molecule due to noncovalent forces, hydrophobic and ionic interactions, and H-bonding; and quarternary - the association of several identical or non-identical chains. Proteins have ionizable groups: amino acid side chain, the N- and C- termini, and sometimes other attached groups. The charge of each particular group, and hence, the net charge of a protein molecule, depends on the pK_a values of the ionizable groups in relation to the pH of the solution. At low pH, there are more positively-charged groups than negatively-charged groups, while at high pH, the negatively-charged groups predominate. The pH at which there are equal numbers of positively- and negatively-charged groups, is known as the isoelectric point. As the charge repulsion between protein molecules is least at the isoelectric point, proteins are least soluble at this pH. Isoelectric point varies between proteins.

Besides pH, structure and ionic strength also affect the solubility of proteins. Globular proteins are generally soluble in water or dilute salt solution but are less soluble in strong salt solution, while fibrous proteins are generally water-insoluble. NaCl enhances protein solubility while most salts cause proteins to precipitate at ionic levels higher than 0.15 M (Franks 1993).

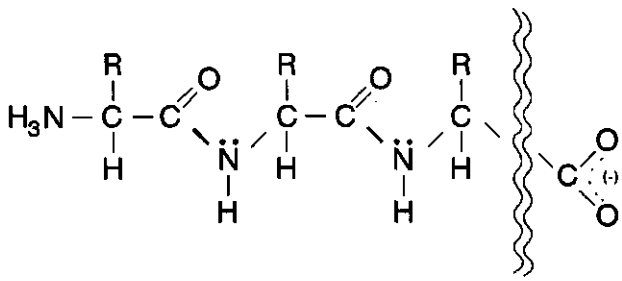

Figure 1.3: Illustration of a protein molecule: a chain of amino acids linked by amide bonds, i.e., the peptide bonds (--), between the carboxyl group of one amino acid and the α-amino group of another. R is an alkyl, an aryl, or a heterocyclic group

The natural, biological conformation of a protein is known as its native state. It is normally the most stable conformation into which the protein molecule spontaneously folds. Proteins can be denatured, i.e., unfolded or altered, under extreme pH conditions, high temperatures, and presence of denaturing agents such as, concentrated urea, guanidine hydrochloride, ionic detergents, and organic solvents. The denaturing conditions weaken the non-covalent bonds that maintain the native tertiary structure, and stabilize a different denatured conformation. Denaturation may be reversible or irreversible.

Table 1.4: Long-chain fatty acid composition of fish lipids (g/100 g lipids)

Common name	Abbre-viation	Atlantic Herring	Pacific Herring	Atlantic Mackerel	Chub Mackerel	Horse Mackerel	Menhaden	Atlantic Salmon	Chinook Salmon	Chum Salmon	Pink Salmon	Sockeye Salmon	Albacore Tuna	Bluefin Tuna	Skipjack Tuna	Yellowfin Tuna
Lauric	12:0	nd	nd	0.1	nd	nd	nd	nd	nd	nd	0.0	nd	0.0	0.0	nd	nd
Myristic	14:0	nd	nd	5.5	nd	nd	8.0	nd	nd	nd	3.4	nd	3.7	4.5	nd	nd
Pentadecanoic	15:0	nd	nd	0.5	nd	nd	0.5	nd	nd	nd	1.0	nd	1.0	0.6	nd	nd
Palmitic	16:0	nd	nd	17.3	nd	nd	28.9	nd	nd	nd	10.2	nd	29.3	22.1	nd	nd
Margaric	17:0	nd	nd	0.3	nd	nd	1.0	nd	nd	nd	1.6	nd	1.2	0.8	nd	nd
Stearic	18:0	nd	nd	3.3	nd	nd	4.0	nd	nd	nd	4.4	nd	6.1	6.2	nd	nd
Myristoleic	14:1	0.5	0.4	0	0.0	0.7	nd	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0
Palmitoleic	16:1	7.5	8.3	5.6	5.3	7.8	7.9	4.4	9.2	7.8	5.0	6.7	5.0	2.8	4.1	3.5
Oleic	18:1n-9	18.3	22.7	14.2	17.4	14.4	13.4	23.5	29.1	25.7	17.6	17.6	17.9	21.7	15.1	15.1
Gadoleic	20:1n-9	8.7	10.6	8.8	6.8	6.5	0.9	3.9	4.7	5.5	4.0	17.0	2.8	6.3	2.0	1.0
Erucic	22:1n-9	10.1	11.7	12.8	9.2	7.8	1.7	5.1	3.6	9.5	3.5	10.8	1.6	5.4	0.7	2.0
Linoleic	18:2n-6	1.6	1.5	1.6	1.9	1.3	1.1	3.0	1.1	1.9	1.6	4.9	2.0	0.8	1.8	1.1
Linolenic	18:3n-3	1.2	0.4	0.9	0.3	1.0	0.9	5.1	0.9	1.0	1.1	1.2	3.5	0.0	0.8	0.0
Moroctic	18:4n-3	2.6	1.9	1.6	2.6	1.0	1.9	1.5	1.5	2.0	2.9	1.3	1.9	0.9	0.5	0.7
Arachidonic	20:4n-6	0.7	0.7	nd	1.1	0.8	1.2	4.6	1.6	0.9	0.7	1.2	2.2	1.0	3.0	3.8
Timnodonic	20:5n-3	8.6	7.6	6.5	6.6	9.4	10.2	5.6	8.2	7.2	13.5	6.6	7.7	6.4	8.2	5.1
Clupanodonic	22:5n-3	0.7	1.3	1.7	1.3	2.6	1.6	5.0	2.4	2.3	3.1	0.5	0.6	1.4	1.5	1.8
Docosahexaenoic	22:6n-3	10.4	5.4	12.9	13.4	7.8	12.8	19.4	5.9	9.2	18.9	8.3	22.5	17.1	21.3	25.1
saturated		24.6	25.4	27.0	28.9	32.0	42.4	17.1	26.1	25.3	20.6	19.1	27.9	34.2	37.7	32.6
monounsaturated		45.1	53.7	41.4	38.8	37.2	23.9	36.9	46.6	48.4	30.1	52.6	27.3	36.2	21.9	21.5
polyunsaturated		25.7	18.9	25.2	27.1	23.9	29.7	44.2	21.6	24.5	41.8	24.0	40.4	27.6	37.0	37.6

Source: Kinsella 1987; Spotte 1992; nd = no data

Table 1.5: Amino acid composition of fish proteins (g/100 g fish protein)

Fish	Ala	Arg	Asp	Cys.	Glu	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Trp	Tyr	Val
Finfishes:																		
Capelin[a]	5.6	6.0	8.9	1.4	13.2	5.3	2.4	4.7	8.2	8.5	3.1	3.8	3.7	4.2	4.3	1.1	3.3	5.7
Cod[b]	5.9	6.2	10.5	0.8	19.9	3.1	1.9	6.1	9.4	9.8	2.8	3.5	2.7	3.9	4.0	0.7	2.5	4.8
Atlantic herring[c]	nd	1.2	nd	6.2	nd	nd	2.7	5.1	9.6	8.6	2.0	4.7	nd	nd	5.3	11.0	4.1	6.0
Pacific herring[c]	nd	5.5	nd	1.1	nd	nd	2.2	4.8	7.4	8.4	2.7	3.9	nd	nd	4.4	0.9	nd	5.2
Atlantic mackerel[c]	nd	5.6	nd	nd	nd	nd	4.0	4.7	7.3	8.2	2.8	3.3	nd	nd	4.7	0.9	nd	5.2
Chub mackerel[c]	nd	6.0	nd	1.1	nd	nd	3.2	6.8	7.7	9.2	3.0	4.4	nd	nd	5.0	1.2	3.9	7.1
Yellowfin tuna[c]	nd	5.5	nd	1.2	nd	nd	5.3	4.0	6.2	8.5	2.6	3.1	nd	nd	3.5	1.7	4.2	7.2
Sardine[d]	6.6	6.2	10.1	nd	13.3	5.1	2.3	5.4	8.4	9.9	3.3	4.2	3.6	4.6	5.1	1.2	4.0	6.7
Invertebrates:																		
Blue crab[c]	nd	8.6	nd	1.5	nd	nd	1.8	4.1	7.0	7.3	2.4	3.5	nd	nd	3.7	1.3	3.3	4.3
Dungenese crab[c]	nd	11.0	nd	0.6	nd	nd	2.2	3.5	5.9	8.5	2.5	3.2	nd	nd	3.6	1.8	2.9	4.8
White shrimp[c]	nd	8.0	nd	1.8	nd	nd	2.6	3.8	7.5	7.8	2.7	3.6	nd	nd	3.3	1.2	4.0	1.3
Brown shrimp[c]	nd	9.5	nd	1.2	nd	nd	1.9	4.3	7.7	7.1	2.3	3.3	nd	nd	3.6	1.4	2.4	4.4
Eastern oyster[c]	nd	6.0	nd	nd	nd	nd	1.9	3.5	4.0	5.8	2.0	3.3	nd	nd	4.2	9.9	3.8	6.3
Pacific oyster[c]	nd	6.2	nd	1.4	nd	nd	2.0	4.9	6.7	6.0	2.2	3.5	nd	nd	3.8	0.8	2.8	4.3
Longfinned squid[c]	nd	5.7	nd	nd	nd	nd	1.5	3.7	6.9	7.2	2.3	2.7	nd	nd	3.5	10.7	1.8	3.0
Pacific squid[c]	nd	6.3	nd	nd	nd	nd	2.5	3.7	6.9	7.5	2.2	2.7	nd	nd	3.7	nd	1.8	3.3

[a]Shahidi 1994; [b]Suzuki 1981; [c]Spotte 1992; [d]Amano 1962: nd = no data

4.1. **Fish Lipids**

The majority of lipids in fish are triglycerides (Kinsella 1987). Less than 1% of the lipids are phospholipids and are associated with the cell structure of fish tissues (Kinsella 1987). The fatty acid residues in lipids of different fish species are shown in Table 1.4. The main fatty acid components are palmitic, oleic, gadoleic, erucic, timnodonic, and docosahexaenoic acids. Most of the fatty acids are unsaturated. The average percentages of saturated, monounsaturated, and polyunsaturated fatty acids are 29, 40, and 31, respectively.

4.2. **Fish Proteins and Non-Protein Nitrogenous (NPN) Compounds**

The muscles of fish contain three main classes of proteins: sarcoplasmic, myofibrillar, and stroma which are 18-25%, 70-79%, and 3-5%, respectively, of the total amount of proteins in fish (Suzuki 1981). Sarcoplasmic proteins are soluble in water or dilute salt solutions (Suzuki 1981, Sikorski 1994[a]) while myofibrillar proteins are generally non-water soluble and can be extracted only using stronger salt solutions. Stroma proteins are those that form connective tissues and cannot be extracted by water, acid, alkaline solution, or neutral 0.01-0.1 M salt solution. The proportions of these three classes of proteins vary between species.The sarcoplasmic proteins in fish are (1) myoglobin, a globular heme protein having a molecular weight of about 18 kDa, (2) enzymes of physiological functions in fish, (3) anti-freeze proteins, e.g., in fish of the Arctic and Antarctic waters, and (4) hydrolytic enzymes, e.g. lipases, proteinases, and polysaccharide-degrading enzymes in the digestive organs and tissues of fish (Haard *et al.* 1994). The main myofibrillar proteins in fish are myosin, actin, tropomyosin, and troponins (Suzuki 1981, Sikorski 1994[b]). The main amino acid component of fish proteins are glutamate, aspartate, lysine, and leucine (Table 1.5). The fraction of each amino acid in fish proteins, except tryptophan, does not vary much between species.

The protein content in foods is generally given as 6.25x(total N). This value includes both protein and non-protein nitrogenous (NPN) compounds, and is therefore sometimes referred to as 'crude protein'. The 'true' protein-to-nitrogen ratio, with no correction for the non-protein N in the total N, in fish is 5.72-5.82 (Sikorski 1994[a]). The NPN components in seafoods are free amino acids, peptides, guanidino compounds, urea, betaines, nucleotides, and quaternary ammonium compounds (Haard *et al.* 1994, Sikorski 1994[a]). Free amino acids in aquatic organisms contribute to osmoregulation (Haard *et al.* 1994). Some unique free amino acids in seafoods are taurine, sarcosine, β-alanine, methyl-histidine, and α-amino-*n*-butyric acid. The NPN fraction of the total N in marine animals varies between species: 16-18% in tuna, sardines, and mackerel, and 20-25% in molluscs and crustaceans, e.g., squids, clams, and abalones (Haard *et al.* 1994, Sikorski 1994[a]). Free amino acids comprise 50-65% of the NPN compounds in shrimp and squids while ca. 25% of those in mackerel. Betaines are especially present in molluscs and crustaceans, e.g. 5% and 10% of the NPN compounds in squid and shrimps, respectively.

5. Anaerobic Digestion

Anaerobic digestion is biodegradation in the absence of oxygen (O_2) resulting in the formation of CH_4 and CO_2 when ions like NO_3^- and SO_4^{2-} are absent. Its advantages over the aerobic method include (1) no or little net energy consumption because of the production of methane which can serve part of the energy requirement of the treatment plant, (2) lower excess sludge production, (3) more dewaterable and stabilized excess sludge, and (4) possible recovery of useful products, e.g. NH_3 and sulphur, via post-treatment (Lettinga and Hulshoff Pol 1991, Verstraete *et al.* 1996). Significant research has broadened the knowledge on the technology and hence lessened the presumed drawbacks in employing the process. The stability of the process is improved with a better understanding of the design, operation, and control of anaerobic reactors (see Hulshoff Pol and Lettinga 1986, Lettinga *et al.* 1983, Lettinga *et al.* 1984, Lettinga *et al.* 1987, Lettinga and Hulshoff Pol 1991). The emergence of high-rate systems which allow efficient treatment at relatively high COD loading rates is a major breakthrough in the technology. At present, the UASB reactor is the most widely applied anaerobic treatment (Verstraete *et al.* 1996) for wastewaters containing organic matters. A high concentration of active biomass is maintained in the UASB reactor, as aggregates of sludge, usually granular, develop because of a selection process effected by the gas and hydraulic surface loads. With a gas-liquid-solid (GLS) separation device, biogas is recovered and solids are separated from the effluent and retained in the reactor because they return to the digestion compartment of the reactor.

The possible application of an anaerobic method for the treatment of wastewaters with high levels of proteins and lipids, such as fish processing wastewater, is interesting because the amount of theoretically recoverable methane from the digestion of these compounds is high. Moreover, anaerobic waste treatment comprises a low-cost method, and, in view of its possible other advantages over conventional aerobic methods, it represents an attractive option for many industries. A relevant background on anaerobic treatment of these types of wastes is presented in the following sections.

5.1. Biochemical Processes in Anaerobic Treatment of Wastes

The anaerobic digestion of complex wastes, such as those involving particulate or suspended matter, comprises a series of sequential biochemical processes (Fig. 1.4). Organic polymers are firstly hydrolysed into smaller subunits that can be assimilated by bacterial cells. This process is catalyzed by extracellular enzymes from acidogenic bacteria, e.g. lipids are broken down to long-chain fatty acids (LCFAs) by lipases; proteins to amino acids by protease and peptidases; cellulose to polysaccharides by cellulases; and polysaccharides to sugar monomers. The monomers or smaller molecules from hydrolysis are converted by acidogenic bacteria into short- or branched-chain fatty acids, alcohols, lactic acids, CO_2, H_2, and NH_3. The fatty acids, including LCFAs from lipid hydrolysis, and other products of acidogenesis are further converted into acetate, CO_2, and H_2. Finally, methane is formed via two types of reaction: hydrogenotrophic ($4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$) and acetotrophic ($CH_3COOH \rightarrow CH_4 + CO_2$). The reactions in the series are carried out by different groups of bacteria, some of which coexist in syntrophic relations, i.e., the product of one species assimilated by another group of bacteria. For instance, interspecies hydrogen transfer between the obligate H_2-producing acetogens and the H_2-consuming methanogens is crucial for the degradation of short- and long-chain fatty acids.

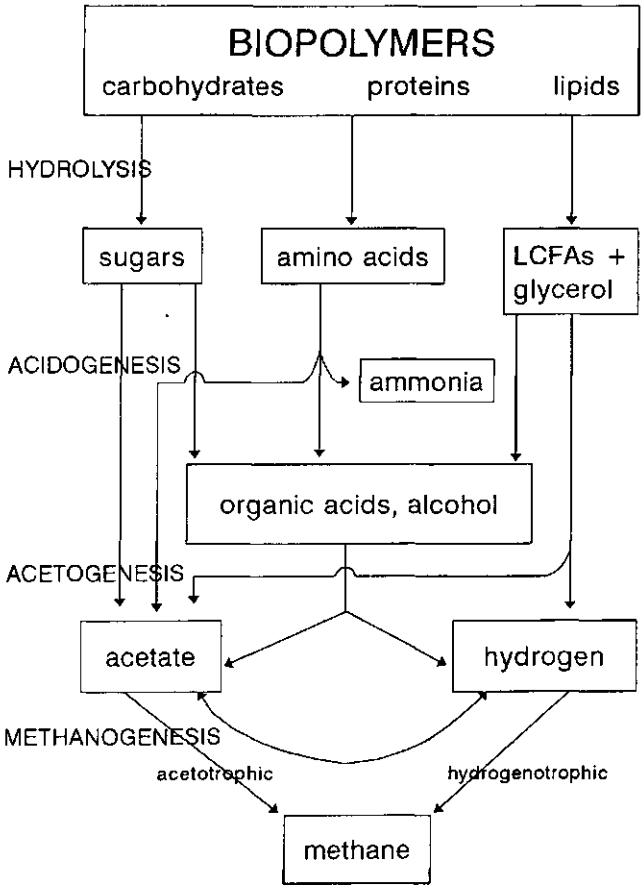

Figure 1.4: Conversion steps in anaerobic degradation of complex wastes (adapted from Gujer and Zehnder 1983)

Hydrolysis is generally the rate-limiting step in the overall anaerobic process (Eastman and Ferguson 1981, Ghosh 1987, Sayed *et al.* 1988, Pavlostathis and Giraldo-Gomez 1991, Pavlostathis and Gossett 1988). It is considered as first-order (Eqn. 1.1) with respect to the concentration of biodegradable solids (Eastman and Ferguson 1981, McInerney 1988, Pavlostathis and Giraldo-Gomez 1991). The individual rates of the succeeding conversion processes generally fit Monod kinetics as shown in Eqn. 1.2 (Pavlostathis and Giraldo-Gomez 1991).

For each type of biopolymer, the range in the reported values of the hydrolysis first-order rate constant, k_h, is rather wide: 0.04-2.88 d^{-1} for cellulose and cellulose-containing materials, 0.02-0.69 d^{-1} for different proteins and nitrogenous compounds, and 0.08-1.7 d^{-1} for lipids (Pavlostathis and Giraldo-Gomez 1991). These variations in the literature values of k_h within each type of waste is due to differences between studies in the culture used, pH and temperature conditions, the origin of the material, and the considered biodegradable fraction of the waste.

Eqn. 1.1. First-order hydrolysis rate equation

$$-dF/dt = k_h F$$

where F = concentration of insoluble substrate, $M.L^{-3}$
k_h = first-order hydrolysis rate constant, θ^{-1}
t = time, θ

Eqn. 1.2. Monod kinetics

$$\mu = \mu_m S/(K_s+S) - b; \qquad -dS/dt = X\mu_m/Y$$

where μ = specific growth rate of microorganism, θ^{-1}
μ_m = maximum specific growth rate of microorganism, θ^{-1}
K_s = half-saturation constant, $M.L^{-3}$
S = substrate concentration, $M.L^{-3}$
X = microorganism concentration, $M.L^{-3}$
Y = growth yield coefficient $M.M^{-1}$
b = specific decay rate of microorganism, θ^{-1}
θ, M, L^3 ≡ units of time, mass, and volume, respectively

5.2. **Degradation of Hydrolysis Products from Proteins and Lipids**

Degradation of LCFAs

LCFA degradation proceeds via β-oxidation (Angelidaki and Ahring 1995, Hanaki *et al.* 1981, Heukelekian and Mueller 1958, Novak and Carlson 1970, Weng and Jeris 1976), i.e. the sequential removal of 2 carbon atoms from the LCFA via oxidation at the β-carbon. The products are acetate and hydrogen from even-carbon LCFAs and, in addition, propionate from odd-carbon LCFAs. Unsaturated LCFAs undergo hydrogenation before β-oxidation (Heukelekian and Mueller 1958). In anaerobic digestion of saturated and unsaturated LCFAs, β-oxidation is the rate-limiting step (Heukelekian and Mueller 1958, Novak and Carlson 1970, Rinzema *et al.* 1994). The rate of degradation of saturated LCFAs decreases with increasing chain length (Loehr and Roth 1968, Novak and Carlson 1970). Unsaturated LCFAs are more rapidly degraded than the corresponding saturated LCFAs (Loehr and Roth 1968).

As in the degradation of shorter chain fatty acids, the conversion of LCFAs also requires an efficient removal of H_2 since it is thermodynamically feasible only at very low H_2 partial pressure, e.g. lower than 10^{-4} atm. Thus, the degradation of LCFAs depends on methane formation. Hence, the H_2-producing acetogenic bacteria that carry out the β-oxidation normally grow in syntrophic co-culture with H_2-consuming methanogens (Angelidaki and Ahring 1995, Roy *et al.* 1986). Examples of LCFA degraders are *Syntrophomonas sapovorans*, *Syntrophomonas wolfei*, and *Thermosyntropha lipolytica* (Hwu 1997).

Degradation of amino acids

Amino acids from protein hydrolysis are degraded via oxidation-reduction reactions performed mainly by different *Clostridium* species, producing mainly short- and branched-

chain fatty acids, NH_4^+, S^{2-}, CO_2, and in some pathways, H_2 (McInerney 1988, Barker 1961, Barker 1981). In anaerobic reductive reactions, the electron acceptors are amino acids, α- and β-keto acids, α, β-unsaturated acids or their coenzyme A derivatives, and protons.

Most of the proteolytic *Clostridium* species carry out Stickland reactions, i.e., electron transfer between two amino acids, in which alanine, histidine, or valine act as an electron donor while arginine, glycine, proline, hydroxyproline, or ornithine act as an electron acceptor. Leucine, isoleucine, phenylalanine, tryptophan, and tyrosine can act both as an electron acceptor or donor depending on the other amino acids present. Branched-chain and aromatic amino acids are oxidatively deaminated and decarboxylated to the corresponding fatty acids, e.g., alanine to acetate, valine to isobutyrate, isoleucine to 2-methylbutyrate, leucine to isovalerate, tyrosine to hydroxyphenylacetate, phenylalanine to phenylacetate, tryptophan to indoleacetate. Some of these oxidative reactions are coupled to the reduction of some amino acids in the Stickland reaction. The other degradation products of aromatic amino acids are phenylpropionate from phenylalanine; hydroxyphenyllactate, hydroxyphenylpropionate, phenol, and p-cresol from tyrosine; indole and indolepropionate from tryptophan. The aromatic ring in amino acids is not altered by acidogenic bacteria but by H_2-producing syntrophs or sulfate-reducing bacteria (McInerney 1988). Individual amino acids are degraded via specific pathways, e.g., ornithine from arginine; propionate and n-butyrate from threonine; acetate from glycine via serine and pyruvate; glutamate to acetate, butyrate, and H_2 via methylaspartate or hydroxyglutarate pathway; acetate and propionate from alanine via the acrylate pathway; acetate and succinate from aspartate; pyruvate and S^{2-} from cystein; formamide and glutamate from histidine; and pyruvate from serine.

Some amino acids can be degraded via H_2-producing reactions. Whether the H_2-producing pathway will prevail or not over alternative degradation reactions would be dependent on the H_2 partial pressure. The results of Nanninga and Gottschal (1985), Örlygsson *et al.* (1994), Stams and Hansen (1984) show the importance of interspecies H_2 transfer in the degradation of some amino acids, e.g. alanine, phenylalanine, methionine, valine, leucine, isoleucine, serine, glutamate, and aspartate. The degradation of these amino acids is enhanced by the presence of methanogenic or sulfate-reducing bacteria.

5.3. **Inhibition and Other Problems Caused by Lipids and LCFAs**

Lipids are present in many wastewaters: fish processing wastewater, sewage, wastewaters from slaughterhouses, meat processing (Borja *et al.* 1995, Sayed *et al.* 1987), dairy industry (Öztürk *et al.* 1993, Perle *et al.* 1995), ice-cream factory (Hawkes *et al.* 1995), cafeteria (Hanaki *et al.* 1990), palm oil mill (Borja *et al.* 1996), olive oil mill (Beccari *et al.* 1996, Boari *et al.* 1993, Tsonis and Grigoropoulos 1993), and wool scouring (Mercz and Cord-Ruwisch 1997). Among the problems encountered in the anaerobic treatment of these wastewaters are sludge flotation resulting to washout due to the strong tendency of lipids to adhere on surfaces, reduction in sludge methanogenic activity, and as a result, reduction in methane conversion efficiency. The inhibitory effect of lipids is commonly attributed to the LCFAs from lipid hydrolysis (Angelidaki *et al.* 1990, Angelidaki and Ahring 1992, Hanaki *et al.* 1981, Perle *et al.* 1995). Neutral lipids are less inhibitory (Angelidaki and Ahring 1992).

Inhibition by LCFAs is generally attributed to their adsorption to bacterial membranes, which may hinder either the transport of substrates and products across the membrane or the protective function of the membrane, leading to cell death. This implies that LCFA inhibition is a function of the surface area covered per molecule of LCFA, the accessibility and susceptibility of the cell membrane towards adsorption which may vary between microorganisms. Gram-positive bacteria are more vulnerable to LCFA inhibition than gram-negative bacteria (Rinzema 1988). Hwu *et al.* (1996[a]) found that LCFA inhibition is dependent also on the specific surface area of sludge particles. They found that the acetotrophic methanogens in granular sludge are less susceptible to inhibition by oleate than those in suspended and flocculent sludge. Rinzema *et al.* (1994) found that LCFA inhibition is more related to the concentration of the LCFA than to the LCFA/biomass ratio. They found similar LCFA toxicity threshold levels, i.e. LCFA concentration at which specific methanogenic activity of sludge becomes inhibited, at different values of LCFA/biomass ratio. Hwu *et al.* (1996[b]) showed that the degree of inhibition on methanogenesis, as indicated by the length of methanogenic lag period, is related to the adsorbed LCFA in the sludge surface, the amount of which depends on the initial LCFA concentration. In addition, they found that in continuous reactors, sludge flotation caused by the adsorption of LCFAs to sludge particles is more related to LCFA loading rate than to LCFA concentration. Thus, in practical conditions, LCFA adsorption may affect more the sludge retention than the sludge activity.

LCFAs inhibit methanogenesis at millimolar concentrations. Their inhibitory threshold levels vary among different bacteria and LCFAs (Koster and Cramer 1987). Lauric (C_{12}:0) and myristic (C_{14}:0) acids are the strongest inhibitors in various bacteria (Koster and Cramer 1987). Among LCFAs, oleate inhibition is most studied because it is among the most abundant LCFA in wastewaters (Hwu 1997). The extent of inhibition by unsaturated fatty acids increases with the number of double bonds (Demeyer and Henderickx 1967). Koster and Cramer (1987) found a synergistic inhibitory effect of lauric acid on myristic and capric acids, i.e. the net inhibition by the mixture of LCFAs is greater than the combined effect of the individual LCFAs. Lecithin is also synergistic to capric acid (Rinzema *et al.* 1994).

Several ways to abate the problems caused by the presence of lipids and/or LCFAs were studied. LCFA inhibition can be antagonized via precipitation by calcium (Angelidaki *et al.* 1990, Demeyer and Henderickx 1967, Hanaki *et al.* 1981, Koster 1987, Rinzema *et al.* 1993[a]) or via adsorption by bentonite (Angelidaki *et al.* 1990). Calcium, however, does not prevent wash-out of granular sludge from UASB reactors (Rinzema 1988), and cannot abate inhibition when the microorganisms are already exposed to LCFAs for a few minutes (Koster 1987). Rinzema *et al.* (1993[b]) suggested the use of an expanded granular sludge bed (EGSB) reactor, i.e. a reactor similar to a UASB reactor but operated at higher upflow velocities, in order to increase the rate of degradation of lipids via enhanced mixing, and thus, abate their inhibitory effect. Hwu *et al.* (1997) suggested the application of thermophilic anaerobic treatment for lipid containing wastewater because, although thermophilic methanogens are more susceptible towards LCFA inhibition than the mesophilic ones, LCFA degradation rates are higher under thermophilic conditions, and thus, when inhibition occurs, the recovery period is shorter than that in mesophilic conditions.

Adaptation of sludge to LCFAs was considered in some studies. The results of Angelidaki and Ahring (1992), Hanaki *et al.* (1981), and Rinzema *et al.* (1994) indicate that mesophilic and thermophilic bacteria cannot adapt to LCFAs. Methanogenic sludge already pre-exposed to LCFAs are still inhibited by LCFAs. Hwu (1997) suggested that during start-up of continuous reactors, the LCFA concentration in the influent must be low enough to allow retention of LCFA degraders in the reactor, hence, prevent LCFA accumulation.

Little is known about the effects of LCFAs and neutral lipids on hydrolysis of suspended solids in anaerobic reactors although LCFAs are known to inhibit growth of various microorganisms, including several non-methanogenic bacteria (Koster and Cramer 1987). LCFAs are inhibitory to the β-oxidation of themselves (Hanaki *et al.* 1981).

5.4. **Two-phase Anaerobic Treatment Systems**

To prevent sludge flotation and inhibition of methanogenesis caused by lipids, especially in high-rate anaerobic reactors, two-phase anaerobic systems or pre-removal of lipids are suggested for the treatment of wastewaters containing high lipid levels (Hanaki *et al.* 1990, Kumatsu *et al.* 1991, Perle *et al.* 1995, Hawkes *et al.* 1992, Sayed *et al.* 1993). Two-phase systems are employed also for the following purposes: (1) optimizing the environmental conditions for the different anaerobic processes, i.e. hydrolysis and acidogenesis in the first phase and acetogenesis and methanogenesis in the second phase, so that an imbalance between the different groups of anaerobic bacteria, which may take place in one-phase systems, could be prevented (Ghosh 1987); (2) preventing digester foaming which could lead to many serious operational problems (Ghosh 1991); (3) enhancing sludge granulation in methanogenic reactor (Shin *et al.* 1992); and (4) preventing shockloads in terms of hydraulic and/or organic loading rates, temperature, and pH (Norman and Frostell 1977). Phase separation can be achieved by controlling the pH to acidic levels (Shin *et al.* 1992) or by applying short hydraulic retention time (Lin and Ouyang 1993) or solid retention time (Zhang and Noike 1991).

As in the treatment of raw sewage in a two-phase UASB system (Wang 1994), the first phase raises the level of dissolved COD and volatile fatty acids (VFAs) in the influent of the second phase and stabilizes the produced sludge to some extent. With wastewaters containing high lipid levels, the first phase serves mainly to remove the suspended solids, especially the lipids. However, hardly any lipid degradation occurs in the first-phase reactor (Kumatsu *et al.* 1991, Hanaki *et al.* 1990). The removal of lipids in acidogenic reactors is probably due to lipid adsorption or co-precipitation with other settleable or coagulable components of the wastewater. For instance, in the treatment of dairy wastewater under acidogenic conditions in an upflow anaeorobic reactor, Zeeman *et al.* (1997) hypothesized that lipids are removed via co-settling with casein, the main protein in the wastewater, that precipitates at its isoelectric point. In the degradation of dairy wastewater, a pH as low as the isoelectric point of casein can be attained due to the production of volatile fatty acids from lactose degradation.

5.5. Inhibition by NH_4^+ and NaCl

High amounts of NH_4^+ may be generated from the degradation of proteins and NPN compounds in fish processing wastewaters. Low NH_4^+ concentrations have a beneficial effect on anaerobic processes as NH_4^+ is an important nutrient source for the anaerobic bacteria (McInerney 1988). At high concentrations, it inhibits methanogenesis (Hobson and Shaw 1976, Koster and Lettinga 1984, Soto *et al.* 1991, Van Velsen 1979, Zeeman 1991). This inhibition effect is attributed to the free NH_3 (present in equilibrium with NH_4^+) which is toxic at concentrations beyond 80-150 $mg.l^{-1}$ (De Baere *et al.* 1984, McCarty and McKinney 1961). Since NH_3-NH_4^+ equilibrium is pH and temperature dependent, NH_4^+ inhibition is also related to these factors (Angelidaki and Ahring 1994, Koster and Koomen 1988). The NH_4^+ tolerance level of sludge can be improved by adaptation (De Baere *et al.* 1984, Koster 1986, Koster and Lettinga 1988). The results of Zeeman (1991) and Van Velsen (1981) on the digestion of animal wastes indicate that NH_4^+ also inhibits the hydrolysis of suspended solids.

NaCl may affect biochemical processes via its effect on enzyme activity and the bacteria which carry out these processes using intracellular and extracellular enzymes. NaCl inhibition on non-halophilic bacteria is hypothesized to be due to (1) dehydration as a consequence of increased osmotic pressure, (2) inhibition of the activity of intracellular enzymes thus affecting bacterial metabolism, and (3) a physical effect on bacterial cell walls that leads to inhibited cell wall functions (Larsen 1962). Salt tolerance vary between microorganisms (Larsen 1962). De Baere *et al.* (1984) and Lema *et al.* (1987) showed the possibility of adapting the methanogenic sludge to high concentrations of NaCl.

6. Hydrolysis of Lipids and Proteins

6.1. Factors Affecting Hydrolysis

Several factors are found to affect the rate of hydrolysis of different biopolymers: pH, temperature, oxygen concentration, seeding, and the occurrence of methanogenesis.

pH

The optimum pH for the hydrolysis of particulates is near neutral. The hydrolysis rates of both nitrogenous and carbohydrate COD increase at increasing pH, e.g. the digestion of primary sewage sludge at 35°C as compared at pH range 5.1-6.7 (Eastman and Ferguson 1981) and brewery wastewater at pH range 4-7 (Suzuki *et al.* 1997). At 30°C and in the pH range 5.3 to 7.0, Breure and Van Andel (1984), found that the rate of hydrolysis of gelatin and the specific hydrolytic activity in an anaerobic reactor increase at increasing pH. Hydrolysis rate is negatively affected by very low pH (Henry *et al.* 1987). In the acid-phase digestion of mixed primary and waste activated sludge, the optimum pH for hydrolysis and acidogenesis, is found to be 5.5-6.5 by Ghosh (1987) and Henry *et al.* (1987) for both mesophilic (35°C) and thermophilic (55°C) cultures, respectively. Within the pH and temperature ranges of 5-7 and 35-55°C, Perot *et al.* (1988) determined the optimum

conditions for the hydrolysis of a mixture of primary and waste activated sludge at pH 6.8 and 50°C.

Temperature

The growth rate of microorganisms is generally higher under thermophilic conditions than at mesophilic and psychrophilic conditions. Hydrolysis rates of proteins and lipids are higher at thermophilic than those at mesophilic conditions (Ghosh 1987, Henry *et al.* 1987). Within the mesophilic temperature range, the rate of hydrolysis of particulates increases at increasing temperature, e.g. suspended COD in sewage at temperature range 10-30°C (Wang *et al.* 1995) and carbohydrate in brewery wastewater at the range 20-40°C (Suzuki *et al.* 1997).

Oxygen concentration

At 20°C and 30°C, Henze and Mladenovski (1991) and Wang *et al.* (1995), respectively, showed that the hydrolysis rate of particulate matter in sewage is higher at aerobic conditions than that at anaerobic conditions.

Seeding and methanogenesis

The occurrence of methanogenesis appears to enhance the hydrolysis of proteins and lipids. Wang *et al.* (1995) found that the hydrolysis of seeded sewage is faster than that of unseeded sewage and attributed this to a higher concentration of methanogenic bacteria that reduce the concentration of intermediate products. During the acidification stage in the digestion of unseeded sewage sludge, i.e. the period where mainly formation of acids occurs and there is hardly any methanogenesis, only a very limited reduction in total lipids, i.e. neutral plus LCFAs, occurs (Heukelekian and Mueller 1958).

6.2. **Aspects of Hydrolysis**

Hydrolysis may be considered a multi-step process that includes production of extracellular enzymes by the acidogens, and catalysis involving these enzymes and complex substrates. It is reasonable to expect that the above factors, e.g. pH, temperature, and seeding, affect hydrolysis via their effect on the acidogenic bacteria, bacterial production of hydrolytic enzymes, enzyme activity, and the nature of the macromolecules, i.e. the susceptibility of the molecule to enzymatic attack.

Acidogenic bacteria

Acidogens probably play an important role in hydrolysis. They produce the necessary extracellular enzymes and degrade the products of hydrolysis. However, it is not known whether the acidogenic population and their growth are related to enzyme activities. Although enzymes are regenerated after a catalytic process, they may be irreversibly deactivated. The acidogens are probably important in replenishing the required enzymes. The stimulus for the excretion of lipases and proteases by bacteria is not known. The most logical reason is their need for monomers as carbon and nutrient source. Therefore, scarcity in assimilable substrate may stimulate them to produce hydrolytic enzymes in the presence of the macromolecules.

According to the review of Holzer and Heinrich (1980), deprivation of amino acids as a carbon source increases the rate of protein degradation in bacteria. The degradation of these monomers, e.g. amino acids, must be related to the growth of the acidogens.

The acidogens that predominate in anaerobic environments are of the genera *Bacteroides*, *Clostridium*, *Butyrivibrio*, *Eubacterium*, *Bifidobacterium*, and *Lactobacillus* (McInerney 1988, Siebert and Toerien 1969, Toerien and Hattingh 1969). The known lipolytic bacteria are *Anaerovibrio lipolytica*, *Butyrivibrio fibrisolvens*, *Clostridium botulinum*, *C. perfringens*, *Eubacterium* spp., *Treponema phagedenia*, *T. denticola*, *T. refringins*, *T. minutum*, *T. vicentii*, and *Borrelia* spp. (McInerney 1988). The known proteolytic bacteria include different species: *Bacteroides* spp., *Bifidobacterium* spp., *Butyrivibrio* spp., *Clostridium* spp., *Eubacterium* spp., *Lachnospira* spp., *Peptococcus anaerobicus*, *Selenomonas* spp., *Staphylococcus* spp., and *Streptococcus* spp. (in rumen, digesters, eutrophic lake, and sewage sludge); *Thermobacteroides proteolyticus* (tannery waste and cattle manure); and *Thermofilum pendens* and *Thermococcus celer* (casein, peptides, tryptone) (McInerney 1988). The predominant proteolytic bacteria are the *Clostridium* spp. (McInerney 1988, Siebert and Toerien 1969). Some of them are associated with the spoilage of meat and canned products, e.g. *C. perfringens*, *C. bifermentans*, *C. histolyticum*, *C. botulinum*, and *C. sporogenes* (Amano 1962, McInerney 1988). Some *Lactobacillus*, *Flavobacterium*, *Bacillus*, *Micrococcus*, and *Pediococcus* species are found responsible for the hydrolysis of proteins and lipids and giving the typical flavor in the preparation of fermented fish products (Amano 1962, Sikorski and Ruiter 1994). Fish fermentations are done under anaerobic conditions and high NaCl concentrations, i.e. 10-20 weight percent. Some bacteria, e.g. *Pseudomonas* sp. (Adachi *et al.* 1993), produce lipoprotein lipase.

As in ruminant animals, symbiotic microbial digestion occurs in the forestomach of marine animals. Most of the indigenous bacteria found in the forestomach of minke whale are lipolytic, gram-positive and strict anaerobes, e.g. mostly *Lactobacillus* spp., *Streptococcus* spp., and *Ruminococcus* spp., and also some *Coprococcus* spp., *Peptostreptococcus* strains, *Eubacterium* spp., and *Sarcina* strain (Olsen *et al.* 1994). It is reasonable to expect that proteins and lipids in fish processing wastes are partly hydrolysed by extracellular enzymes from acidogenic bacteria in the raw fish itself. The acid producing bacteria found by Olsen *et al.* (1994) in freshly caught and homogenized herring (*Clupea harengus*) are mainly of the genera *Pediococcus, Aerococcus, Lactobacillus, Streptococcus*, and *Bacteroides*. They are facultatively or strictly anaerobes, 60% and 40% respectively of the strains, mostly gram-positive, mostly cocci, and able to grow at 35°C. The viable population of anaerobic acidogenic bacteria in homogenized fresh herring is very low (57-95 cells per gram) compared to that found in anaerobic digesters, e.g. 2.55-7.0 x 10^4 lipolytic and 1.0 x 10^7 proteolytic bacteria per ml sewage sludge (Novaes 1986).

The optimum pH, temperature, and nutrient conditions for the growth of lipolytic and proteolytic acidogenic bacteria are not very well known. Toxicity effects of ions and other compounds in wastewaters on acidogens are not known. While some ions may serve as nutrients for acidogens, some ions, e.g. Na^+ and Cl^-, may have negative effects on cell walls or physiological activities. The inhibition mechanism of LCFAs on methanogens, i.e. via adsorption on bacterial membrane, may similarly affect some acidogenic bacteria.

Enzyme production

Accumulation of hydrolysis products may indirectly affect the rate of hydrolysis. Accumulation of amino acids from protein hydrolysis suppresses the production of protease (Doi 1972, Glenn 1976), while deprivation of amino acids or a carbon source induces protease production in bacteria (Holzer and Heinrich 1980). The production of protease by *B. megaterium* and *B. licheniformis* is controlled via repression by end products, e.g. isoleucine or threonine, or via repression by catabolites, e.g. glucose, respectively (Bull 1972). Similarly, the synthesis and excretion of lipases are regulated either via end product repression or end product inhibition, i.e., LCFAs from lipid hydrolysis either represses or inhibits lipase production (Bull 1972). Lipase production is not a function of temperature, cell growth, or cell concentration (Kramer 1971).

When hydrolysis products such as LCFAs and amino acids inhibit the activity or production of enzymes, the hydrolysis process may benefit from the syntrophic association between H_2-consuming methanogens and H_2-producing acetogens that are responsible for the degradation of LCFAs and some amino acids. When the growth of H_2-producing acetogens is stimulated, the removal rates of these hydrolysis products are hastened, and as a consequence, prevent an inhibitory build-up of LCFAs and amino acids.

Enzymes and enzyme activity

As enzymes are also protein molecules, their tertiary structure may be similarly affected by pH, temperature, and ionic concentrations. Some compounds, extremes of pH and temperature conditions alter the tertiary structure of enzymes and may render enzymes inactive. Proteases vary with respect to their pH optima and ability to stand temperature changes or extremes in temperature.

It is interesting to note that proteases of each pH group can be found in fish tissues: pepsin, cathepsin, carboxypeptidase A, trypsin, and chymotrypsin (Haard *et al.* 1994). The predominant proteases in fish are those found in the digestive tract, e.g. trypsin and chymotrypsin which are alkaline proteases (Haard *et al.* 1994, Sikorski and Ruiter 1994). Lipase activity is also found in fish, predominantly at the digestive tract. The postmortem changes in fish and the proteolysis in the preparation of fish hydrolysates are attributed to these hydrolytic enzymes (Haard *et al.* 1994).

Lipolytic activity generally takes place at interfaces. In water, lipases have a very low activity towards dissolved substrates (Martinelle and Hult 1994). Studies on lipases are mostly done outside the environment of the microorganisms that produced them. Lipase kinetics that are derived from these studies are described by Semereva and Desnuelle (1979) and Martinelle and Hult (1994) as follows. The hydrolysis of lipids by lipases starts with adsorption, generally considered to be reversible and occurring in equilibrium. The adsorbed enzyme is activated at the interface, followed by the binding of the substrate at the surface to the enzyme. The second step involves orientation and conformational changes in the enzyme. Lipase activity and specificity vary among microorganisms, particularly with respect to the type of LCFA component and its position in the triglyceride (Kramer 1971).

As an interfacial phenomenon, lipolysis is a function of the 'concentration' and the quality of the interface, e.g. the hydration of the lipid molecules lying at the interface. Lipase activity increases at increasing surface pressure (Semeriva and Desnuelle 1979). Considering the above catalytic mechanism, lipase activity may be inhibited by an accumulation of hydrolysis products at the interface which may induce changes in the physical and chemical properties of the interface (Martinelle and Hult 1994). At low concentrations, bile salts and surfactants prevent lipase denaturation but at high concentrations, they inhibit the binding of the enzyme at the interface. Some proteins such as bovine serum albumin also inhibit the adsorption of lipase at interface (Martinelle and Hult 1994).

The adsorption of extracellular enzymes, i.e. lipases as well as proteases, on surfaces is determined by the enzyme-surface interaction, dehydration of the interface, and structural changes in the enzyme upon adsorption. Enzyme-surface interactions include electrostatic attractions which are dependent on pH and ionic concentrations. Dehydration of interface promotes adsorption (Duinhoven 1992, Semereva and Desnuelle 1979).

Considering the mechanisms of hydrolysis by enzymes, which generally act on surfaces, it is expected that small particles with a high surface-to-volume ratio will be hydrolysed faster than large particles, and slowly-degrading or non-degradable materials will retard the hydrolysis of particulates to which they are adsorbed or associated. The possibility of enhancing hydrolysis of proteins and lipids by increasing the concentration of the interface, such as decreasing particle size and using emulsifiers, in anaerobic reactors are not yet explored.

The feasibility of using purified and immobilized hydrolytic enzymes, as in many food industries, to augment the lipase and protease activities are not yet tested in anaerobic reactors. The fate of extracellular enzymes, i.e. whether they are retained or washed-out, in high-rate anaerobic reactors such as UASB, FB, and AF reactors is not known. Possibilities, e.g. suitable reactor design, for preventing accumulation of hydrolysis products are yet to be explored.

Nature of complex waste

The degradation of complex wastes depend on their characteristics such as polarity in the case of lipids (i.e. polar lipids are more fastly degraded than non-polar lipids, Henze and Harremoes 1983), and, solubility, kind of end group, and tertiary structure in the case of proteins (McInerney 1988). Less soluble proteins are more slowly degraded than those that are highly soluble. Modification of the tertiary structure of proteins by chemical treatment or heating prevents protein degradation in rumen (McInerney 1988). Coagulation of proteins, as well as lipids, decreases their hydrolysis rates (Dentel and Gossett 1982). This effect in protein degradation can be due to the change in the tertiary structure of the protein molecule involved in the coagulation. The structure and solubility of proteins both depend on some environmental factors: temperature, pH, and ionic concentrations (see Section 4).

Among the different macromolecules, lipids are considered to be the most difficult to degrade (Eastman and Ferguson 1981, Sarada and Joseph 1993). Protein hydrolysis is usually slower than that of carbohydrates (Pavlostathis and Giraldo-Gomez 1991). Among carbohydrates,

cellulose is more difficult to degrade than soluble starch and glucose (Pavlostathis and Giraldo-Gomez 1991).

7. Scope of this Dissertation

The importance of fish processing industries in the Philippines was earlier discussed (Section 1). While there are research activities geared towards conversion of the presently considered solid and slurry wastes into valuable by-products, there is hardly any study in the country that deals with finding more cost-effective ways of minimizing the polluting effects of fish processing wastewaters on receiving lakes and coastal waters. Since these wastewaters contain high concentrations of biodegradable organic matter, a treatment system composed of an anaerobic process as the main treatment step followed by an appropriate post-treatment may be more feasible than the conventional aerobic processes, considering the prospects of net energy production and the other advantages of the former over the latter. For fish canneries operating on limited profit and plant space, high-rate treatment systems such as UASB reactors are suitable alternatives. Hydrolysis is considered the rate-limiting step in the overall anaerobic digestion process, as earlier discussed, but there is little knowledge on the effects of environmental factors and operating parameters on this step, especially in treating wastewaters containing high concentrations of lipids and proteins. Hence, this study considers the possibilities for treating fish processing wastewater via an anaerobic method and gives special emphasis on the hydrolysis of suspended solids in the wastewater.

Results of batch experiments on the effects of pH, presence of seed sludge, occurrence of methanogenesis, lipid level, NH_4^+, and NaCl concentrations on lipid and protein hydrolysis at mesophilic conditions are presented in the next three chapters (Chapters 2, 3, and 4). The particular case of fish canning industries in the Philippines is considered in selecting temperature conditions, ranges in ammonium and NaCl concentrations, and wastewater composition.

In Chapter 5, the performance of single-stage UASB reactors is compared for fish wastewater having different lipid and NaCl levels. The results of Chapters 2, 3, and 5 strongly suggest the need to remove lipids from the wastewater prior to treatment in a UASB reactor. In Chapter 6, the results of continuous experiments on the application of an upflow substrate precipitation and entrapment system as a pre-treatment step or as the first stage of a two-phase anaerobic process are presented. The roles of pH and solid retention time in the removal of lipids are discussed.

REFERENCES:

Adachi S., Okumura K., Ota Y. and Mankura M. (1993) Acidolysis of sardine oil by lipase to concentrate Eicosapentaenoic and Decosahexaenoic acids in glycerides. *J. Ferment. Bioeng.*, **75**, 259-264.

Aguiar A. L. C., Sant'Anna G. L. Jr. (1988) Liquid effluents of the fish canning industries of Rio de Janeiro State - treatment alternatives. *Envi. Tech. Lett.*, **9**, 421-428.

Amano K. (1962) The influence of fermentation in the nutritive value of fish with special reference to fermented fish products of South-East Asia. In: *Fish in Nutrition: Influence of Processing*, Heen E. and Kreuzer R. (eds.), pp. 180-200. Fishery News Ltd., London.

Angelidaki I., Petersen S. P. and Ahring B. K. (1990) Effects of lipids on thermophilic anaerobic digestion and reduction of lipid inhibition upon addition of bentonite. *Appl. Microbiol. Biotechnol.*, **33**, 469-472.

Angelidaki I. and Ahring B. K. (1992) Effects of free long-chain fatty acids on thermophilic anaerobic digestion. *Appl. Microbiol. Biotechnol.*, **37**, 808-812.

Angelidaki I. and Ahring B. K. (1994) Anaerobic thermophilic digestion of manure at different ammonia loads: effect of temperature. *Wat. Res.*, **28**, 727-731.

Angelidaki I. and Ahring B. K. (1995) Establishment and characterization of an anaerobic thermophilic (55°C) enrichment culture degrading long-chain fatty acids. *Appl. Environ. Microbiol.*, **61**, 2442-2445.

Aprieto V. L. (1995) *Philippine Tuna Fisheries: Yellowfin and Skipjack*. University of the Philippines Press, Quezon City, Philippines.

Aquaculture Watch (1987) Chitin from fishery waste. *Aquaculture Watch*, December 1987, **1** (3), 13. Asia-Pacific Info. Consultants, Philippines.

Balsev-Olesen P., Lynggaard-Jensen A. and Nickelsen C. (1990) Pilot-scale experiments on anaerobic treatment of wastewater from a fish processing plant. *Wat. Sci. Technol.*, **22**, 463-474.

Barker H. A. (1961) Fermentations of nitrogenous organic compounds. In: *The Bacteria*, Vol. 2, Gunsalus I. C. and Stanier R. Y. (eds.), pp. 151-207. Academic Press, New York.

Barker H. A. (1981) Amino acid degradation by anaerobic bacteria. *Ann. Rev. Biochem.*, **50**, 23-40.

BAS (Bureau of Agric. Statistics) (1988) *Selected Fishery Statistics 1979-1988*. Department of Agriculture, Quezon City, Philippines.

BAS (Bureau of Agric. Statistics) (1992) *Annual Report 1992*. Department of Agriculture, Quezon City, Philippines.

BAS (Bureau of Agric. Statistics) (1993) *Fishery Statistics 1993*. Department of Agriculture, Quezon City, Philippines.

Battistoni P., Fava G. and Gatto A. (1992) Fish processing wastewater: emission factors and high load trickling filters evaluation. *Wat. Sci. Technol.*, **25**, 1-8.

Beccari M., Bonemazzi F., Majone M. and Riccardi C. (1996) Interaction between acidogenesis and methanogenesis in the anaerobic treatment of olive oil mill effluents. *Wat. Res.*, **30**, 183-189.

BFAR (Bureau of Fisheries and Aquatic Resources) Reports (1985-1993) *Annual Reports 1985, 1986, 1987, 1988, 1989, 1990, 1991, 1992, 1993*. Department of Agriculture, Quezon City, Philippines.

Boardman G. D., Tisinger J. L. and Gallagher D. L. (1995) Treatment of clam processing wastewaters by means of upflow anaerobic sludge blanket technology. *Wat. Res.*, **29**, 1483-1490.

Boari G., Mancini I. M. and Trulli E. (1993) Anaerobic digestion of olive oil mill effluent pretreated and stored in municipal solid waste sanitary landfills. *Wat. Sci. Technol.*, **28**, 27-34.

Borja R., Banks C. J. and Wang Z. (1995) Effect of organic loading rate on anaerobic treatment of slaughterhouse wastewater in a fluidised-bed reactor. *Bioresource Technology*, **52**, 157-162.

Borja R., Banks C. J. and Sanchez E. (1996) Anaerobic treatment of palm oil mill effluent in a two-stage upflow anaerobic sludge blanket (UASB) system. *J. Biotechnology*, **45**, 125-135.

Brett Borup M. and Fenhaus S. L. (1990) Food processing wastes. *J. Wat. Pollut. Control Fed.*, **62**, 461-465.

Breure A. M. and Van Andel J. G. (1984) Hydrolysis and acidogenic fermentation of a protein, gelatin, in an anaerobic continuous culture. *Appl. Microbiol. Biotechnol.*, **20**, 40-45.

Bull A. T. (1972) Environmental control influencing the synthesis and excretion of exocellular macromolecules. *J. Appl. Chem. Biotechnol.*, **22**, 261-292.

Chen H. M. and Meyers S. P. (1982) Extraction of astaxanthin pigment from crawfish waste using a soy oil process. *J. Food Sci.*, **47**, 892.

Civit E. M., Parin M. A. and Lupin H. M. (1982) Recovery of protein and oil from fishery bloodwater waste. *Wat. Res.*, **16**, 809-814.

De Baere L. A., Devocht M., Van Assche P. and Verstraete W. (1984) Influence of high NaCl and NH_4Cl salt levels on methanogenic associations. *Wat. Res.*, **18**, 543-548.

Demeyer D. I. and Henderickx H. K. (1967) The effect of C_{18} unsaturated fatty acids on methane production *in vitro* by mixed rumen bacteria. *Biochim. Biophys. Acta.*, **137**, 484-497.

Dentel S. K. and Gossett J. M. (1982) Effect of chemical coagulation on anaerobic digestibility of organic materials. *Wat. Res.*, **16**, 707-718.

Doi R. H. (1972) Role of proteases in sporulation. *Curr. Top. on Cell Regul.*, **6**, 1-20.

DOST (1990) DOST technology turns food wastes into new products. *Animal Husbandry and Agricultural J.*, Nov. 1990, p.28.

DTI (Department of Trade and Industry) (1989) *Tuna: Product Profile.* Product Research and Strategy Group, Bureau of Export Trade Promotion, DTI, Philippines.

DTI (Department of Trade and Industry) (1994) *Phil. Agribusiness Factbook and Directory 1993-1994.* Department of Trade and Industry, Philippines.

Duinhoven S. (1992) *Enzyme adsorption at solid-liquid interfaces.* Ph.D. Thesis. Wageningen Agricultural University, Wageningen, The Netherlands.

Eastman J. A. and Ferguson J. F. (1981) Solubilization of particulate organic carbon during the acid phase of anaerobic digestion. *J. Wat. Pollut. Control Fed.*, **53**, 352-366.

Edwards D., Street P. and Clucas I. (1981) *Economic Aspects of Small-Scale Fish Canning.* Tropical Product Institute, London.

Frankel R. J. and Phongsphetraratana A. (1986) Effects of water reuse, recycling and resource recovery on food processing waste treatment in Thailand. *Chem. Abs.*, **104**, 229863x.

Franks F. (1993) *Protein Biotechnology: Isolation, Characterization, & Stabilization.* Humana Press, Inc., New Jersey.

Fukuda H. and Nakatani H. (1979) Treatment of waste water from fish meat processing plant I. Effects of salt and sugar on condensation treatment of waste water. *Chem. Abs.*, **90**, 60713c.

Ghosh S. (1987) Improved sludge gasification by two-phase anaerobic digestion. *ASCE. J. Environ. Engng.*, **113**, 1265-1284.

Ghosh S. (1991) Pilot-scale demonstration of two-phase anaerobic digestion of activated sludge. *Wat. Sci. Technol.*, **23**, 1179-1188.

Glenn A. R. (1976) Production of extracellular proteins by bacteria. *Ann. Rev. Microbiol.*, **30**, 41-62.

Guida V. G. and Kugelman I. J. (1988) Treatment of clam processing wastewater by activated sludge and salt marsh polishing: Results of microcosm scale experiments. In: *Proc. 1988 Food Processing Waste Conf.*, Georgia Tech. Research Inst., Atlanta, U.S.A.

Gujer W. and Zehnder A. J. B. (1983) Conversion processes in anaerobic digestion. *Wat. Sci. Technol.*, **15**, 127-167.

Haard N. F., Simpson B. K. and Sun Pan B. (1994) Sarcoplasmic proteins and other nitrogenous compounds. In: *Seafood Proteins*, Sikorski Z. E., Sun Pan B. and Shahidi F. (eds.), pp. 13-39. Chapman & Hall, New York.

Hanaki K., Matsuo T. and Nagase M. (1981) Mechanism of inhibition caused by long-chain fatty acids in anaerobic digestion process. *Biotechnol. and Bioengg.*, **23**, 1591-1610.

Hanaki K., Matsuo T. and Kumazaki K. (1990) Treatment of oily cafeteria wastewater by single-phase and two-phase anaerobic filter. *Wat. Sci. Technol.*, **22**, 299-306.

Hawkes F. R., Rozzi A., Black K., Guwy A. and Hawkes D. L. (1992) The stability of anaerobic digesters operating on a food-processing wastewater. *Wat. Sci. Technol.*, **25**, 73-82.

Hawkes F. R., Donnelly T. and Anderson G. K. (1995) Comparative performance of anaerobic digesters operating on ice-cream wastewater. *Wat. Res.*, **29**, 525-533.

Henry M. P., Sajjad A. and Ghosh S. (1987) The effects of environmental factors on acid-phase digestion of sewage sludge. In: *42nd Purdue Industrial Waste Conference Proceedings*, pp. 727-737. Lewis Publishers, Inc., Chelsea, Michigan, U.S.A.

Henze M. and Harremoes P. (1983) Anaerobic treatment of wastewater in fixed film reactors - a literature review, *Wat. Sci. Technol.*, **15**, 1-101.

Henze M. and Mladenovski C. (1991) Hydrolysis of particulate substrate by activated sludge under aerobic, anoxic, and anaerobic conditions. *Wat. Res.*, **25**, 61-64.

Heukelekian H. and Mueller P. (1958) Transformation of some lipids in anaerobic sludge digestion. *Sew. Ind. Wastes.*, **30**, 1108-1120.

Hobson P. N. and Shaw B. G. (1976) Inhibition of methane production by *Methanobacterium Formicicum*. *Wat. Res.*, **10**, 849-852.

Holzer H. and Heinrich P. C. (1980) Control of proteolysis. *Ann. Rev. Biochem.*, **49**, 63-91.

Hozumi H. (1988) Flocculation of fishery processing wastewater. *Chem. Abs.*, **108**, 209560a.

Hudson J. W., Pohland F. G. and Pendergrass R. P. (1978) Anaerobic packed column treatment of shellfish processing wastewaters. In: *33rd Purdue Industrial Waste Conference Proceedings*, pp. 560-574, Lewis Publishers, Inc., Chelsea, Michigan, U.S.A.

Hulshoff Pol L. W. and Lettinga G. (1986) New technologies for anaerobic wastewater treatment. *Wat. Sci. Technol.*, **18**, 41-53.

Hwu C. S. (1997) *Enhancing Anaerobic Treatment of Wastewaters Containing Oleic Acid*. Ph.D. Thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

Hwu C. S., Donlon B. and Lettinga G. (1996[a]) Comparative toxicity of long-chain fatty acid to anaerobic sludges from various origins. *Wat. Sci. Technol.*, **34**, 351-358.

Hwu C. S., Tseng S. K., Yuan C. Y., Kulik Z. and Lettinga G. (1996[b]) Adsorption of long-chain fatty acids onto granular sludges and its effect on upflow anaerobic sludge bed reactor operation. In: *Proc. 1st IAWQ Specialized Conf. on Adsorption in Water Environment and Treatment Processes*, 5-8 Nov. 1996, Shirahama, Wakayama, Japan, pp. 91-98.

Hwu C. S., Van Lier J. B. and Lettinga G. (1997) Anaerobic toxicity and degradability of oleic acid under mesophilic and thermophilic conditions. Paper presented in *Forum for Applied Biotechnology*, 25-26 Sept. 1997, Gent, Belgium.

Iggleden G. J. and Van Staa R. (1984) The use of chemical dosing and dissolved air flotation in the recovery of protein from waste waters. *Inst. Chem. Eng. Symp. Ser.*, **84**, 381.

ITDI-DOST (Industrial Technology Development Institute - Department of Science and Technology of the Philippines) (1993) *Annual Report 1993*. ITDI-DOST, Philippines.

Johnson R. A. and Lindley K. L. (1982) Use of hydrocyclones to treat seafood-processing wastewaters. *J. Wat. Pollut. Control Fed.*, **54**, 1607-1612.

Johnson R. A. and Gallanger S. M. (1984) Use of coagulants to treat seafood processing wastewaters. *J. Wat. Pollut. Control Fed.*, **56**, 970-976.

Johnson E. L. and Peniston Q. P. (1982) Utilization of shellfish waste for chitin and chitosan production. In: *Chemistry and Biochemistry of Marine Food Products*, Martin R. E. (ed.), AVI Publ. Co., U.S.A.

Kinsella J. E. (1987) *Seafoods and Fish Oils in Human Health and Diseases*. Marcel Dekker, Inc., New York.

Kitabayashi K. (1983[a]) Protein aggregating agents for treatment of protein-rich wastewater. *Chem. Abs.*, **98**, 33414q.

Kitabayashi K. (1983[b]) Coagulation of proteins in wastewater. *Chem. Abs.*, **98**, 203854c.

Kobayashi H., Fuutai N., Nakamura K., Dazai M., Miyatake M., and Aizawa M. (1985) Phosphorus removal by batch activated sludge process in a pilot plant. *Chem. Abs.*, **103**, 200321n.

Koster I. W. (1986) Characteristics of the pH-influenced adaptation of methanogenic sludge to ammonium toxicity. *J. Chem. Tech. Biotech.*, **36**, 445-455.

Koster I. W. (1987) Abatement of long-chain fatty acid inhibition of methanogenesis by calcium addition. *Biol. Wastes.*, **22**, 295-301.

Koster I. W. and Cramer A. (1987) Inhibition of methanogenesis from acetate in granular sludge by long-chain fatty acids *Appl. Environ. Microbiol.*, **53**, 403-409.

Koster I. W. and Koomen E. (1988) Ammonia inhibition of the maximum growth rate (μ_m) of hydrogenotrophic methanogens at various pH levels and temperatures. *Appl. Microbiol. Biotechnol.*, **28**, 500-505.

Koster I. W. and Lettinga G. (1984) The influence of ammonium-nitrogen on the specific activity of pelletized methanogenic sludge. *Agric. Wastes.*, **9**, 205-216.

Koster I. W. and Lettinga G. (1988) Anaerobic digestion at extreme ammonia concentration. *Biol. Wastes.*, **25**, 51-59.

Kramer G. R. (1971) Hydrolysis of lipids in wastewater. *ASCE J. Sanitary Engineering Division.*, **97**, 731-744.

Krvaric-Skare M. (1955) On seasonal variations in the chemical composition and nutritive value of sardine (*Sardina Pilchardus* Walb.) of the Central Adriatic. In: *Proceedings and Technical Papers*, No. 3, p.441-447, General Fisheries Council for the Mediterranian, FAO, Rome.

Kumatsu T., Hanaki K. and Matsuo T. (1991) Prevention of lipid inhibition in anaerobic processs by introducing a two-phase system. *Wat. Sci. Technol.*, **23**, 1189-1200.

Larsen H. (1962) Halophilism. In: *The Bacteria*, Vol. 1, Gunsalus I. C. and Stanier R. Y. (eds.), pp. 297-342. Academic Press, New York.

Lema J. M., Soto M., Veiga M. C. and Mendez R. (1987) Mesophilic and thermophilic anaerobic treatment of wastewaters from industrial processing of mussel. In: *Biomass for Energy and Industry*, Grassi G., Delmon B., Molle J. F. and Zibetta H. (eds.), pp. 872-876. Elsevier Applied Science, London.

Lettinga G., Hobma S. W., Hulshoff Pol L. W., De Zeeuw W., De Jong P., Grin P. and Roersma R. (1983) Design operation and economy of anaerobic treatment. *Wat. Sci. Technol.*, **15**, 177-195.

Lettinga G., Hulshoff Pol L. W., Koster I. W., Wiegant W. M., de Zeeuw W. J., Rinzema A., Grin P. C., Roersma R. E. and Hobma S. W. (1984) High-rate anaerobic waste-water treatment using the UASB reactor under a wide range of temperature conditions. *Biotechnol. and Gen. Engg. Rev.*, **2**, 253-283.

Lettinga G., De Man A., Grin P. and Hulshoff Pol L. W. (1987) Anaerobic waste water treatment as an appropriate technology for developing countries. *Trib. Cebedeau*, **40**, 21-32.

Lettinga G. and Hulshoff Pol L. W. (1991) UASB-process design for various types of wastewaters. *Wat. Sci. Technol.*, **24**, 87-107.

Lin H. Y. and Ouyang C. F. (1993) Upflow anaerobic sludge digestion in a phase separation system. *Wat. Sci. Technol.*, **28**, 133-138.

Litchfield J. H. (1980) Meat, fish, and poultry processing wastes. *J. Wat. Pollut. Control Fed.*, **52**, 1325-1329.

Litchfield J. H. (1983) Meat, fish, and poultry processing wastes. *J. Wat. Pollut. Control Fed.*, **55**, 683-686.

Loehr R. C. and Roth J. C. (1968) Anaerobic degradation of long-chain fatty acid salts. *J. Wat. Pollut. Control Fed.*, **40**, 385.

Martinelle M. and Hult K. (1994) Kinetics of triglyceride lipases. In: *Lipases: Their Structure, Biochemistry and Application*, Petersen S. B. and Woolley P. (eds.), pp. 159-180. Cambridge University Press., U.K.

McCarty P. L. and McKinney R. E. (1961) Salt toxicity in anaerobic digestion. *J. Wat. Pollut. Control Fed.*, **33**, 399-415.

McComis W. T. and Litchfield J. H. (1985) Meat, fish, and poultry processing wastes. *J. Wat. Pollut. Control Fed.*, **57**, 570-573.

McComis W. T. and Litchfield J. H. (1986) Meat, fish, and poultry processing wastes. *J. Wat. Pollut. Control Fed.*, **58**, 549-552.

McInerney J. M. (1988) Anaerobic hydrolysis and fermentation of fats and proteins. In: *Biology of Anaerobic Microorganisms*, Zehnder A. J. B. (ed.), pp. 373-416. John Wiley & Sons, Inc., New York.

Mendez R., Omil F., Soto M., and Lema J. M. (1992) Pilot plant studies on the anaerobic treatment of different wastewaters from fish-canning factory. *Wat. Sci. Technol.*, **25**, 37-44.

Mercz T. I. and Cord-Ruwisch R. (1997) Treatment of wool scouring effluent using anaerobic biological and chemical flocculation. *Wat. Res.*, **31**, 170-178.

Milazzo A. (1983) Use of chitosan as a flocculant in industrial effluents from lobster-processing plants. *Chem Abs.*, **99**, 76179c.

Miyata Y. (1984) Concentration of proteins from the wash water of red meat fish by ultrafiltration membrane. *Chem. Abs.*, **101**, 22164n.

Nagamine F. (1986) Sedimentation treatment test of blood-containing wastewater from sardine processing plant. *Chem. Abs.*, **105**, 213682x.

Nair C. (1990) Pollution control through water conservation and wastewater reuse in the fish processing industry. *Wat. Sci. Technol.*, **22**, 113-121.

Nakamura K. (1986) Performance and application of batch-type activated sludge process with limited aeration. *Chem. Abs.*, **104**, 173800e.

Nanninga H. J. and Gottschal J. C. (1985) Amino acid fermentation and hydrogen transfer in mixed cultures. *FEMS Microbiology Ecology*, **31**, 261-269.

Norman J. and Frostell B. (1977) Anaerobic wastewater treatment in a two-stage reactor of a new design. In: *32nd Purdue Industrial Waste Conference Proceedings*, pp. 387-395. Lewis Publishers, Inc., Chelsea, Michigan, U.S.A.

Novaes R. F. V. (1986) Microbiology of Anaerobic Digestion. *Wat. Sci. Technol.*, **12**, 1-14.

Novak J. T. and Carlson D. A. (1970) The kinetics of anaerobic long-chain fatty acid degradation. *J. Wat. Pollut. Control Fed.*, **42**, 1932-1943.

NSO (1993) *Foreign Trade Statistics*. National Statistics Office, Philippines.

Okubo T. (1984[a]) Biological treatment of wastewater. *Chem. Abs.*, **101**, 136422a.

Okubo T. (1984[b]) Biological treatment of wastewater. *Chem. Abs.*, **101**, 136423b.

Okubo T., Ishihara J. E. and Matsumoto J. (1980) Fundamental studies on the treatment of fish-processing wastewater by the activated sludge process. In: *Treatment and Disposal of Liquid and Solid Industrial Wastes*, Curi K. (ed)., pp.345-355, Pergamon Press, England.

Olsen M. A., Aagnes T. H. and Mathiesen S. D. (1994) Digestion of herring by indigenous bacteria in the Minke whale forestomach. *Appl. Environ. Microbiol.*, **60**, 4445-4455.

Örlygsson J., Houwen F. P. and Svensson, B. H. (1994) Influence of hydrogenothrophic methane formation on the thermophilic anaerobic degradation of protein and amino acids. *FEMS Microbiology Ecology*, **13**, 327-334.

Öztürk I., Eroglu V., Ubay G. and Demir I. (1993) Hybrid upflow anaerobic sludge blanket reactor (HUASBR) treatment of dairy effluents. *Wat. Sci. Technol.*, **28**, 77-85.

Palenzuela A. M. (1993) *Anaerobic Treatment of Fish-Processing Wastewater Using Expanded Granular Sludge Bed Reactor in One-Phase and Two-Phase Systems*. M.Sc. Thesis E.E. 98, Wageningen Agricultural University, Wageningen, and International Institute for Infrastructural, Hydraulic, and Environmental Engineering, Delft, The Netherlands.

Palomares T. S., Gonzalez O. N., Sonsing R. and Tanyag L. M. (1986) Fish processing in the Philippines. In: *Proceedings of the First ASEAN Workshop on Fish and Fish Waste Processing and Utilization*, 22-24 Oct. 1986, Jakarta, pp. 250-274.

Pavlostathis S. G. and Giraldo-Gomez E. (1991) Kinetics of anaerobic treatment. *Wat. Sci. Technol.*, **24**, 35-59.

Pavlostathis S. G. and Gossett J. M. (1988) Preliminary conversion mechanism in anaerobic digestion of biological sludges. *ASCE. J. Environ. Engng.*, **114**, 575-592.

Perle M., Kimchie S. and Shelef G. (1995) Some biochemical aspects of the anaerobic degradation of dairy wastewater. *Wat. Res.* **29**, 1549-1554.

Perot C., Sergent M., Richard P., Phan Tan Luu R. and Milot N. (1988) The effects of pH, temperature and agitation speed on sludge anaerobic hydrolysis-acidification. *Envi. Tech. Lett.*, **9**, 741-752.

Pigott G. M. (1996) Edible oil and fats products: application and technology. In: *Bailey's Industrial Oil and Fat Products*, Vol. 3, Hui Y. H. (ed.), John Wiley & Sons, Inc., U.S.A.

Pohland F. G. and Hudson J. W. (1976) Aerobic and anaerobic microbial treatment alternatives for shellfish processing wastewaters in continuous cultures. *Biotechnol. and Bioengg.*, **18**, 1219-1247.

Prasertsan P., Jung S. and Buckle K. A. (1994) Anaerobic filter treatment of fishery wastewater. *World J. Microbiol. and Biotechnol.*, **10**, 11-13.

Raa J. and Hansen F. (1983) Isolation of astaxanthin from crawfish or shrimp waste for use as a coloring agent in fish feed. *Chem. Abs.*, **98**, 177859m.

Reece P. (1988) Recovery of protease from fish waste. *Process Biochem.*, **23**, 62.

Rinzema A. (1988) *Anaerobic Treatment of Wastewater with High Concentrations of Lipids or Sulfate*. Ph.D. Thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

Rinzema A., Van Veen H. and Lettinga G. (1993[a]) Anaerobic digestion of triglyceride emulsions in expanded granular sludge bed upflow reactors with modified sludge separators. *Envi. Tech.* **14**, 423-432.

Rinzema A., Alphenaar A. and Lettinga G. (1993[b]) Anaerobic digestion of long-chain fatty acids in UASB and expanded granular sludge bed reactors. *Biochem.*, **28**, 527-537.

Rinzema A., Boone M., Van Knippenberg K., and Lettinga G. (1994) Bactericidal effect of long-chain fatty acids in anaerobic digestion. *Water Environ. Res.*, **66**, 40-49.

Roy F., Samain. E., Dubourguier H. C. and Albagnac G. (1986) *Syntrophomonas sapovorans* sp. nov., a new obligately proton reducing anaerobe oxidizing saturated and unsaturated long-chain fatty acids. *Arch. Microbiol.*, **145**, 142-147.

Sandberg M. and Ahring B. K. (1992) Anaerobic treatment of fish meal process wastewater in a UASB reactor at high pH. *Appl. Microbiol. Biotechnol.*, **36**, 800-804.

Sarada R. and Joseph R. (1993) Biochemical changes during anaerobic digestion of tomato processing waste. *Process Biochem.*, **28**, 461-466.

Sayed S., Van Campen L. and Lettinga G. (1987) Anaerobic treatment of slaughterhouse waste using a granular sludge UASB reactor. *Biol. Wastes*, **21**, 11-28.

Sayed S., Van der Zanden J., Wijffels R. and Lettinga G. (1988) Anaerobic degradation of the various fractions of slaughterhouse wastewater. *Biol. Wastes*, **23**, 117-142.

Sayed S. K. I., Van der Spoel H. and Truijen G. J. P. (1993) A complete treatment of slaughterhouse wastewater combined with sludge stabilization using two stage high rate UASB process. *Wat. Sci. Technol.*, **27**, 83-90.

Semereva M. and Desnuelle P. (1979) Pancreatic lipase and colipase. An example of heterogeneous biocatalysis. In: *Advances in Enzymology and Related Areas of Molecular Biology*, Vol. 48., Meister A. (ed.), pp. 319-370, John Wiley & Sons, Inc., U.S.A.

Shahidi F. (1994) Proteins from seafood processing discards. In: *Seafood Proteins*, Sikorski Z. E., Sun Pan B. and Shahidi F. (eds.), pp. 171-193. Chapman & Hall, New York.

Shimizu H. and Nishioka F. (1979) Protein recovery from meat or fish rinse. *Chem. Abs.*, **90**, 28673d.

Shin H. S., Bae B. U., Lee J. J. and Paik B. C. (1992) Anaerobic digestion of distillery wastewater in a two-phase UASB system. *Wat. Sci. Technol.*, **25**, 361-371.

Siebert M. L. and Toerien D. F. (1969) The proteolytic bacteria present in the anaerobic digestion of raw sewage sludge. *Wat. Res.*, **3**, 241-250.

Sikorski Z. E. (1994[a]) The contents of proteins and other nitrogenous compounds in marine animals. In: *Seafood Proteins*, Sikorski Z. E., Sun Pan B. and Shahidi F. (eds.), pp. 6-12. Chapman & Hall, New York.

Sikorski Z. E. (1994[b]) The myofibrillar proteins in seafoods. In: *Seafood Proteins*, Sikorski Z. E., Sun Pan B. and Shahidi F. (eds.), pp. 41-57. Chapman & Hall, New York.

Sikorski Z. E. and Ruiter A. (1994) Changes in proteins and nonprotein nitrogen in cured, fermented, and dried seafoods. In: *Seafood Proteins*, Sikorski Z. E., Sun Pan B. and Shahidi F. (eds.), pp. 113-126. Chapman & Hall, New York.

Soto M., Mendez R. and Lema J. M. (1991) Biodegradability and toxicity in the anaerobic treatment of fish canning wastewaters. *Envi. Tech.*, **12**, 669-677.

Soto M., Mendez R. and Lema J. M. (1992) Characterization and comparison of biomass from mesophilic and thermophilic fixed bed anaerobic digesters. *Wat. Sci. Technol.*, **25**, 203-212.

Soto M., Mendez R. and Lema J. M. (1993) Sodium inhibition and sulphate reduction in the anaerobic treatment of mussel processing wastewaters. *J. Chem. Tech. Biotech.*, **58**, 1-7.

Spotte S. (1992) *Captive Seawater Fishes Science and Technology*, John Wiley & Sons, Inc., U.S.A.

Stams A. J. M. and Hansen T. A. (1984) Fermentation of glutamate and other compounds by *Acidaminobacter hydrogenoformans* gen. nov. sp. nov., an obligate anaerobe isolated from black mud. Studies with pure cultures and mixed cultures with sulfate-reducing and methanogenic bacteria. *Arch. Microbiol.*, **137**, 329-337.

Suzuki H., Yoneyama Y. and Tanaka T. (1997) Acidification during anaerobic treatment of brewery wastewater. *Wat. Sci. Technol.*, **35**, 265-274.

Suzuki T. (1981) *Fish and Krill Protein Processing Technology*. Applied Science Publishers, Great Britain.

Toerien D. F. and Hattingh W. H. J. (1969) Anaerobic digestion I. The microbiology of anaerobic digestion. *Wat. Res.*, **3**, 385-416.

Tsonis S. P. and Grigoropoulos S. G. (1993) Anaerobic treatability of olive oil mill wastewater. *Wat. Sci. Technol.*, **28**, 35-44.

Van Velsen A. F. M. (1979) Adaptation of methanogenic sludge to high ammonia-nitrogen concentrations. *Wat. Res.*, **13**, 995-999.

Van Velsen A. F. M. (1981) *Anaerobic Digestion of Piggery Waste*. Ph.D. Thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

Vega R. E. and Brennan J. G. (1988) Enzymic hydrolysis of fish offal without added water. *J. Food Engg.*, **8**, 201.

Veiga M. C., Mendez R.J. and Lema J. M. (1992) Treatment of tuna processing wastewater in laboratory and pilot scale DSFF anaerobic reactors. In: *46th Purdue Industrial Waste Conference Proceedings*, pp. 447-453. Lewis Publishers, Inc., Chelsea, Michigan, U.S.A.

Veiga M. C., Mendez R. J. and Lema J. M. (1994) Wastewater treatment for fisheries operations. In: *Fisheries Processing: Biotechnological Applications*, Martin A. M. (ed.), pp. 344-369. Chapman & Hall, London.

Verscharen J. A. (1989) An analysis of seafood processing wastewater and potential pretreatment methodology for small seafood processors. In: *Proc. 1989 Food Process. Waste Conf.*, p. 398, Georgia Tech. Res. Inst., U.S.A.

Verstraete W., De Beer D., Pena M., Lettinga G. and Lens P. (1996) Anaerobic bioprocessing of organic wastes. *World J. Microbiol. and Biotechnol.*, **12**, 221-238.

Wang K. (1994) *Integrated Anaerobic and Aerobic Treatment of Sewage*. Ph.D. Thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

Wang K., Zeeman G. and Lettinga G. (1995) Alteration in sewage characteristics upon aging. *Wat. Sci. Technol.*, **31**, 191-200.

Watanabe Y. (1985) Precoat filter. *Chem. Abs.*, **102**, 119039j.

Welsh F. W. and Zall R. R. (1984) An ultrafiltration activated carbon treatment system for renovating fishery refrigeration brines. *Can. Inst. Food Sci. Technol. J.*, **17**, 92.

Weng C. N. and Jeris J. S. (1976) Biochemical mechanisms in the methane fermentation of glutamic and oleic acids. *Wat. Res.*, **10**, 9-18.

Wheaton F. W., Brinsfield R. B., Lomax K. M., Geiger E. L. (1984) Blue crab processing plant effluent treatment. *J. Wat. Pollut. Control Fed.*, **56**, 482-486.

Wheaton F. W. and Lawson T. B. (1985) *Processing Aquatic Food Products*. John Wiley & Sons, Inc., U.S.A.

Zhang T. C. and Noike T. (1991) Comparison of one-phase and two-phase anaerobic digestion processes in characteristics of substrate degradation and bacterial population levels. *Wat. Sci. Technol.*, **23**, 1157-1166.

Zeeman G. (1991) *Mesophilic and Psychrophilic Digestion of Liquid Manure*. Ph.D. Thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

Zeeman G., Sanders W. T .M., Wang K. Y. and Lettinga G. (1997) Anaerobic treatment of complex wastewater and waste activated sludge - application of an upflow solid removal (UASR) reactor for the removal and pre-hydrolysis of suspended COD. *Wat. Sci. Technol.*, **35**, 121-128.

Chapter 2

Anaerobic hydrolysis of proteins and lipids in fish processing wastewater: Effects of pH and methanogenesis

ABSTRACT

Fish processing wastewaters contain high levels of organic matter which are mainly suspended solids. In the anaerobic digestion of such complex wastes, hydrolysis is usually the rate-limiting step. The effects of pH and the presence of methanogenic seed sludge on their degradation may be significant in fish wastewater. Thus, the course of anaerobic digestion was followed in batch reactors with wastewater prepared using Indian sardines (*Sardinella longiceps*) simulating the wastewater generated in sardine cannery. Protein hydrolysis occurred at all the applied pH values (4-9) with or without methanogenic seed sludge. The rate of protein hydrolysis was higher at pH $\geq$ 6 than at lower pH values. Protease activity increased at increasing pH except at pH 8 where a significant decrease is shown. It appears that lipid hydrolysis was enhanced by methanogenesis as lipid hydrolysis occurred in the presence of methanogenesis and at pH $\geq$ 6. pH affected both the occurrence and the rate of lipid acidification. In an experiment where long-chain fatty acids and total lipids were measured, hardly any lipid hydrolysis occurred at pH 4 while both lipid hydrolysis and acidification occurred at pH 8.

Keywords - acidification, anaerobic wastewater treatment, fish processing wastewater, hydrolysis, lipids, long-chain fatty acids, methanogenesis, pH, proteins

INTRODUCTION

The composition of fish processing wastewater varies with respect to the levels of soluble and insoluble organic matter (proteins, fats, and carbohydrates), and the levels of cations and anions, such as Cl^-, SO_4^{2-}, PO_4^{3-} (Nair 1990, Soto *et al.* 1991, Soto *et al.* 1993, Lema *et al.* 1987, Veiga *et al.* 1994). Application of high-rate anaerobic systems for this type of wastewater may require a pre-removal or pre-hydrolysis of suspended solids in order to abate problems in the methanogenic reactor caused by the insoluble fraction, such as sludge flotation and inhibition of methanogenic activity. In order to determine how such systems should be designed and operated, knowledge of the effect of different factors on hydrolysis is necessary. So far, no studies yet focussed on anaerobic hydrolysis of lipids and proteins in fish wastewater.

Anaerobic digestion of proteins and lipids

Anaerobic digestion of proteins and lipids is carried out by different types of bacteria coexisting in syntrophic interdependence, i.e., the product of one is consumed by another bacteria. Proteins are firstly hydrolysed by extracellular enzymes, e.g. peptidases and proteases produced by acidifying bacteria, to amino acids. Amino acids are degraded to short-chain or branched-chain fatty acids with the production of ammonia via different pathways. Pairs of amino acids are degraded via coupled oxidation-reduction known as Stickland reactions carried out by the majority of the known *Clostridium* species. Single amino acids, e.g. glutamate and leucine, are also degraded via hydrogen-producing pathways (Barker 1961, Barker 1981, McInerney 1988). The importance of interspecies hydrogen transfer between hydrogen-producing and hydrogen-consuming bacteria for the complete degradation of some amino acids, e.g. valine, leucine, and isoleucine, was proposed by Örlygsson *et al.* (1994). Some bacteria can acidify amino acids via the Stickland reaction or via the hydrogen-producing pathway (Örlygsson *et al.* 1994). Other studies on this syntrophic relation are included in the review of McInerney (1988). Produced short-chain and branched-chain fatty acids are further converted to acetate.

Lipids are hydrolysed by extracellular enzymes produced by acidifying bacteria to long-chain fatty acids (LCFA) which are degraded via β-oxidation by hydrogen-producing acetogenic bacteria. Methane is produced from acetate by acetotrophic methanogens and from hydrogen and carbon dioxide by hydrogenotrophic methanogens (Hanaki *et al.* 1981, McInerney 1988, Pavlostathis and Giraldo-Gomez 1991). The presence of these hydrogenotrophic methanogens is known to enhance the growth of hydrogen-producing acetogens such as the β-oxidizers.

Hydrolysis is usually considered as the rate-limiting step in the overall process (Eastman and Ferguson 1981, Pavlostathis and Giraldo-Gomez 1991). Nature of proteins, i.e. solubility, tertiary structure, and kind of end group, and pH affect protein hydrolysis (McInerney 1988). Little is known about the effect of different factors on lipid hydrolysis. This study aimed to determine the effects of pH and methanogenesis on hydrolysis of proteins and lipids in fish wastewater. Considering the known interdependence between groups of bacteria, it may be hypothesized that the effect of pH on hydrolysis may be direct or indirect. It may directly affect growth of acidogenic and methanogenic bacteria, enzyme activity, and stability of protein structure. As hydrogenotrophic methanogenesis can stimulate growth of hydrogen-producing bacteria, it may also have an important role in the complete hydrolysis of lipids and proteins.

MATERIALS AND METHODS

Set-up of the batch experiments

The pH, initial (day 0) wastewater compositions and amounts of inoculant for the different experiments are summarized in Table 2.1. Experiment I was performed to determine the effect of pH on hydrolysis in the absence of methanogenesis. Sludge from an anaerobic reactor treating brewery wastewater (Asia Brewery, Philippines) was used. Bromoethane sulfonic acid (BESA) was used as a specific methanogenic inhibitor (Florencio *et al.* 1994). Continuously-stirred 12-liter batch reactors were placed in a room where temperature is

controlled at 30±2°C. In 4 reactors, pH was controlled manually at 5, 6, 7, and 8 every day using 12 M NaOH and 12 M HCl solutions. In one reactor, pH was not controlled nor buffer was added. Inoculant sludge and BESA were also not added in order to determine the rate and extent of hydrolysis without a seed sludge. Soluble COD, volatile fatty acids (VFA) and total NH_4^+-N were measured at designated sampling days.

Experiment II was performed to determine the effect of pH on hydrolysis when methanogenesis is present. The same seed sludge was used as in experiment I but BESA was not added. pH was controlled at 4, 5, 6, 7, and 8 in the same way as in experiment I. Methane was measured by displacement of 3% NaOH solution using a Mariotte flask. The same analytical tests were done in the samples.

Table 2.1: Initial composition of the wastewater, inoculant sludge and BESA used in the batch experiments

experiment #	soluble COD, $mg.l^{-1}$			SS-COD[a], $mg.l^{-1}$		total COD	seed sludge[e]	BESA
	total N	VFA	SNA[b]	protein[c]	lipid[d]	$mg.l^{-1}$	$g\ VS.l^{-1}$	$g.l^{-1}$
I: pH 5-8	343	240 (4%)	353 (5%)	2060 (32%)	3700 (58%)	6360	1	5.3
I: no pH control	343	240 (4%)	353 (5%)	2060 (32%)	3700 (58%)	6360	0	0
II: pH 4-8	312	88 (2%)	255 (4%)	2070 (33%)	3780 (61%)	6190	1	0
IV: pH 4 and 8					450 (12%)	3740	1	0

[a]SS-COD = suspended solids COD = total COD - soluble COD; [b]soluble non-acidified COD = soluble COD - VFA COD; [c]7.74 * total N - soluble COD (Haard *et al.* 1994, Sikorski 1994, Suzuki 1981); [d]2.89 * lipids (Kinsella 1987); [e]specific methanogenic activity = 0.7 g COD. g^{-1} VS. d^{-1}; values in parentheses are percentages of total COD.

In experiment III, protease activity was determined in mixtures of (a) fish wastewater and anaerobically digested primary sludge and (b) same mixture but with dissolved casein added at different pH conditions. pH was firstly adjusted in each of the 250-ml serum flasks containing tap water by addition of 6 M NaOH and 6 M HCl before adding fish slurry. Equal amounts of fish slurry (stored for 1 day at 4°C) were added to all flasks in order to obtain 12.6 $g.l^{-1}$ (wet weight) fish meat. The same amounts of sludge were added. Readjustment of the pH was done after addition of the fish slurry and sludge. On one pair of sample, casein was added, while on another pair, demineralized water was added instead of casein. For the measurement of protease activity, the samples were incubated for 2 hours at 30°C and the hydrolysed substrate before and after incubation was spectrophotometrically measured at 280 nm through 1-cm light path using tryptophan as reference. Toluene was added before incubation to inhibit both the consumption of hydrolysis products and the production of enzymes during incubation (Boschker *et al.* 1995). At the end of incubation, trichloroacetic acid was added to have a resulting 9.1% concentration in order to inhibit enzyme activity.

Lipid hydrolysis and acidification were compared at extreme pH values (4 and 8) in experiment IV. To avoid loss in lipids due to adsorption to reactor walls, the whole content of a replicate flask was taken as a sample. Two sets of 18 serum flasks containing wastewater of the same volume (0.50 l) and concentration were kept at 30±2°C. In each set, pH was

controlled manually every day as in experiments I and II. Each sampling day, 2 flasks of every set were randomly selected for total lipids and LCFA analyses. After filtering the whole content, the flask was dried (103°C) and washed by shaking with extraction solvent. Lipids were extracted from the washings and the dried filtered solids.

Wastewater preparation and nutrients

All the wastewaters used in the experiments were prepared using raw Indian sardines (*Sardinella longiceps*) to simulate the soluble and the insoluble components of wastewaters generated from fish (sardines) canneries. Raw fish (all parts included) were cut into small pieces using a blender equipped with blades in 1 minute with water (160 g fish/400 ml water). The resulting mixture was sieved (1-mm pores) and was diluted with tap water to the desired COD concentration. In all the batch reactor experiments, macro-nutrients (concentrations in $mg.l^{-1}$: 280 NH_4Cl, 250 KH_2PO_4, 100 $MgSO_4.7H_2O$, 10 $CaCl.2H_2O$) and trace elements (concentrations in $\mu g.l^{-1}$: 483 Fe[III], 450 Co, 140 Mn, 11 Cu[II], 24 Zn, 25 Ni) were added.

Analytical methods

Total chemical oxygen demand (COD) was determined by the dichromate reflux method (APHA 1992). Samples for soluble COD analyses were filtered (0.45 μm, MFS cellulose nitrate) and digested in 20-ml covered digestion tubes with proportionally similar amounts of reagents as in the above method. Total N was measured according to the Kjeldahl method (APHA 1992). Total NH_4^+-N was determined by nesslerization (APHA 1992) of diluted filtered (0.45 μm) samples. Absorbance was measured at 425 nm through 1-cm light path. Analyses were duplicated. For each set of measurements, the same procedures were applied to a set of 2-5 blanks. Volatile fatty acids (VFA) were determined on diluted samples (1:1 with 3% formic acid) using gas chromatograph with flame ionization detector, equipped with 6mm x 2mm x 2m glass column with 10% Fluorad 431 on Supelcro - port 100-120 mesh. The carrier gas was N_2 (30 $ml.min^{-1}$, $\pm$ 2 bar) saturated with formic acid. Operating temperatures at the oven, detector, and injector were 130°C, 280°C, and 200°C, respectively.

Lipids were determined according to the Soxhlet extraction method (APHA 1992) using petroleum ether (40-60°C boiling point) as solvent and Whatman filter paper No. 40 and diatomaceous-silica for filtering. LCFAs were separated from neutral fats by running lipids (redissolved in chloroform) through aminopropyl columns (Bond Elut, bonded phase: NH_2, 500 mg/column) and analysed on the gas chromatograph using WAX 58, Chrompack C80C24 (Kalunsy *et al.* 1985).

RESULTS

Hydrolysis at different pH values with and without methanogenic seed sludge

The course of hydrolysis, acidification, methanogenesis and NH_4^+-N production in experiments I (without methanogenesis) and II (with methanogenesis) is illustrated in Fig. 2.1. Results show that protein hydrolysis (as depicted by the NH_4^+-N production) occurred

at all the applied pH values, in the presence or in the absence of seed sludge and with or without methanogenesis at rates which are higher at pH $\geq$ 6 than at pH 4 and 5. The pH of the wastewater to which seed sludge was not added and of which pH was not controlled spontaneously decreased from 6.2 to 5.5 on day 5, increased again and reached 6 on day 10 and 6.2 on day 25 (data not shown). The calculated first-order rate constants (k_h) for protein hydrolysis at the different pH and seeding conditions are summarized in Table 2.2. Comparison of these values shows that (a) without methanogenesis, k_h values at pH 6, 7, and 8 are significantly higher ($p<0.05$) than at pH 5; (b) with methanogenesis, k_h value at pH 8 is significantly higher than at pH 4, 5, 6 and 7; (c) increase in k_h when methanogenesis was present (compared to without methanogenesis) was only significant ($p<0.005$) at pH 8. At pH 6, comparison among k_h values without seed sludge, with seed sludge but without methanogenesis, with seed sludge and with methanogenesis shows no significant differences ($p>0.05$). Comparing these k_h values at pH 6 to the k_h values at pH 5 (with and without methanogenesis) shows significant differences ($p<0.05$) between pH 5 and pH 6. Protein biodegradability does not vary significantly among different pH and seeding conditions.

Without methanogenesis, NH_4^+-N production followed the same course as total acidification (Fig. 2.1). However, the presumed acidified protein COD equivalent of NH_4^+-N, i.e. 7.74 * NH_4^+-N (based on composition of fish proteins, Haard *et al.* 1994, Sikorski 1994, Suzuki 1981) was higher than the measured total acidified COD. In day 15 and at pH 7, the former was 1540 mg.l^{-1} while the latter was 1400 mg.l^{-1}. This indicates that, probably, in the degradation of amino acids, NH_4^+ is firstly produced before the branched-chain or short-chain fatty acids. After 15 days, both the hydrolysed and acidified COD only slowly further increased with time while butyrate and propionate (data not shown) were not further converted to acetate, indicating that the conditions are thermodynamically unfavorable to hydrogen-producing acetogenesis such as β-oxidation of LCFA. Thus, the total acidified COD probably represents mainly acidification of proteins.

With methanogenic seed sludge and in absence of BESA, the hydrolysed and the acidified COD beyond day 15 increased further at pH 6, 7, and 8 while the increase in NH_4^+-N production remained nearly the same (and only slowly increased with time) as at the other pH conditions. The total acidified COD exceeds the equivalent acidified protein COD of the produced NH_4^+-N (Fig. 2.1), e.g. at pH 7, the former increased from 29% (percent based on initial total COD) in day 12 to 67% in day 26 while the latter increased from 23% to 31% in the same period. Moreover, the former exceeds the total percent soluble plus protein-SS COD (6%+33%) in the wastewater. This indicates that aside from proteins, other components of the wastewater, i.e. lipids, were hydrolysed and acidified. The rate of hydrolysis, acidification and methanogenesis (as indicated by their respective slopes in Fig. 2.1 and summarized in Table 2.3) increased with increasing pH at pH $\geq$ 6. When lipid hydrolysis was occurring aside protein hydrolysis, VFA concentrations remained low and mainly consisted of acetate, i.e. 90-105 mg COD.l^{-1} at pH 6 and 60-95 mg COD.l^{-1} at pH 7 and 8. These observed VFA levels and lower observed rates of methanogenesis (Fig. 2.1 and Table 2.3) than the methanogenic activity of the seed sludge (Table 2.1) indicate that methanogenesis is not rate-limiting.

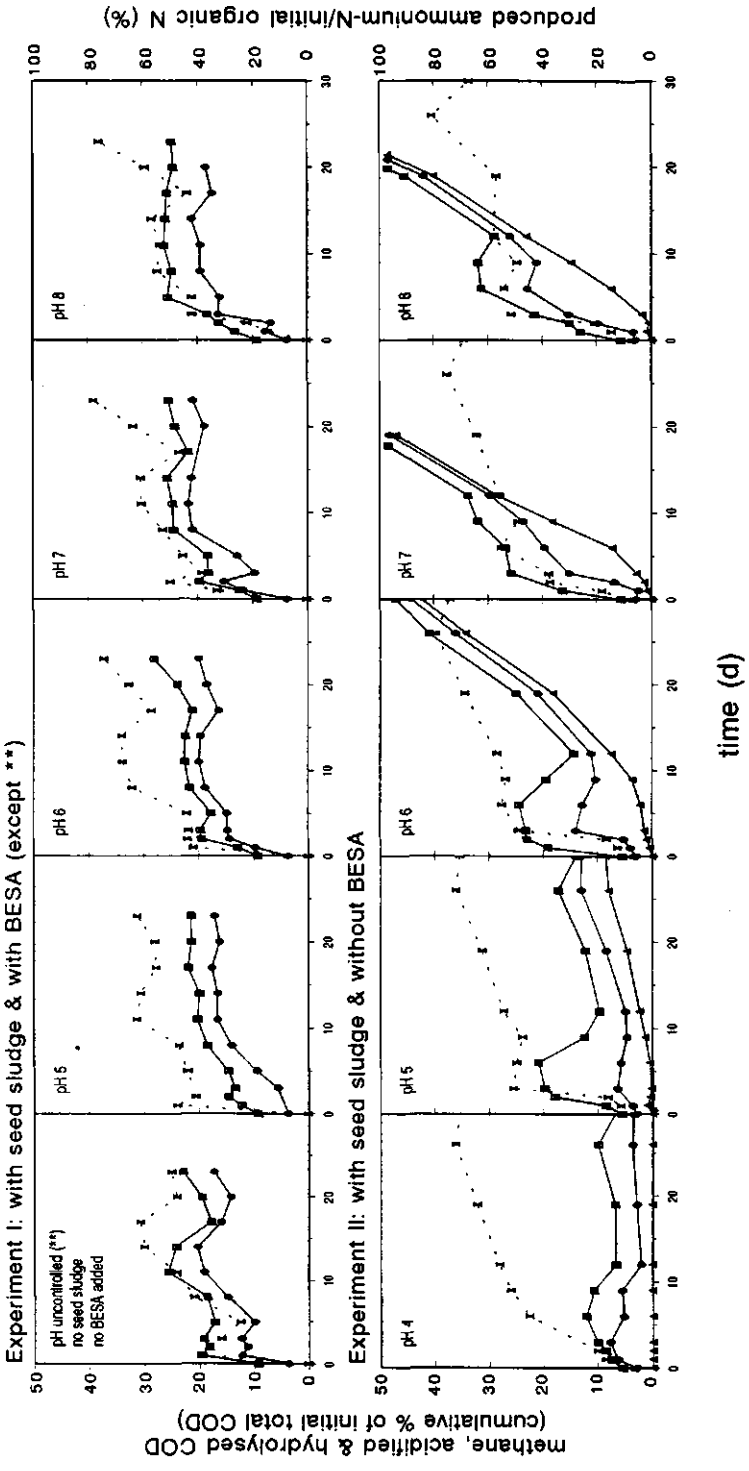

Figure 2.1: Methane (▲), acidified (● = methane + VFA), hydrolysed COD (■ = methane + soluble COD) as cumulative percentage of initial total COD and produced ammonium-N (I) as cumulative percentage of initial organic N at different pH levels without methanogenesis (experiment I: with seed sludge and BESA added) and with methanogenesis (experiment II: with seed sludge and no BESA added). Note: In experiment II, methane conversion, acidification, and hydrolysis continued (not shown in this figure) and were monitored until day 59.

Table 2.2: First-order hydrolysis rate constants (k_h) for proteins in the batch experiments at different pH and seeding conditions

pH	k_h (d^{-1}) no methanogenic seed sludge no BESA no pH control	k_h (d^{-1}) with methanogenic seed sludge with BESA with pH control	k_h (d^{-1}) with methanogenic seed sludge no BESA with pH control
4	--	--	0.086 (c)
5	--	0.081 (c)	0.083 (c)
6	0.092 (b)[#]	0.119 (b)	0.111 (b)
7	--	0.139 (b)	0.094 (bc)
8	--	0.110 (b)	0.177 (a)
time[*] (d)	0-23	0-23	0-26
S_0[#]	0.79	0.79	0.81

[*]time range in the batch experiment and [#]biodegradable fraction (maximum produced NH_4^+-N/organic N) used in k_h calculations; [#]letters in parentheses indicate significant differences between k_h values ($p<0.05$) with a>b>c. Values with the same letters are not significantly different.

Table 2.3: Rates (g $COD.g^{-1}VS.d^{-1}$) of methanogenesis, acidification, and hydrolysis, and levels (mg $COD.l^{-1}$) of VFA and SNA COD in days 20-30[#] in experiment II.

pH	hydrolysis	acidification	methanogenesis	SNA COD	VFA COD
4	nil	0.002	none	90	90
5	0.003	0.020	0.019	48	220
6	0.118	0.130	0.133	75	118
7	0.168	0.164	0.165	218	70
8	0.188	0.200	0.204	45	81

[#]In these period, lipid hydrolysis and acidification occur aside protein hydrolysis at pH 6, 7, and 8.

The observed drop in percent cumulative hydrolysed COD at pH 5 and 6 in days 6-9 of experiment II was probably due to a precipitation of soluble proteins as possibly caused by the addition of acid for pH adjustment. An unmeasured methane COD may explain some of this decrease but such loss is minimal. Gas production is visually slow in this period. After 30 days of digestion at pH 5, gas production ceased. The decrease in acidified COD at pH 6 (experiment II) can not be explained by an error in methane measurement since the initial total COD was completely recovered as methane after 59 days.

Effect of pH on protease activity

The protease activities at different pH values are shown in Fig. 2.2. With (a) only fish proteins and with (b) both fish proteins and casein as substrates, protease activity increased with increasing pH (4-9) except at pH 8 where a significant decrease occurred.

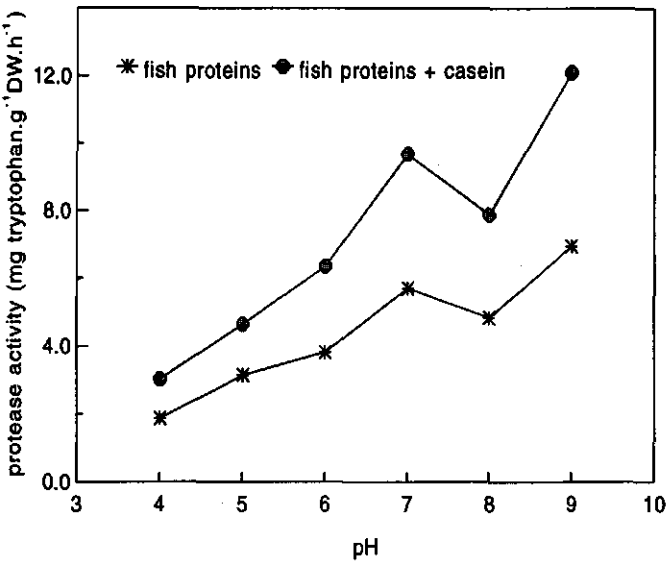

Figure 2.2: Protease activity (mg tryptophan.g^{-1} dry weight.h^{-1}) in a mixture of fish wastewater and sludge at different pH (4-9), with and without casein added (experiment III: incubation temperature = 30°C; hydrolysed substrate measured at 280 nm; the plotted values are averages of duplicate measurements)

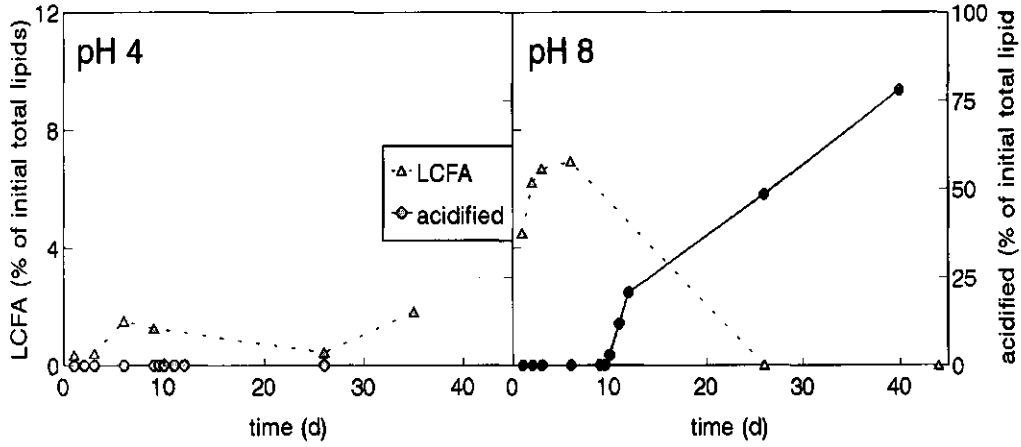

Figure 2.3: Long-chain fatty acids (▲ = LCFA) and acidified lipids (●) as cumulative percentage of initial total lipids at pH 4 and 8, experiment IV (acidified lipids = [total lipids]$_{initial}$ - [total lipids]$_{sampling\ time}$)

Lipid hydrolysis at pH 4 and 8

The amount of LCFA-COD and the calculated amount of acidified lipids at pH 4 and 8 are shown in Fig. 2.3. While lipid acidification has not yet commenced, the percent hydrolysed lipids, as depicted by the percent LCFA-COD, is higher at pH 8 than at pH 4. The percent LCFA-COD remained at 6-7% at days 3-6 while at pH 4, it remained almost constant at 1-2% at days 6-35. At pH 8, hydrolysis continued at a much higher rate once lipid acidification commenced while at pH 4, acidification and further hydrolysis did not occur. During lipid acidification (day 27 and 44), the amount of LCFA was negligible indicating that lipid hydrolysis and not acidification was rate-limiting.

DISCUSSION

Protein hydrolysis occurs at all the applied pH values regardless of the presence of methanogenic inoculum. The hydrolysis rate of proteins is higher at pH $\geq$ 6 than at lower pH values. At pH 8, the rate of protein hydrolysis is higher with than without methanogenesis. The effect of pH on protein hydrolysis can be the net effect of different factors. The low pH values, 4 and 5, are below the iso-electric point (IEP) of myosin (Franks 1993) which is the main fish protein (Suzuki 1981). At the IEP, the secondary structure of proteins is unfolded causing precipitation or decreased solubility. The effect of solubility on the rate of hydrolysis could not be quantified within this research. Less soluble proteins have been found to be more slowly degraded than the more soluble ones (McInerney 1988). At lower pH, there is a higher amount of non-degraded lipids which diminish the accessibility of proteins to hydrolytic enzymes. Similarly, as pH can alter the secondary structure of proteins, it can also inactivate enzymes. The presented results show an increase in protease activity as pH increases from 4 to 9. The reason for the relatively low enzyme activity found at pH 8 is not known. Possibly, the measured activity is a result of the activities of different enzymes having different pH optima. Among the acid and neutral enzymes in fish are pepsin, cathepsin and collagenase (Haard *et al.* 1994). The activity of the enzymes found in fish digestive organs such as trypsin and chymotrypsin (alkaline proteases) is much higher than that of the other proteases found in fish (Sikorski and Ruiter 1994).

As the ratio of tryptophan to total fish proteins is not known, a comparison cannot be made between the measured protease activities in experiment IV and the observed hydrolysis rates in experiments I and II. Thus, whether protein structure or protease activity is rate-limiting could not be assessed in the present research.

As methanogenesis and the β-oxidation of LCFA are inhibited at low pH and in the absence of methanogenesis respectively, acidification of lipids only occurs in the presence of methanogenic inoculum and at pH $\geq$ 6. Not only the occurrence but also the rate of lipid acidification is affected by pH. It appears that lipid hydrolysis is inhibited at pH 4 while at pH 8, it temporarily stagnated but continued at a higher rate once acidification started. It can be hypothesized that methanogenesis enhances lipid hydrolysis by enhancing the β-oxidation of LCFAs. In our follow-up research with fish wastewater (Chapter 3), and in the research of Sanders and Zeeman (pers. comm.) with model lipids, it appeared that in the absence of methanogenesis, lipid hydrolysis is inhibited.

The results of the batch experiments presented here illustrate that when applying a two-phase continuous treatment system to fish processing and other lipid and protein containing wastewaters, hydrolysis of proteins will occur but lipid hydrolysis cannot be expected in the first reactor in absence of methanogenesis.

CONCLUSIONS

Protein hydrolysis occurs at all the applied pH values (4-8) regardless of the presence of methanogenic inoculum. The rate of hydrolysis of proteins is higher at pH $\geq$ 6. Lipid hydrolysis is enhanced by the presence of methanogenesis. pH not only affects the occurrence but also the rate of lipid acidification.

ACKNOWLEDGEMENT

This study was financially supported by the Environmental Ecotechnology Project, a cooperation between International Institute for Infrastructural, Hydraulic and Environmental Engineering (IHE, Delft, The Netherlands), Wageningen Agricultural University (WAU, Wageningen, The Netherlands), and University of the Philippines (Philippines).

REFERENCES

American Public Health Association (APHA), American Waterworks Association (AWWA) and Water and Environment Federation (WEF) (1992) *Standard Methods for the Examination of Water and Wastewater*, 18th edn. Washington DC.

Barker H. A. (1961) Fermentations of nitrogenous organic compounds. In: *The Bacteria*, Vol. 2, Gunsalus I. C. and Stanier R. Y. (eds.), pp. 151-207. Academic Press, New York.

Barker H. A. (1981) Amino acid degradation by anaerobic bacteria. *Ann. Rev. Biochem.* **50**, 23-40.

Boschker H. T. S., Bertilsson S. A., Dekkers E. M. J. and Cappenberg T. E. (1995) An inhibitor based method to measure initial decomposition of naturally occurring polysaccharides in sediments. *Appl. Envi. Microbiol.* **61**, 2165-2192.

Eastman J. A. and Ferguson J. F. (1981) Solubilization of particulate organic carbon during the acid phase of anaerobic digestion. *J. Wat. Pollut. Control Fed.* **53**, 352-366.

Florencio L., Field J. A. and Lettinga G. (1994) The importance of cobalt for individual trophic groups in an anaerobic methanol degrading consortium. *Appl. Environ. Microbiol.* 60, 227-234.

Franks F. (1993) *Protein Biotechnology: Isolation, Characterization, & Stabilization*. Humana Press, Inc., New Jersey.

Haard N. F., Simpson B. K. and Sun Pan B. (1994) Sarcoplasmic proteins and other nitrogenous compounds. In: *Seafood Proteins*, Sikorski Z. E., Sun Pan B. and Shahidi F. (eds.), pp. 13-39. Chapman & Hall, New York.

Hanaki K., Matsuo T. and Nagase M. (1981) Mechanism of inhibition caused by long-chain fatty acids in anaerobic digestion process. *Biotechnol. and Bioengg.* **23**, 1591-1610.

Kalunsy M. A., Duncan L. A., Merill M. V. and Epps D. E. (1985) Rapid separation of lipid classes in high yield and purity using bonded phase columns. *J. Lipid Res.* **25**, 135-140.

Kinsella J. E. (1987) *Seafoods and Fish Oils in Human Health and Diseases*. Marcel Dekker, Inc., New York.

Lema J. M., Soto M., Veiga M. C. and Mendez R. (1987) Mesophilic and thermophilic anaerobic treatment of wastewaters from industrial processing of mussel. In: *Biomass for Energy and Industry*, Grassi G., Delmon B., Molle J. F. and Zibetta H. (eds.), pp. 872-876. Elsevier Applied Science, London.

McInerney J. M. (1988) Anaerobic hydrolysis and fermentation of fats and proteins. In: *Biology of Anaerobic Microorganisms*, Zehnder A. J. B. (ed.), pp. 373-416. John Wiley & Sons, Inc., New York.

Nair C. (1990) Pollution control through water conservation and wastewater reuse in the fish processing industry. *Wat. Sci. Technol.* **22**, 113-121.

Örlygsson J., Houwen F. P. and Svensson, B. H. (1994) Influence of hydrogenothrophic methane formation on the thermophilic anaerobic degradation of protein and amino acids. *FEMS Microbiol. Ecol.* **13**, 327-334.

Pavlostathis S. G. and Giraldo-Gomez E. (1991) Kinetics of anaerobic treatment. *Wat. Sci. Technol.* **24**, 35-59.

Sanders W. and Zeeman G. (pers. comm.) Department of Environmental Technology, Wageningen Agricultural University, Bomenweg 2, 6703 HD (P.O. Box 8129, 6700 EV), Wageningen, The Netherlands

Sikorski Z. E. (1994) The contents of proteins and other nitrogenous compounds in marine animals. In: *Seafood Proteins*, Sikorski Z. E., Sun Pan B. and Shahidi F. (eds.), pp. 6-12. Chapman & Hall, New York.

Sikorski Z. E. and Ruiter A. (1994) Changes in proteins and nonprotein nitrogen in cured, fermented and dried seafoods. In: *Seafood Proteins*, Sikorski Z. E., Sun Pan B. and Shahidi F. (eds.), pp. 113-126. Chapman & Hall, New York.

Soto M., Mendez R. and Lema J. M. (1991) Biodegradability and toxicity in the anaerobic treatment of fish canning wastewaters. *Envi. Tech.* **12**, 669-677.

Soto M., Mendez R. and Lema J. M. (1993) Sodium inhibition and sulphate reduction in the anaerobic treatment of mussel processing wastewaters. *J. Chem. Tech. Biotech.* **58**, 1-7.

Suzuki T. (1981) *Fish and Krill Protein Processing Technology*. Applied Science Publishers, Great Britain.

Veiga M. C., Mendez R. J. and Lema J. M. (1994) Wastewater treatment for fisheries operations. In: *Fisheries Processing: Biotechnological Applications*, Martin A. M. (ed.), pp. 344-369. Chapman & Hall, London.

Chapter 3

Anaerobic hydrolysis of proteins and lipids in fish processing wastewater: Effect of lipid concentration

ABSTRACT

The concentration of lipids can be a significant factor affecting the hydrolysis of both proteins and lipids in fish processing wastewaters. Thus, the course of anaerobic digestion was followed in batch reactors with wastewater prepared using Indian sardines (*Sardinella longiceps*) simulating the wastewater generated in fish canneries. Results showed that increasing the lipid concentration to $\geq$ 4300 mg COD.l^{-1}, the first-order rate constant for proteins significantly decreased ($p < 0.05$). At these concentrations, the biodegradability of proteins also decreased. Increasing levels of initial total lipids increased the length of methanogenic lag periods indicating an inhibition towards methanogenesis. The amounts of lipids which were initially hydrolysed remained constant until methanogenesis had started. At this time, further hydrolysis and acidification of lipids occurred. It appears that methanogenesis enhances lipid hydrolysis. Thus, by inhibiting methanogenesis, high levels of lipids ($\geq$ 4300 mg COD.l^{-1}) will also negatively affect their own hydrolysis.

Keywords - acidification, anaerobic wastewater treatment, fish processing wastewater, hydrolysis, lipids, long-chain fatty acids, methanogenesis, proteins

INTRODUCTION

In anaerobic waste treatment, the hydrolysis of organic polymers such as lipids, proteins and carbohydrates is an important subject of research because it is generally considered as the rate-limiting step in the digestion process (Eastman and Ferguson 1981, Pavlostathis and Giraldo-Gomez 1991). Hydrolysis of proteins is affected by solubility, kind of end group, tertiary structure and pH (McInerney 1988). Little is known about the effects of different factors on lipid hydrolysis. With fish processing wastewater, it was shown in Chapter 2 that pH and the presence of methanogenesis both affected protein and lipid hydrolysis. Protein hydrolysis was found to occur at all the applied pH conditions (4-9) regardless of the presence of methanogenic seed sludge, with higher rate at pH $\geq$ 6 than at lower pH values. It was shown that lipid hydrolysis was enhanced by the occurrence of methanogenesis.

The levels of proteins and lipids in fish processing wastewater vary according to the involved unit operations and the raw materials used (Nair 1990, Veiga *et al.* 1994, Wheaton and Lawson 1985). In finfish species (sardines, herring, mackerel and tuna) which are generally

canned (Wheaton and Lawson 1985, Palomares *et al.* 1986), lipids and proteins constitute 1-16% and 16-25% respectively of the total wet weight of fish (Kinsella 1987). Fatty acid components of these finfish lipids are of 14- to 22- carbon chains most of which are unsaturated (61-81% of the total lipids): 22-54% mono-unsaturated and 19-44% poly-unsaturated (Kinsella 1987) while total fish protein is composed of 18-25% sarcoplasmic (water soluble or extractable by dilute salt solutions), 70-79% myofibrillar (soluble at higher salt concentrations), and 3-5% stroma proteins which are normally insoluble (Suzuki 1981). As lipids can be remarkably high in the raw fish used, the lipid level in the generated wastewater can also be considerably high.

In high-rate anaerobic treatment systems, sludge flotation and deterioration of sludge methanogenic activity are problems often associated with the presence of lipids (Rinzema 1988, Sayed *et al.* 1988). Long-chain fatty acids (LCFAs) which are produced from lipid hydrolysis are found to inhibit methanogenesis (Demeyer and Henderickx 1967, Hanaki *et al.* 1981, Rinzema 1988). Different inhibition mechanisms were hypothesized, one of which is adsorption to sludge thereby hindering substrate and product transport through the bacterial membrane (Demeyer and Henderickx 1967). As both non-hydrolysed lipids and LCFAs have a strong tendency towards adsorption, both may have similar inhibitory effects on methanogens and acidogens. By adsorption, both may hinder attack of non-hydrolysed protein surfaces by extracellular hydrolytic enzymes. Furthermore, as syntrophic interdependence is found to exist between different anaerobic bacteria, e.g. β-oxidizers of LCFAs and hydrogenotrophic methanogens (McInerney 1988), amino acid fermenting bacteria and hydrogenotrophic methanogens (McInerney 1988, Stams and Hansen 1984), inhibited methanogenesis may also result in inhibited hydrolysis of proteins and lipids. Therefore, this study aimed to determine the effect of lipid concentration on the hydrolysis of both proteins and lipids in fish processing wastewater.

MATERIALS AND METHODS

Set-up of the batch experiments

The initial (day 0) wastewater compositions in the batch experiments are summarized in Table 3.1. In experiment I, three dilutions of the same wastewater were prepared and brought into continuously-stirred 12-liter batch reactors, placed at 30±2°C. $NaHCO_3$ (400 $mg.l^{-1}$) was added for buffering at neutral pH. Sludge from an anaerobic reactor treating brewery wastewater (Asia Brewery, Philippines) was used for seeding (0.67 g $VS.l^{-1}$ with specific methanogenic activity of 0.75 $g.g^{-1}$ $VS.d^{-1}$). Soluble COD, volatile fatty acids (VFA) and total NH_4^+-N were measured at designated sampling days. Methane (CH_4) was measured by displacement of 3% NaOH using Mariotte flasks. To avoid inaccuracies in measurement of total lipids and LCFAs due to their adsorption to reactor walls, three sets of 0.50-l flasks (each set containing the same wastewater as its corresponding batch reactor) were prepared at the same time and kept at the same temperature as the batch reactors. Each sampling day, 2 flasks of every set were randomly taken for total lipids and LCFA analyses. After filtering the whole content, the flask was dried at 103°C and washed with extraction solvent. Lipids in the washings and in the dried filtered solids were determined. Experiment II was carried out in the same way as experiment I except that raw fish of the same species but having different lipid contents were used in the preparation of the wastewater.

Table 3.1: Initial wastewater composition in the batch experiments with different dilutions of the same wastewater (I) and with wastewater prepared with fish of different lipid contents (II)

experiment		soluble COD, $mg.l^{-1}$		SS-COD, $mg.l^{-1}$		total COD	%protein	%lipid
and reactor #	total N	VFA	SNA[a]	protein[b]	lipid[c]	$mg.l^{-1}$	COD	COD
I-1	298	83	243	1980	2160	4460	44	48
I-2	602	166	486	4010	4310	8970	44	48
I-3	895	226	752	5950	6470	13390	44	48
II-1	449	130	439	2910	260	3740	78	7
II-2	441	132	406	2880	1040	4460	65	23
II-3	590	145	455	3970	2560	7120	56	36

[a]soluble non-acidified COD = soluble COD - VFA COD; [b]7.74 * total N - soluble COD (Haard *et al.* 1994, Sikorski 1994, Suzuki 1981); [c]2.89 * lipids (Kinsella 1987); SS = suspended solids

Wastewater preparation and nutrients

All the wastewaters used in the experiments were prepared using raw Indian sardines (*Sardinella longiceps*) to simulate the soluble and the insoluble components of wastewaters generated from the fish canneries. Raw fish (all parts, i.e. viscera, head, scale, bones, and flesh, included) were cut into small pieces using a blender equipped with blades (1 minute, 160 g fish/400 ml water). The resulting mixture was sieved (1-mm pores) and was diluted with tap water to the desired COD concentration. In all the batch reactor experiments, macro-nutrients (concentrations in $mg.l^{-1}$: 280 NH_4Cl, 250 KH_2PO_4, 100 $MgSO_4.7H_2O$, 10 $CaCl.2H_2O$) and trace elements (concentrations in $\mu g.l^{-1}$: 483 Fe[III], 450 Co, 140 Mn, 11 Cu[II], 24 Zn, 25 Ni) were added.

Analytical methods

Total chemical oxygen demand (COD) was determined using the dichromate reflux method (APHA 1992). For soluble COD, membrane-filtered (0.45 μm, MFS cellulose nitrate) samples were digested in 20-ml covered digestion tubes with proportionally similar amounts of reagents as in the above method. Total N was measured according to the Kjeldahl method (APHA 1992). Total ammonium-N was determined by nesslerization (APHA 1992) of diluted filtered (0.45 μm) samples. Absorbance was measured at 425 nm through 1-cm light path. Analyses were duplicated. For each set of measurements, the same procedures were applied to a set of 5 blanks. Volatile fatty acids (VFA) were determined on diluted samples (1:1 with 3% formic acid) using gas chromatograph with flame ionization detector and glass column (2 m x 6 mm x 2mm: 10% Fluorad 431 on Supelcro-port, 100-120 mesh). The carrier gas was N_2 (30 $ml.min^{-1}$, $\pm$ 2 bar) saturated with formic acid. Operating temperatures were 130°C, 280°C, and 200°C at the oven, detector, and injector, respectively.

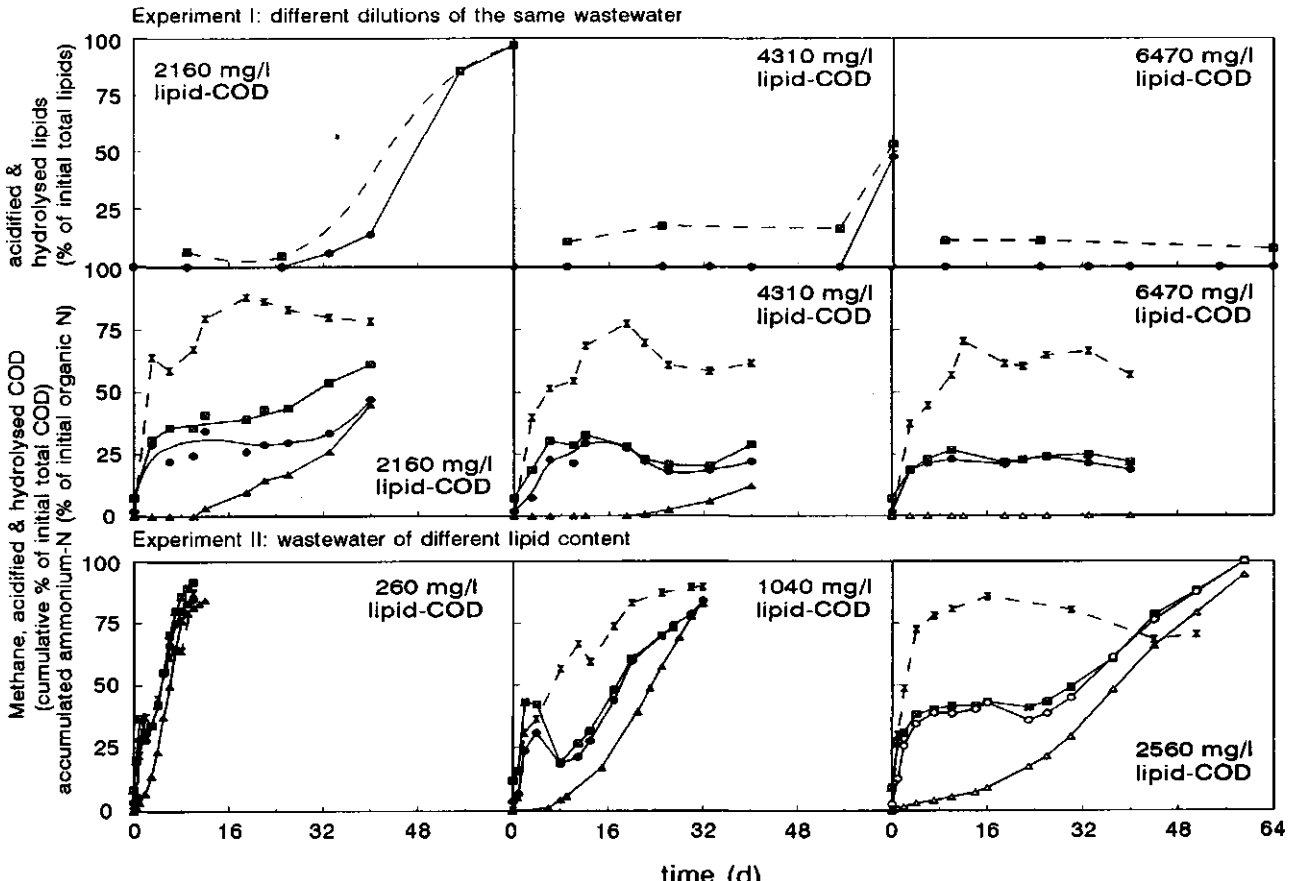

Figure 3.1: (a) Acidified lipids (● = [total lipids]$_{initial}$ - [total lipids]$_{sampling\ time}$), hydrolysed lipids (■ = LCFA COD + acidified lipid COD) as cumulative percent of initial total lipid COD (top: experiment I); (b) Methane (▲), total acidified (● = methane COD + VFA COD), hydrolysed COD (■ = methane COD + soluble COD) as cumulative percent of initial total COD and produced ammonium-N (⧗) as cumulative percent of initial organic N at the different initial total lipid concentrations: experiment I and II

Lipids were determined according to the Soxhlet extraction method (APHA 1992) using petroleum ether (40-60°C boiling point) as solvent and Whatman filter paper No. 40 and diatomaceous-silica for filtering. LCFAs were separated from neutral fats by running lipids (redissolved in chloroform) through aminopropyl columns (Bond Elut, bonded phase: NH_2, 500 mg/column) and analysed on gas chromatograph using WAX 58, Chrompack C80C24 (Kaluzny *et al.* 1985).

RESULTS

The course of hydrolysis, acidification, methanogenesis and ammonium-N production in experiment I and II is illustrated in Fig. 3.1 while the calculated first-order hydrolysis rate constants for protein at different initial lipid concentrations are compared in Table 3.2. At an initial total lipid concentration < 4310 mg.l^{-1}, the course of protein hydrolysis (as depicted by the ammonium-N production) and the k_h for protein hydrolysis (Table 3.2) did not vary significantly at an increasing initial total lipid concentration. At an initial total lipid concentration ≥ 4310 mg.l^{-1}, the k_h of protein hydrolysis was significantly lower ($p < 0.05$) than at lower lipid concentrations. The maximum amount of ammonium-N produced was also lower indicating decreased biodegradability of proteins at these lipid levels. At lower lipid concentrations (<4310 mg.l^{-1}), the hydrolysed and acidified COD increased further while the produced ammonium-N remained almost constant with time indicating that lipids, aside from proteins, were also hydrolysed and acidified. At higher lipid concentrations (≥ 4310 mg.l^{-1}), the course of hydrolysis and acidification remained the same as the course of ammonium-N production, i.e. both became 'stagnant' after the initial fast phase, which means that within 40 days, only proteins were degraded. This observation agrees with the lipid measurements shown in the same figure (Fig. 3.1).

Table 3.2: First-order hydrolysis rate constants (k_h) for protein at different initial protein and lipid concentrations

experiment and reactor #	COD (mg.l^{-1})			k_h (d^{-1})	t[a] (d)	S_o[b]
	protein-SS	lipid	total			
I-1	1980	2160	4460	0.186	0-19	0.90
I-2	4010	4310	8970	0.109 **	0-19	0.90
I-3	5950	6470	13390	0.086 **	0-19	0.90
II-1	2910	260	3740	0.177	0-10	0.90
II-2	2880	1040	4460	0.152	0-32	0.90
II-3	3970	2660	7120	0.189	0-16	0.87

[a]time range where k_h was calculated; [b]the biodegradable fraction used in k_h calculation (obtained maximum ammonium-N as a fraction of initial organic N); ** indicates that k_h value is significantly lower than the other values shown in the same table ($p<0.05$)

It is shown (Fig. 3.1) that there was no increase in the hydrolysed lipids during days 10-25 in the wastewater with 2160 mg.l^{-1} lipid COD and during days 25-55 in the wastewater with 4310 mg.l^{-1}. In these two wastewaters, further hydrolysis and acidification of lipids started

(at day 25 and day 55 respectively) when methanogenesis had started. The amount of lipids hydrolysed when no methanogenesis yet occurred was very low, i.e. 4-17% in the two wastewaters, compared to the amount hydrolysed when methanogenesis was occurring. In the wastewater of 6470 mg.l^{-1} lipid COD, the amount of hydrolysed lipids remained at 10-11% and no lipid acidification was observed within the 64 days of batch digestion. In this wastewater, methanogenesis was not observed within 40 days. The length of the methanogenic lag period increased with increasing initial total lipid COD (Fig. 3.2).

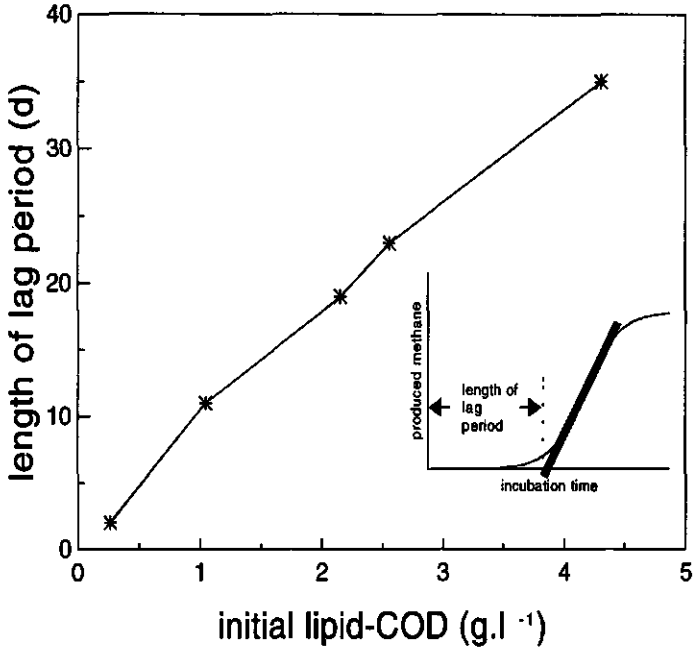

Figure 3.2: Length of the lag period in methanogenesis at the different initial total lipid concentrations. (At initial total lipid concentration of 6.470 g COD.l^{-1}, no methanogenesis occurred in 40 days.)

DISCUSSION

When the initial lipid concentration increased to ≥ 4300 mg.l^{-1}, the rate of protein hydrolysis decreased and the complete degradation of proteins was prevented within 40 days of digestion time. This may be due to physical hindrance by non-hydrolysed lipids and probably also by LCFAs, i.e., covering surfaces of non-hydrolysed proteins so that their accessibility to enzymatic attack is decreased. In our previous study on protein and lipid hydrolysis in fish wastewater (Chapter 2), lipid hydrolysis appeared to be inhibited in the absence of methanogenesis while the protein hydrolysis was not affected by the occurrence of methnanogenesis except at pH 8 (Chapter 2). This indicates that the effect of the lipid concentration on protein hydrolysis is a direct effect (physical hindrance) and not due to the inhibition of methanogenesis.

Increasing initial lipid concentration increased the methanogenic lag period, indicating an inhibitory effect towards methanogenesis. Whether this inhibition was due to either LCFAs or non-hydrolysed lipids, or both of them, was not determined in this research. In batch experiments performed at 37±1°C, Hanaki *et al.* 1981 found increasing methanogenic lag periods of up to 35 days at increasing LCFA (oleate) concentrations of 0.25-2.00 $g.l^{-1}$. In thermophilic (50°C) batch digestion of glyceride trioleate (GTO) at different concentrations (using seed sludge unadapted to oil), Angelidaki *et al.* (1990) observed decreased methane production at GTO concentrations ≥ 2.0 $g.l^{-1}$. At 5.0 $g.l^{-1}$ GTO, no methanogenesis was observed within 60 days incubation period. In another research, Angelidaki and Ahring (1992) observed initial inhibitory levels (also indicated by increased methanogenic lag period) of 0.1-0.2 $g.l^{-1}$ for oleate which is much lower than the initial inhibitory level of GTO. With this observation, they attributed the inhibitory effect of GTO to the free fatty acids produced during its hydrolysis. Under thermophilic conditions, Hwu (1997) similarly found an increasing methanogenic lag period as initial oleate concentration increases. The fraction of the oleate adsorbed to biomass increases as the initial oleate concentration increases and the specific methanogenic activity of the sludge decreases as the amount of adsorbed oleate increases. In the present experiment with different dilutions of the same wastewater, the LCFA concentration reached only about 100 $mg.l^{-1}$ COD (≈ 40 $mg.l^{-1}$ LCFA) and 700-750 $mg.l^{-1}$ COD (≈ 240-250 $mg.l^{-1}$ LCFA) in the wastewater with initial lipid concentration of 2160 and 4310 $mg.l^{-1}$ initial lipid COD respectively. The 40 $mg.l^{-1}$ LCFA level is too low to explain the >10 days methanogenic lag period and the 240-250 $mg.l^{-1}$ LCFA level may be near the above cited initial inhibition concentration but the observed methanogenic lag period of >20 days was relatively long (compared to those observed by Hanaki *et al.* 1981). It must be noted, however, that the above cited studies were performed at higher temperatures. The possibility that both LCFA and non-hydrolysed lipids were causing the inhibitory effect in the present experiment cannot be ruled out.

The results of this chapter and Chapter 2 on hydrolysis of fish proteins and lipids showed that lipid hydrolysis is enhanced by the occurrence of methanogenesis. If methanogenesis is inhibited at higher lipid concentrations, lipid hydrolysis will be inhibited. The mechanism by which methanogenesis enhances lipid hydrolysis could not be determined from these results. It can only be hypothesized that when methanogenesis occurs, the growth of acetogens which carries out the β-oxidation of LCFAs is stimulated. Thus, LCFAs are consumed thereby shifting any equilibrium (if it exists) between lipids and LCFAs towards further hydrolysis.

The results of this study suggest the need to abate the inhibitory effect of lipids on methanogenesis. With two-phase systems, complete lipid degradation cannot be expected in the acid-phase reactor. In a continuous methanogenic reactor, accumulation of lipids in the sludge bed must be avoided. For wastewater of high lipid concentrations, alternative treatment or pre-treatment processes must be explored. A possible option is physical-chemical lipid removal and recovery as fish oil which can be a commercially valuable by-product or digestion or co-digestion at thermophilic conditions.

CONCLUSIONS

Increasing the total lipid concentration (e.g. to ≥ 4300 mg $COD.l^{-1}$ at the temperature and seed sludge conditions in this experiment) decreases both the rate of hydrolysis and the

biodegradability of proteins. At increasing initial total lipid concentration, the degree of inhibition towards methanogenesis also increases. As the hydrolysis of lipids is enhanced by the occurrence of methanogenesis and as methanogenesis is inhibited by high lipid concentrations, higher lipid concentrations will inhibit their own hydrolysis.

ACKNOWLEDGEMENT

This study was financially supported by the Environmental Ecotechnology Project, a cooperation between IHE (Delft, The Netherlands), WAU (Wageningen, The Netherlands), and UP (The Philippines).

REFERENCES

American Public Health Association (APHA), American Waterworks Association (AWWA) and Water and Environment Federation (WEF) (1992) *Standard Methods for the Examination of Water and Wastewater*, 18th edn. Washington DC.

Angelidaki I. and Ahring B. K. (1992) Effects of free long-chain fatty acids on thermophilic anaerobic digestion. *Appl. Microbiol. Biotechnol.* **37**, 808-812.

Angelidaki I., Ahring B. K. and Petersen S. P. (1990) Effects of lipids on thermophilic anaerobic digestion and reduction of lipid inhibition upon addition of bentonite. *Appl. Microbiol. Biotechnol.* **37**, 808-812.

Demeyer D. I. and Henderickx H. K. (1967) The effect of C_{18} unsaturated fatty acids on methane production *in vitro* by mixed rumen bacteria. *Biochim. biophys. Acta* **137**, 484-497.

Eastman J. A. and Ferguson J. F. (1981) Solubilization of particulate organic carbon during the acid phase of anaerobic digestion. *J. Wat. Pollut. Control Fed.* **53**, 352-366.

Haard N. F., Simpson B. K. and Sun Pan B. (1994) Sarcoplasmic proteins and other nitrogenous compounds. In: *Seafood Proteins*, Sikorski Z. E., Sun Pan B. and Shahidi F. (eds.), pp. 13-39. Chapman & Hall, New York.

Hanaki K., Matsuo T. and Nagase M. (1981) Mechanism of inhibition caused by long-chain fatty acids in anaerobic digestion process. *Biotechnol. and Bioengg.* **23**, 1591-1610.

Hwu, Ching-Shyung (1997) *Enhancing Anaerobic Treatment of Wastewaters Containing Oleic Acid*. Ph.D. Thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

Kaluzny M. A., Duncan L. A., Merill M. V. and Epps D. E. (1985) Rapid separation of lipid classes in high yield and purity using bonded phase columns, *J. Lipid Res.* **25**, 135-140.

Kinsella J. E. (1987) *Seafoods and Fish Oils in Human Health and Diseases*. Marcel Dekker, Inc., New York.

McInerney J. M. (1988) Anaerobic hydrolyis and fermentation of fats and proteins. In: *Biology of Anaerobic Microorganisms*, Zehnder A. J. B. (ed.), pp. 373-416. John Wiley & Sons, Inc., New York.

Nair C. (1990) Pollution control through water conservation and wastewater reuse in the fish processing industry. *Wat. Sci. Technol.* **22**, 113-121.

Palomares T. S., Gonzalez O. N., Sonsing R. and Tanyag L. M. (1986) Fish processing in the Philippines. In: *Proceedings of the First ASEAN Workshop on Fish and Fish Waste Processing and Utilization*, pp. 250-273. Jakarta.

Pavlostathis S. G. and Giraldo-Gomez E. (1991) Kinetics of anaerobic treatment. *Wat. Sci. Technol.* **24**, 35-59.

Rinzema A. (1988) *Anaerobic Treament of Wastewater with High Concentrations of Lipids or Sulfate*. Ph.D. Thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

Sayed S., Van der Zanden J., Wijffels R. and Lettinga G. (1988) Anaerobic degradation of the various fractions of slaughterhouse wastewater. *Biological Wastes*, 23, 117-142.

Sikorski Z. E. (1994) The contents of proteins and other nitrogenous compounds in marine animals. In: *Seafood Proteins*, Sikorski Z. E., Sun Pan B. and Shahidi F. (eds.), pp. 6-12. Chapman & Hall, New York.

Stams A. J. M. and Hansen T. A. (1984) Fermentation of glutamate and other compounds by *Acidaminobacter hydrogenoformans* gen. nov. sp. nov., an obligate anaerobe isolated from black mud. Studies with pure cultures and mixed cultures with sulfate-reducing and methanogenic bacteria. *Arch. Microbiol.* **137**, 329-337.

Suzuki T. (1981) *Fish and Krill Protein Processing Technology*. Applied Science Publishers, Great Britain.

Veiga M. C., Mendez R. J. and Lema J. M. (1994) Wastewater treatment for fisheries operations. In: *Fisheries Processing: Biotechnological Applications*, Martin A. M. (ed.), pp. 344-369. Chapman & Hall, London.

Wheaton F. W. and Lawson T. B. (1985) *Processing Aquatic Food Products*. John Wiley & Sons, Inc., U.S.A.

Chapter 4

The effects of NaCl and NH_4^+ on the anaerobic degradation of proteins and lipids in fish processing wastewater

ABSTRACT

Wastewaters generated by fish processing industries can contain high levels of NaCl when sea water is used in the unit operations. Considerable amounts of NH_4^+ are also produced from the degradation of proteins and non-protein nitrogenous compounds from fish. As these wastewaters contain high concentrations of organic matter, anaerobic treatment is energetically more attractive than aerobic treatment and therefore may be economically more feasible. The hydrolysis of non-dissolved organic matter is generally considered as the rate-limiting step of the whole anaerobic process. Thus, it is important to determine the effects of NaCl and NH_4^+ on the hydrolysis of the non-dissolved components, i.e. proteins and lipids, in fish processing wastewater. Artificially generated fish wastewaters having different NaCl and NH_4^+ concentrations were digested in batch flasks at 30±2°C. The results show that at increasing NH_4^+-N concentrations (0-1.5 $g.l^{-1}$), lipid acidification was not affected. The rate of methanogenesis slowly decreased at increasing NH_4^+-N concentration from 0.6 to 1.5 $g.l^{-1}$. The initial rate of protein hydrolysis was not affected but at 0.6-1.5 $g.l^{-1}$ NH_4^+-N, the amino acid levels were high, indicating an inhibition of the acidogens. When NaCl concentration was increased from 0 to 19.9 $g.l^{-1}$, the rate of protein hydrolysis decreased. As protease activity was not negatively affected by NaCl at the applied concentrations, this effect must be an inhibition of protease production or a direct effect on the protein acidifying bacteria. Higher concentration of soluble non-acidified COD at 19.9 $g.l^{-1}$ NaCl, indicates a direct effect on the acidogens. The resulting high concentration of amino acids probably inhibited protease production. At increasing NaCl concentration, the length of the lag periods in methanogenesis and lipid acidification gradually increases but the rates of these two processes do not change.

Keywords - ammonium, anaerobic wastewater treatment, chlorides, fish processing wastewater, hydrolysis, lipids, methanogenesis

INTRODUCTION

Fish processing wastewater may contain high levels of salts, e.g. Na^+, SO_4^{2-}, K^+, Ca^{2+}, Mg^{2+} (Soto *et al.* 1991, Soto *et al.* 1993, Veiga *et al.* 1994) in addition to high levels of soluble and insoluble organic matter. As these salts can come from both the raw material and the water used in the involved manufacturing processes, their levels in the wastewater vary

between factories and between different product lines. High amounts of NH_4^+ may be generated due to the degradation of proteins and non-protein nitrogenous (NPN) compounds from fish. The levels of these salts may have implications on the choice of the wastewater treatment technology. The possibility of the application of anaerobic processes and the toxicity or inhibition of some of the present salts have been recently considered for fish processing wastewaters by Boardman *et al.* (1995), Soto *et al.* (1991) and Soto *et al.* (1993).

Low NH_4^+ concentrations should have a beneficial effect on anaerobic processes as NH_4^+ is an important nutrient source for the anaerobic bacteria (McInerney 1988). At high concentrations, it was found to inhibit methanogenesis. Different inhibition threshold levels were reported. Hobson and Shaw (1976) observed partial and complete inhibition of methane production at 3.0 and 4.0 $g.l^{-1}$ NH_4^+-N respectively at pH 7.1 with a methanogenic bacteria isolated from a piggery waste digester operating at an average NH_4^+-N concentration of 1.5 $g.l^{-1}$. Soto *et al.* (1991) observed 50% and 80% inhibition of methanogenesis at 2.8 and 4.4 $g.l^{-1}$ NH_4^+-N respectively at pH 7.4 with sludge adapted to 1.1-1.2 $g.l^{-1}$ NH_4^+-N. Van Velsen (1979) observed gradually decreasing maximum gas production rate at increasing NH_4^+ concentration from 0.6 to 3.1 $g.l^{-1}$ NH_4^+-N at pH 7.5-7.6 for sludge acclimated to 2.4 $g.l^{-1}$ NH_4^+-N while with a sludge acclimated to 0.8 $g.l^{-1}$ NH_4^+-N, he also found increasing lag phase in methane formation at increasing NH_4^+ concentration from 0.7 to 5.0 $g.l^{-1}$ at pH 7.2-7.4. With a seed sludge from a cow slurry digester in which NH_4^+-N was 2.5 $g.l^{-1}$, Zeeman (1991) also observed decreasing methane production from a mixture of animal wastes with increasing NH_4^+ concentration from 1.2 to 4.9 $g.l^{-1}$ at pH 7.6-7.9. These inhibition effects have been attributed to the free NH_3 (present in equilibrium with NH_4^+) which is inhibitory at concentrations beyond 80-150 $mg.l^{-1}$ (De Baere *et al.* 1984, McCarty and McKinney 1961). The NH_4^+ tolerance level of sludge is dependent on its adaptation. De Baere *et al.* (1984) observed that 50% inhibition of methanogenic sludge occurred at 11.9 and 9.2 $g.l^{-1}$ NH_4^+-N in sludge receiving gradually increasing NH_4^+ and sludge receiving shock concentrations, respectively. The research of Zeeman (1991) on mesophilic digestion of animal wastes indicates that the hydrolysis of suspended solids is also inhibited at increased NH_4^+-N concentrations in the range of 1.2 to 4.9 $g.l^{-1}$. The results of Van Velsen (1981) on piggery waste digestion, with seed sludge adapted to 1.9 $g.l^{-1}$ NH_4^+-N, also indicated decreasing hydrolysed COD at increasing NH_4^+-N concentration from 2.1 to 5.3 $g.l^{-1}$ (pH 7.5-7.9).

NaCl may affect biochemical processes in different ways as it may directly affect protein solubility, enzyme activity, and the bacteria which carry out these processes using intracellular and extracellular enzymes. NaCl enhances protein solubility while most salts cause proteins to precipitate at ionic levels > 0.15 M (Franks 1993). NaCl inhibition on non-halophilic bacteria is hypothesized to be due to (1) dehydration as a consequence of increased osmotic pressure, (2) inhibition of the activity of intracellular enzymes thus affecting bacterial metabolism, and (3) a physical effect on bacterial cell walls that leads to inhibited cell wall functions (Larsen 1962). Some microorganisms are more salt tolerant than others (Larsen 1962). Some studies (De Baere *et al.* 1984, Lema *et al.* 1987) showed the possibility of adapting methanogenic sludge to high concentrations of NaCl.

Most of the studies on the effects of salts on anaerobic wastewater treatment processes focussed on their effects on methanogenesis. Little is known about the effects of different anions and cations on hydrolysis which is commonly considered as the rate-limiting step in

the whole anaerobic digestion process (Eastman and Ferguson 1981, Pavlostathis and Giraldo-Gomez 1991). This study aimed to determine the effect of NaCl and NH_4^+ on the hydrolysis of proteins and lipids at the concentration range of these ions in the wastewater from the canning of finfishes, e.g. sardines, mackerels, and tuna.

MATERIALS AND METHODS

Set-up of the batch experiments

In experiment I, different amounts of NaCl were added to wastewater in 1.0-liter serum flasks and kept at 30±2°C. Of each salt concentration, four 1.0-liter replicates were prepared. In 2 flasks, methane production was measured by displacement of 3% NaOH solution using Mariotte flasks. In the other 2 flasks, samples were taken for soluble COD, volatile fatty acids (VFA) and total NH_4^+-N analyses. Time series samples for total lipids analyses could not be accurately obtained from one flask due to the adsorption of lipids to the flask walls. Thus, additionally, 5 sets of 0.50-l serum flasks (each set containing wastewater of the same NaCl concentration) were kept under the same temperature conditions. At each sampling time, 2 flasks of each set were randomly taken (each as one whole sample) for total lipids analyses. Each flask, after emptying, was dried at 103°C and washed thoroughly with extraction solvent. The washings of each flask were added to the solvent used to extract lipids from the dried filtered solids of the respective sample. Every day, each serum flask was shaken for about 30 seconds. A separate experiment (II) was similarly carried out as experiment I except that varying amounts of NH_4Cl were added instead of NaCl.

Wastewater composition, sludge and nutrients

All the wastewaters used in the experiments were prepared using raw Indian sardines (*Sardinella longiceps*) to simulate the soluble and the insoluble components of the wastewaters generated from fish canneries. Raw fish (all parts included) were cut into small pieces (using a blender equipped with blades) for 1 minute with water (160 g fish/400 ml water). The resulting slurry was sieved (1-mm pores) and was diluted with tap water to the desired COD concentration. The initial wastewater composition is shown in Table 4.1. Macro-nutrients (concentrations in $mg.l^{-1}$): 280 NH_4Cl (different amounts were added in experiment II), 250 KH_2PO_4, 100 $MgSO_4.7H_2O$, 10 $CaCl_2.2H_2O$) and trace elements (concentrations in $\mu g.l^{-1}$: 483 Fe[III], 450 Co, 140 Mn, 11 Cu[II], 24 Zn, 25 Ni) were added to each wastewater. $NaHCO_3$ (0.400 $g.l^{-1}$) was added for buffering at neutral pH. The seed sludge used was taken from an anaerobic reactor treating brewery wastewater in which NaCl was negligible. No specific procedure was done to acclimate the seed sludge to high salt concentrations before seeding. The NH_4^+-N concentrations in the sludge was 46 $mg.l^{-1}$. To remove any residual soluble COD and other salts, the seed sludge was washed with tap water, settled and decanted (repeated 2-3 times).

Table 4.1: Initial composition[#] of the wastewater and amount of seed sludge

	N (mg.l^{-1})		soluble (mg.l^{-1})		SS-COD (mg.l^{-1})		total	% of totalCOD			pH	seed
Experiment	total	NH_4^+	VFA	SNA[a]	protein[b]	lipids[c]	COD	sol	pr	lip		gVS.l^{-1}
I ("NaCl)	521	15	170	447	3420	3270	7300	8	47	45	6.5-7.5	0.7
II ("NH_4^+)	413	7	142	396	2660	1400	4600	12	58	30	6.7-7.0	0.7

[#]before addition of NaCl or NH_4Cl; ["]variable concentration in the experiment (initial NaCl concentration is 0.20 g.l^{-1}); [a]soluble non-acidified COD = soluble COD - VFA COD; [b]7.74 * total N - soluble COD (Haard *et al.* 1994, Sikorski 1994, Suzuki 1981); [c]lipids * 2.89 (Kinsella 1987); sol = soluble; pr = proteins; lip = lipids

Analytical methods

Total chemical oxygen demand was determined by the dichromate reflux method (APHA 1992). Samples for soluble COD analyses were filtered (0.45 μm MFS cellulose nitrate) and digested in 20-ml covered digestion tubes with proportionally similar amounts of reagents as in the above method. Total N was measured according to the Kjeldahl method (APHA 1992). Total NH_4^+-N was determined by nesslerization (APHA 1992) of diluted filtered (0.45 μm) samples. Absorbance was measured at 425 nm through 1-cm light path. Analyses were duplicated. For each set of measurements, the same procedures were applied to 2-5 blanks.

Volatile fatty acids (VFA) were determined on diluted samples (1:1 with 3% formic acid) using gas chromatograph (GC) with FID, equipped with 2 m (6mm x 2mm) glass column with 10% Fluorad 431 on Supelcro - port 100-120 mesh. The carrier gas was N_2 (30 ml.min^{-1}, $\pm$ 2 bar) saturated with formic acid. Operating temperatures at the oven, detector, and injector were 130°C, 280°C, and 200°C, respectively. Lipids were determined according to the Soxhlet extraction method (APHA 1992) using petroleum ether (40-60°C boiling point) as solvent and Whatman filter paper No. 40 and diatomaceous-silica for filtering.

Effect of NaCl on protease activity

A separate experiment was performed to determine the effect of NaCl on protease activity of a mixture of fish wastewater and anaerobically digested primary sludge. To the flasks containing the same amounts of fish slurry (stored for 2 days at 4°C) and sludge, different amounts of NaCl were added. Two pairs of samples were obtained from these flasks: on one pair, casein was added while on another pair, demineralized H_2O was added instead. For the measurement of protease activity, toluene was added to the samples to inhibit both the consumption of hydrolysis products and the production of enzymes during incubation (Boschker *et al.* 1995). After 2 hours of incubation at 30°C, trichloroacetic acid was added to have a resulting 9.1% concentration in order to inhibit enzyme activity. The produced hydrolysed substrate was spectrophotometrically measured at 280 nm through 1-cm light path using tryptophan as reference.

RESULTS

The course of lipid conversion is shown in Fig. 4.1. A clear lag phase in lipid conversion occurs in both experiments I and II. The lengths of the lag phases and the maximum rates in lipid conversion and methane production at different NaCl and NH_4^+-N concentrations are summarized in Table 4.2. At increasing NaCl concentration, the length of lag periods in methanogenesis and lipid conversion similarly increases while the rates of these two processes do not vary. At increasing NH_4^+-N concentration, the methane production rate slowly decreases while the length of the lag periods in methanogenesis and lipid conversion, and the lipid conversion rate do not vary.

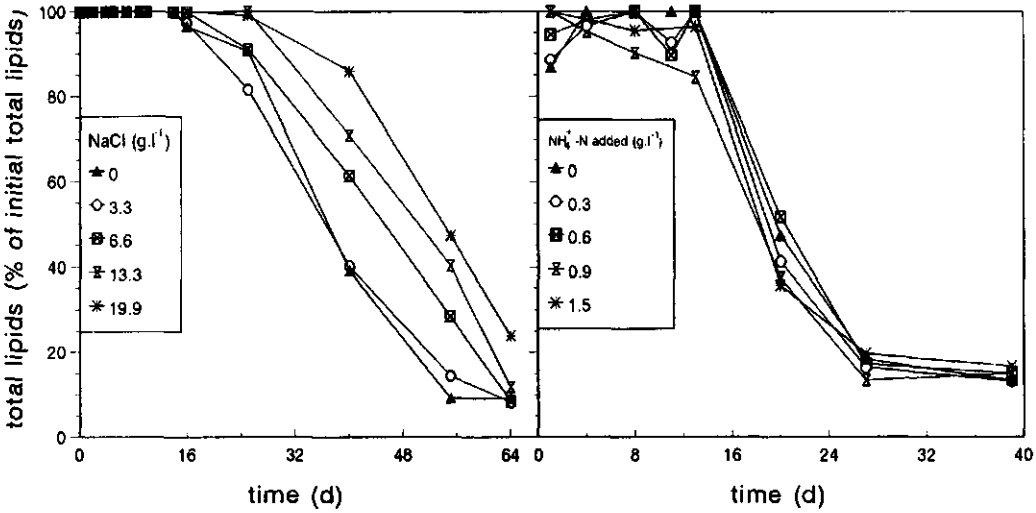

Figure 4.1: Total lipids as percentage of initial total lipids at different levels of NaCl and NH_4^+-N (experiment I and II, respectively)

Table 4.2: Length of lag phase (d) and maximum rates (g COD.g $VS^{-1}.d^{-1}$) in methane production and lipid conversion at different levels of NaCl and NH_4^+

	Expt. I (varying NaCl levels, $g.l^{-1}$)					Expt. II (varying NH_4^+-N levels, $g.l^{-1}$)				
	0	3.3	6.6	13.3	19.9	0	0.3	0.6	0.9	1.5
methanogenic lag	19	22	26	30	34	11	11	12	13	13
lipid conversion lag	23	22	22	28	34	13	13	13	13	13
methane prod'n rate	0.274	0.312	0.310	0.304	0.307	0.345	0.290	0.309	0.276	0.227
lipid conversion rate	0.105	0.105	0.097	0.093	0.118	0.151	0.169	0.137	0.134	0.173

The NH_4^+-N production at different NaCl and NH_4^+-N concentrations is illustrated in Fig. 4.2. At all the applied NaCl levels in experiment I, the acidified protein as depicted by the produced NH_4^+-N reached plateau points after about 10 days of batch digestion. The plateau level in the produced NH_4^+-N was lower at 19.9 $g.l^{-1}$ NaCl than at lower NaCl

concentrations. The NH_4^+-N increased slightly in the following 41 days of digestion, but still the lowest percent of protein acidification was found at NaCl concentration of 19.9 $g.l^{-1}$ (Table 4.3). In experiment II, NH_4^+-N production similarly slowed down after 8 days of digestion at higher levels of NH_4^+-N, indicating that protein acidification is inhibited. Except for the highest NH_4^+ concentration applied, acidification of proteins continued after about 14 days of batch digestion. The first-order rate constant for NH_4^+-N production and the percentage of acidification for proteins are presented in Table 4.3.

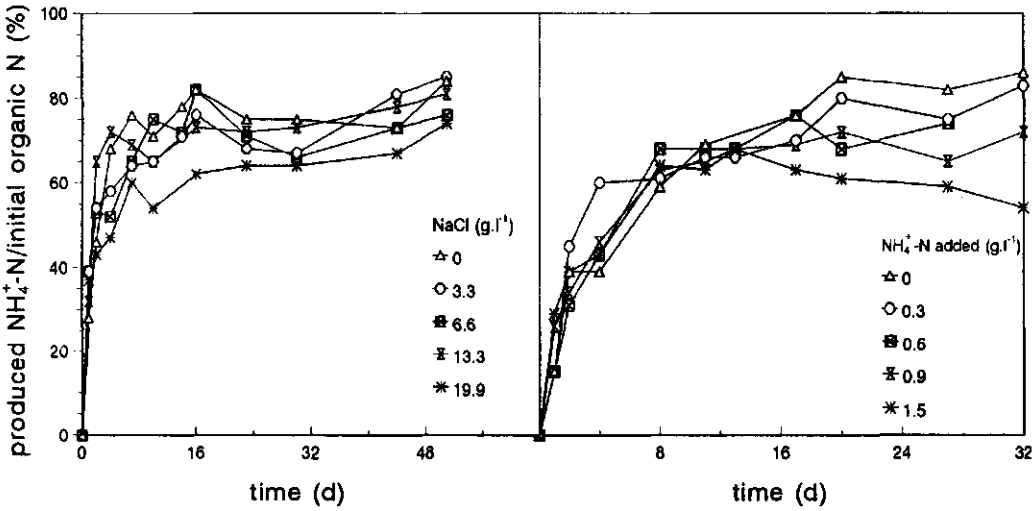

Figure 4.2: Ammonium production as percentage of total N (excluding added NH_4^+-N) at different levels of NaCl and NH_4^+-N (experiment I and II, respectively)

The pH values did not differ among flasks with different amounts of NH_4^+-N initially added. The pH of the digesting fish wastewater mixtures (data not shown) was 6.7-7.0 during the first day, slowly decreased to 6.4-6.6 during day 2 to day 8 and increased afterwards to 6.9-7.3 at days 20-32.

When SS-COD hydrolysis is considered (results not shown), instead of the separate hydrolysis of proteins and lipids, three phases can be considered:

- a rapid increase due to hydrolysis of proteins
- a stagnant period where still no lipid hydrolysis is occurring and protein hydrolysis is also stagnated
- a period with linear increase, occurring simultaneously with methanogenesis, where mainly lipid hydrolysis and also some protein hydrolysis occur.

The first-order rate constant for the hydrolysis of proteins in the first phase and the percentage hydrolysis of SS, during the 'stagnant period' and at the end of batch digestion are presented in Table 4.3. The levels of dissolved products during the 'stagnant period' are shown in Table 4.4. The SNA COD levels were higher while the VFA levels were lower at

higher NaCl levels. The SNA COD levels were higher at higher NH_4^+-N concentrations while the VFA levels were higher at 0.6-0.9 $g.l^{-1}$ NH_4^+-N.

Table 4.3: Initial first-order rate constant for NH_4^+ production (k_N[@]) and hydrolysis of SS-COD (k_h[@]), percent acidified proteins[#] and percent hydrolysed[*] SS-COD at different levels of NaCl and NH_4^+-N

	Expt. I (varying NaCl levels, $g.l^{-1}$)					Expt. II (varying NH_4^+-N levels, $g.l^{-1}$)				
	0	3.3	6.6	13.3	19.9	0	0.3	0.6	0.9	1.5
k_N first 10 (8)[†] d	0.228	0.175	0.212	0.208	0.135	0.156	0.193	0.183	0.175	0.180
(comparison[‡] among k_N)	(a)	(ab)	(ab)	(ab)	(b)	(d)	(c)	(c)	(c)	(c)
% acidified proteins										
after 10[a] (11) d	75	67	75	72	61	69	68	68	66	67
after 51[b] (32) d	85	86	77	82	74	86	83	83	73	57
Hydrolysis of proteins:										
k_h first 10 (8) d	0.271	0.215	0.246	0.224	0.131	0.165	0.180	0.194	0.196	0.192
(comparison[‡] among k_h)	(a)	(ab)	(ab)	(ab)	(b)	(d)	(c)	(c)	(c)	(c)
% hydrolysed SS-COD:										
at plateau phase	34	32	39	38	27	22	21	22	23	20
after 64[c] (32) d	93	96	96	87	73	84	81	83	68	52

[@]Obtained by regression as the slope of the line $\{-\ln(S/S_0) = kt\}$ for the specified period where t = digestion time. k = first-order rate constant (k_N or k_h), S = [(initial biodegradable total N) - (NH_4^+-N)] and S_0 = [(initial biodegradable total N) - (initial NH_4^+-N)] for calculating k_N, while S = [(initial biodegradable protein-SS COD) - (methane COD + soluble COD - initial soluble COD)] and S_0 = (initial biodegradable protein-SS COD) for calculating k_h; The biodegradable fraction of total N and protein-SS COD are the obtained maximum values of the conversion of total N to NH_4^+-N (86%) and the hydrolysis of protein-SS COD (86%) in the digestion experiment. In the calculation of k_h, it was assumed that only proteins are degraded during the lag period in lipid conversion. In calculating for k_N in expt. II, the added NH_4^+-N was excluded in the total N and NH_4^+-N;

[#]{[(NH_4^+-N)-(NH_4^+-N added)]/[total N excluding NH_4^+-N added]}*100;

[*][(soluble COD + methane COD - initial soluble COD)/(initial SS-COD)]*100;

[†]time values in parentheses refer to experiment II;

[‡]letters indicate comparison between k values (a>b and c>d) using the criteria $p<0.05$ for significant difference: Values with common letters are not significantly different.

[a]when NH_4^+ production rate reached plateau levels at higher NaCl or NH_4^+-N concentrations;

[b]last measurement of NH_4^+ (end of batch experiment);

[c]after the linear phase in hydrolysis of total COD

Table 4.4: Different fractions of soluble COD ($mg.l^{-1}$) at different concentrations of NaCl and NH_4^+-N during the 'stagnant period' in NH_4^+ production and SS hydrolysis (days 10-19 and 8-13 in experiments I and II, respectively)

	Expt. I (varying NaCl levels, $g.l^{-1}$)					Expt. II (varying NH_4^+-N levels, $g.l^{-1}$)				
	0	3.3	6.6	13.3	19.9	0	0.3	0.6	0.9	1.5
soluble COD	2383	2321	2836	2685	2235	865	807	1132	1162	1095
SNA COD	116	81	176	435	490	133	210	289	282	308
VFA COD	2268	2240	2660	2250	1745	732	596	843	880	786
acetate	725	634	800	640	508	200	183	240	241	254
propionate	379	412	581	447	268	101	102	158	170	155

Figure 4.3 shows the effect of NaCl on the activity of protease in fish wastewater and sludge with (a) fish proteins and (b) both fish proteins and casein present as substrates. Protease activity does not change significantly with increasing NaCl in the mixture of fish proteins + casein as substrates. With only fish proteins as substrate and at NaCl concentration below 20 $g.l^{-1}$, the protease activity increases linearly at increasing NaCl concentration.

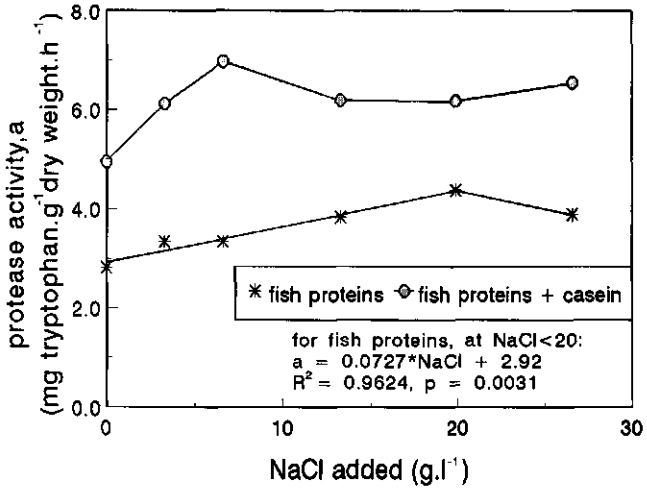

Figure 4.3: Protease activity (mg tryptophan.g^{-1} dry weight.h^{-1}) in a mixture of fish wastewater and sludge (with and without casein added) at different levels of NaCl (incubation temperature = 30°C; pH = 6.2; hydrolysed substrate measured at 280 nm; the plotted values on this graph are averages of duplicate samples)

DISCUSSION

Effect of NaCl and NH_4^+-N on lipid conversion

The extended lag in the lipid acidification at increasing concentration of NaCl probably can be due to inhibition of methanogenesis by NaCl. An inhibition of the latter is indicated by similarly increasing lag in methanogenesis. In Chapters 2 and 3, we found that hardly any hydrolysis of lipids occurs without methanogenesis. Both hydrolysis of lipids and β-oxidation of the LCFAs occur when methanogenesis has started. In Chapter 3, we found that the methanogenic lag increases at increasing lipid concentration. Thus, the observed methanogenic lags in the present experiment can be due to both the lipid and NaCl contents of the wastewater. Based on relation between the lipid level and the methanogenic lag phase as found in Chapter 3, the methanogenic lag phase for the present wastewater that contains 3270 $g.l^{-1}$ lipid COD would be about 25 days when no NaCl is present. At the higher NaCl levels, the length of this lag phase is increased. The maximum methane production rate remains unaffected by the increasing NaCl level indicating that it is possible to adapt the methanogenic sludge to the applied NaCl levels in this experiment.

Lipid acidification remains also unaffected at the imposed NH_4^+ concentrations (0-1.5 $g.l^{-1}$) while the rate of methanogenesis slowly decreases as NH_4^+-N level increases from 0.6 to 1.5

$g.l^{-1}$. With a sludge adapted to 2.4 $g.l^{-1}$ NH_4^+-N, Van Velsen (1979) also observed a gradual decrease in methane production rate at increasing NH_4^+-N concentration from 0.6 to 3.1 $g.l^{-1}$ at pH 7.5-7.6. The mechanism of inhibition of methanogenesis by NH_4^+ was not investigated in this study. As the pH remained quite low, likely the free NH_3 concentration could not reach an inhibitory level. In experiments concerning the thermophilic digestion of cow slurry, Wiegant and Zeeman (1986) hypothesized an inhibition scheme in which the H_2-consuming methanogens are presumed to become inhibited and the resulting increase in H_2 partial pressure then inhibits the propionate degradation to acetate. The accumulated propionate inhibits acetotrophic methane formation. On the contrary, the results of Koster and Lettinga (1984), at NH_4^+-N level of 0.68-2.6 $g.l^{-1}$, indicate that the acetotrophic methanogens are more susceptible to NH_4^+ inhibition than the hydrogenotrophic methanogens. This is confirmed by the results of Borja *et al.* (1996) that, in addition, show that both acetothrophic and hydrogenotrophic methanogens are negatively affected by increasing NH_4^+-N level (0 to 14 $g.l^{-1}$). In the present experiment, the relative concentrations of the VFAs do not show any difference between the susceptibility of hydrogenotrophic and acetotrophic methanogens to NH_4^+ inhibition. Both the concentrations of acetate and propionate during the 'stagnant period' in SS-COD hydrolysis and NH_4^+ production, i.e. before methanogenesis, were higher (by 50-70% and 20-25%, respectively) at 0.6-1.5 $g.l^{-1}$ NH_4^+-N than those at lower levels. When both methanogenesis and lipid conversion proceeded, the concentrations of acetate and propionate were 184-221 and 148-180 $mg.l^{-1}$, respectively, and they did not vary between NH_4^+-N concentrations of 0-0.9 $g.l^{-1}$ (data not shown) while they amounted to 154 and 134 $mg.l^{-1}$, respectively, at 1.5 $g.l^{-1}$ NH_4^+-N. At the imposed NH_4^+-N levels in this study, it is possible that the extent of inhibition of acetotrophic and hydrogenotrophic methanogenesis by NH_4^+ is still the same.

Effect of NaCl and NH_4^+-N on protein degradation

The SS-COD hydrolysed during the lag period in the lipid conversion in both experiments I and II must be mainly protein COD. In this period, the hydrolysed SS-COD and the produced NH_4^+-N increased similarly and they both reached a plateau phase. The plateau phase in the hydrolysis of SS ends as lipid conversion starts. While the produced NH_4^+-N remained almost constant or it only slowly increased with time from day 10 and 8 in experiments I and II, respectively, until the end of the batch digestion, the amount of hydrolysed SS continued to increase, indicating that other components of the wastewater, i.e. the lipids, were also hydrolysed and acidified. The latter was confirmed by the lipid analyses.

The results show a decrease in the protein hydrolysis rate at high NaCl concentration ($\geq$ 19.9 $g.l^{-1}$), as indicated by the k_h value and the percent hydrolysed SS at the plateau phase in the hydrolysis of SS. As there was no observed negative effect of NaCl on the protease activity of the sludge+fish mixture, this decrease in protein hydrolysis rate must be due to an inhibition of protease production or a direct inhibition of the acidogens. The observed higher concentration of SNA COD (Table 4.4) which must be mainly amino acids as there are hardly any carbohydrates and sugars in the wastewater, and the lower value of k_N for NH_4^+-N production at 19.9 $g.l^{-1}$ NaCl (as compared to those at lower NaCl levels), indicate that NaCl inhibits the acidification of amino acids. While the methanogenic lag is longest at 19.9 $g.l^{-1}$ NaCl, the VFA level before the start of methanogenesis, i.e. at the 'stagnant period' in SS-COD hydrolysis, is lower compared to those at lower NaCl concentrations, confirming that the amino acid degrading acidogens are inhibited. When amino acid

concentration builds-up, the production of protease by bacteria is inhibited (Doi 1972, Glenn 1976).

The observed increase in protease activity in the experiment with the fish mixtures without casein at increasing NaCl concentration is probably due to an enhanced solubility of fish proteins caused by NaCl. Soluble proteins are generally hydrolysed faster than insoluble proteins (McInerney 1988). However, in the batch digestion, the inhibitory effect of NaCl on the protein degrading acidogens apparently prevails over its positive effect on the solubility of fish proteins. This indicates that at the imposed NaCl levels, the solubility of fish proteins is not rate-limiting.

The results of the experiment with varying concentrations of NH_4^+-N show that the initial rates of protein hydrolysis and acidification, are not affected by NH_4^+ at all the applied NH_4^+-N levels. However, during the later stages of the degradation and at NH_4^+-N concentrations $\geq$ 0.6 $g.l^{-1}$, the amino acid conversion becomes inhibited. This can likely be attributed to the consequent high concentration of amino acids which probably inhibit the protease production and thereby also reduce the rate of protein hydrolysis. Earlier, the effect of NaCl on protein hydrolysis was similarly explained. This mechanism of inhibition of protein hydrolysis does not manifest during the early stages probably because proteases are already present in the wastewater and their activity is not affected by increasing NH_4^+-N concentration from 0 to 1.5 $g.l^{-1}$.

The longer lag periods and slower rates of methanogenesis and lipid conversion in experiment I without added NaCl compared to those in experiment II without added NH_4Cl (Table 4.2) can be due to the higher lipid level in the wastewater in experiment I compared to that in experiment II. The difference in the k_N and k_h values between these two experiments, (I without NaCl and II without NH_4Cl, Table 4.3) is probably due to a difference in the acidogenic activity between the inocula.

The results of this study suggest that when anaerobic treatment is applied to a similar type of wastewater, NH_4^+ and NaCl will reduce the amount of proteins degraded when inhibition of the acidogens by these ions results in higher amino acid concentration because these compounds inhibit protease production. To avoid high levels of these intermediates, higher solid retention times are required for wastewaters with higher levels of these ions. The possibility to adapt the acidogenic bacteria was not investigated in this study. The NH_4^+-N concentration in fish wastewater in the Philippines normally does not exceed the above levels not even at extremely high protein and NPN concentrations, e.g. 15 $g.l^{-1}$ protein COD. The NH_4^+ tolerance concentration of adapted methanogenic sludge, as reported by De Baere *et al.* 1984, is much higher than the NH_4^+-N concentrations in fish processing wastewaters. The absence of any significant effect of NaCl on the rate of methanogenesis and lipid acidification indicates that it is possible to adapt the methanogenic sludge to the imposed NaCl concentrations (3.3-19.9 $g.l^{-1}$) in this study. The degradation efficiency at higher NaCl concentrations ($\geq$19.9 $g.l^{-1}$) will be limited by the lower rate of protein hydrolysis.

CONCLUSIONS

When using a sludge that is not adapted to high concentrations (3.3-19.9 $g.l^{-1}$) of NaCl, the length of lag phase in methane formation and lipid acidification gradually increases with increasing NaCl concentration, but the rate of methane production remains unaffected. The hydrolysis of fish proteins becomes inhibited at 19.9 $g.l^{-1}$ NaCl, but the protease activity then still is not affected. Thus, the inhibition of protein hydrolysis can be attributed to an inhibition of the acidogens or an inhibition of protease production. High concentrations of non-acidified amino acids indicate that the acidogens become inhibited by NaCl.

At the applied NH_4^+-N concentrations (0-1.5 $g.l^{-1}$), lipid degradation remains unaffected. At 0.6 to 1.5 $g.l^{-1}$ NH_4^+-N, the amino acid degrading acidogens become inhibited, causing high levels of amino acids which could inhibit protease production and thus, inhibit hydrolysis.

ACKNOWLEDGEMENT

This study was financially supported by the Environmental Ecotechnology Project, a cooperation between International Institute of Hydraulic, Infrastructural, and Environmental Engineering (IHE, Delft, The Netherlands), Wageningen Agricultural University (WAU, Wageningen, The Netherlands), and University of the Philippines (U.P. Diliman, Quezon City, Philippines). We acknowledge the cooperation of Engr. Marilou Nabatilan (Department of Chemical Engineering of U.P. Los Baños, Laguna, Philippines) in this research.

REFERENCES

American Public Health Association (APHA), American Waterworks Association (AWWA) and Water & Environment Federation (WEF) (1992) *Standard Methods for the Examination of Water and Wastewater*, 18th edn. Washington DC.

Boardman G. D., Tisinger J. L. and Gallagher D. L. (1995) Treatment of clam processing wastewaters by means of upflow anaerobic sludge blanket technology. *Wat. Res.* **29**, 1483-1490.

Borja R., Sanchéz E. and Weiland P. (1996) Influence of ammonia concentration on thermophilic anaerobic digestion of cattle manure in upflow anaerobic sludge blanket (UASB) reactors. *Process Biochem.* **31**, 477-483.

Boschker H. T. S., Bertilsson S. A., Deckkers E. M. J. and Cappenberg T. E. (1995) An inhibitor based method to measure initial decomposition of naturally occurring polysaccharides in sediments. *Appl. Environ. Microbiol.* **61**, 2165-2192.

De Baere L. A., Devocht M., Van Assche P. and Verstraete W. (1984) Influence of high NaCl and NH_4Cl salt levels on methanogenic associations. *Wat. Res.* **18**, 543-548.

Doi R. H. (1972) Role of proteases in sporulation. *Curr. Top. on Cell Regul.* **6**, 1-20.

Eastman J. A. and Ferguson J. F. (1981) Solubilization of particulate organic carbon during the acid phase of anaerobic digestion. *J. Wat. Pollut. Control Fed.* **53**, 352-366.

Franks F. (1993) *Protein Biotechnology: Isolation, Characterization, & Stabilization*. Humana Press, Inc., New Jersey.

Glenn A. R. (1976) Production of extracellular proteins by bacteria. *Ann. Rev. Microbiol.* **30**, 41-62.

Haard N. F., Simpson B. K. and Sun Pan B. (1994) Sarcoplasmic proteins and other nitrogenous compounds. In: *Seafood Proteins*, (Sikorski Z. E., Sun Pan B. and Shahidi F. (eds.), pp. 13-39. Chapman & Hall, New York.

Hobson P. N. and Shaw B. G. (1976) Inhibition of methane production by *Methanobacterium formicicum*. *Wat. Res.* **10**, 849-852.

Kinsella J. E. (1987) *Seafoods and Fish Oils in Human Health and Diseases*. Marcel Dekker, Inc., New York.

Koster I. W. and Lettinga G. (1984) The influence of ammonium-nitrogen on the specific activity of pelletized methanogenic sludge. *Agric. Wastes.* **9**, 205-216.

Larsen H. (1962) Halophilism. In: *The Bacteria*, Vol. 1, Gunsalus I. C. and Stanier R. Y. (eds.), pp. 297-342. Academic Press, New York.

Lema J. M., Soto M., Veiga M. C. and Mendez R. (1987) Mesophilic and thermophilic anaerobic treatment of wastewaters from industrial processing of mussel. In: *Biomass for Energy and Industry*, (Grassi G., Delmon B., Molle J. F. and Zibetta H. (eds.), pp. 872-876. Elsevier Applied Science, London.

McCarty P. L. and McKinney R. E. (1961) Salt toxicity in anaerobic digestion. *J. Wat. Pollut. Control Fed.* **33**, 399-415.

McInerney J. M. (1988) Anaerobic hydrolysis and fermentation of fats and proteins. In: *Biology of Anaerobic Microorganisms*, Zehnder A. J. B. (ed.), pp. 373-416. John Wiley & Sons, Inc., New York.

Pavlostathis S. G. and Giraldo-Gomez E. (1991) Kinetics of anaerobic treatment. *Wat. Sci. Technol.* **24**, 35-59.

Sikorski Z. E. (1994) The contents of proteins and other nitrogenous compounds in marine animals. In: *Seafood Proteins*, Sikorski Z. E., Sun Pan B. and Shahidi F. S. (eds.), pp. 6-12. Chapman & Hall, New York.

Soto M., Mendez R. and Lema J. M. (1991) Biodegradability and toxicity in the anaerobic treatment of fish canning wastewaters. *Envi. Tech.* **12**, 669-677.

Soto M., Mendez R. and Lema J. M. (1993) Sodium inhibition and sulphate reduction in the anaerobic treatment of mussel processing wastewaters. *J. Chem. Tech. Biotech.* **58**, 1-7.

Suzuki T. (1981) *Fish and Krill Protein Processing Technology*. Applied Science Publishers, Great Britain.

Van Velsen A. F. M. (1979) Adaptation of methanogenic sludge to high ammonia-nitrogen concentrations. *Wat. Res.* **13**, 995-999.

Van Velsen A. F. M. (1981) *Anaerobic Digestion of Piggery Waste*. Ph.D. Thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

Veiga M. C., Mendez R. J. and Lema J. M. (1994) Wastewater treatment for fisheries operations. In: *Fisheries Processing: Biotechnological Applications*, Martin A. M. (ed.), pp. 344-369. Chapman & Hall, London.

Wiegant W. M. and Zeeman G. (1986) The mechanism of ammonia inhibition in the thermophilic digestion of livestock wastes. *Agric. Wastes.* **16**, 243-253.

Zeeman G. (1991) *Mesophilic and Psychrophilic Digestion of Liquid Manure*. Ph.D. Thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

Chapter 5

Treatment of fish processing wastewater with different lipid and chloride contents in an upflow anaerobic sludge blanket (UASB) reactor

ABSTRACT

The performance of an UASB reactor in treating fish processing wastewater of different lipid and chloride levels was compared using artificially generated influent simulating that of the canning of sardines and tuna. The organic loading rates (OLR) and the hydraulic retention times (HRT) were 6-10 g $COD.l^{-1}.d^{-1}$ and 10-12 hours, respectively. In treating a wastewater which contains 3-4 $g.l^{-1}$ total COD of which 5-9% was lipids, the COD removal and conversion to methane were $78\pm8\%$ and $61\pm17\%$, respectively. In treating a wastewater with a higher lipid content (35-60% of the total COD), the COD removal was higher ($92\pm5\%$) but to a considerable part (34% of the total COD) was removed as adhering SS on surfaces near the gas-liquid interface and on the gas-solid-liquid (GLS) separator. Of the total COD, $47\pm19\%$ was converted to methane. A lower percentage hydrolysed COD (53 ± 20) was obtained for the high-lipid wastewater due to a probably limited extent of lipid hydrolysis. The extent of protein degradation was 70-100%. Lipids were degraded in the UASB reactor to some extent but as they tend to float, they are hardly retained in the reactor. Hence, complete degradation of lipids is difficult to achieve. Moreover, the adsorption of lipids on sludge particles threatens the stability of the UASB operation, thus, they must be removed from the wastewater before treatment in a UASB reactor. The presence of NaCl at 4 $g.l^{-1}$ Cl^- does not negatively affect COD reduction and methane conversion but may require a longer start-up period for sludge adaptation.

Keywords - anaerobic, fish processing, hydrolysis, lipids, NaCl, proteins, UASB, wastewater

INTRODUCTION

In general, fish processing wastewaters contain high levels of suspended solids which are mainly proteins and lipids. For the treatment of wastewaters generated from the processing of tuna, herring, mussels, octopus, and fish meal, different types of anaerobic systems were shown feasible at laboratory and pilot-plant scales: upflow anaerobic filter (AF, Balsev-Olesen *et al.* 1990, Lema *et al.* 1987); fluidized bed reactor (AFB, Balsev-Olesen *et al.* 1990); central activity digester (CAD), i.e., a reactor consisting of two co-centric vertical cylinders: the influent goes to the inner cylinder which is equipped with a mixer, while the effluent flows upward through the outer cylinder (Mendez *et al.* 1992); and downflow stationary fixed film (DSFF) reactor (Veiga *et al.* 1992). At the applied hydraulic retention

times (HRT) and organic loading rates (OLR) which range from 0.6 to 7.5 days and 2 to 25 g COD.l^{-1}.d^{-1} respectively, 63-95% COD removal efficiencies were attained for wastewaters having 2-16 g.l^{-1} Cl^-. The application of an UASB system may also be a promising option. In this process, a sludge of good settling characteristics develops at the digestion zone of the reactor while the gas-liquid-solid (GLS) separator enables further settling of sludge, thus, retaining the viable biomass in the digestion zone.

The applicability of a UASB system to wastewater from the fish-canning factories in the Philippines is considered in this study. The fish-processing industry is among the major food industries in the country. At industrial scale, the common raw materials are sardines and tuna for the local and foreign market, respectively. The lipid content of these and other fin-fish which are canned varies between species: 1-7% (wet basis) in Albacore, Bluefin, Skipjack, and Yellow-fin tuna, 12-14% in Atlantic and Chub mackerel, 4-8% in Horse and Japanese Horse mackerel, 9-13% in Atlantic and Pacific mackerel (Kinsella 1987), and 14-18% in Indian oil sardines. These compositions also vary depending on fish size, age, and season (Suzuki 1981). With the varying lipid levels in the fish raw material, the lipid levels in the wastewater can also be proportionately variable. The chloride levels in fish wastewater vary (2-16 g.l^{-1}) between different plant operations depending on the source of the water utilized in the plant (Veiga *et al.* 1994). Indigenous chloride level in raw fish is much lower, e.g., for sardines, about 0.15% of wet weight, than the chlorides contributed by the utility water and the brines used in processing. In the Philippines, the chloride level in the wastewater is relatively low, i.e. <4 g.l^{-1}, since ground water is used in the plant operations (fish canning factories, pers. comm.).

This study aimed to determine the efficiency of an UASB system at 10-12 hours hydraulic retention times (HRT) and organic loading rates (OLR) of 6-10 g.l^{-1}.d^{-1} in the treatment of fish processing wastewaters having different lipid and chloride contents with respect to the removal and conversion of COD to methane, the retention and hydrolysis of suspended solids, and the characteristics of the sludge. The applied OLR was 60-90% of the maximum COD loading rate recommended by Sayed (1987) for the treatment of unsettled slaughterhouse wastewater in a granular sludge UASB reactor operated at 30°C. The efficiency of the system at an influent chloride concentration of 4 g.l^{-1} was tested in this study.

MATERIALS AND METHODS

Influent wastewater preparation

Three continuous UASB reactor runs were performed with different types of artificially generated influent wastewater: high-lipid (run A), low-lipid (run B), and low-lipid with 4 g.l^{-1} Cl^- (run C). The high-lipid wastewater was prepared by cutting raw (all parts included) Indian sardines (*Sardinella longiceps*) into small particles using a blender equipped with blades (160 g fish/0.400 l tap water, 1 minute). The resulting slurry was sieved (1-mm pore size) and was diluted to obtain a mixture of about 4 g.l^{-1} total COD. The low-lipid wastewater was prepared similarly as the high-lipid wastewater except that meat fillet of Yellow-fin tuna (*Thunnus albacares*) was used and for run C, NaCl (4 g.l^{-1} Cl^-) was added. This procedure was designed to obtain a wastewater with similar soluble and insoluble

components as the wastewater generated in the fish canneries in the Philippines. The influent storage tank was refilled daily with new wastewater without discarding any left-over wastewater. In all the runs, nutrients were added (concentration in $mg.l^{-1}$: 280 NH_4Cl, 250 KH_2PO_4, 100 $MgSO_4.7H_2O$, 10 $CaCl.2H_2O$, 0.483 FeIII, 0.450 Co, 0.140 Mn, 0.011 CuII, 0.024 Zn, 0.025 Ni). To control the pH at the neutral range, $NaHCO_3$ (0.4 $g.l^{-1}$) was added as a buffer.

Experimental set-up and operating conditions

The wastewater from a continuously-stirred influent tank kept at 28-32°C was fed by a peristaltic pump to the UASB reactor. The reactor has a rectangular cross-sectional area (0.10 m x 0.10 m), equipped with a gas-liquid-solid (GLS) separator (a 45° inverted cone with a rectangular base), gas bubble deflectors at each side, and U-tube water-locks at effluent ports. The influent and effluent tubes have inside diameters of 0.008 m. The produced methane gas was measured by displacement of NaOH (3%) solution using Mariotte flasks. The UASB reactors were 50-75% filled with a seed sludge withdrawn from a full-scale anaerobic reactor treating brewery wastewater (Asia Brewery, Philippines).

The reactors were started up at 10-12 hours HRT and 6-10 $g.l^{-1}.d^{-1}$ OLR. The reactor of run C was fed with low-lipid wastewater with 4 $g.l^{-1}$ Cl^- during the first day but since many sludge particles were washed out, the same wastewater but without NaCl was fed in days 2-19. The influent NaCl concentration was gradually increased from 0.5 $g.l^{-1}$ Cl^- in day 20 to 4 $g.l^{-1}$ Cl^- in day 30. The operating conditions are summarized in Table 5.1. In all runs, three periods can be distinguished with respect to the progress in conversion efficiencies, i.e., methane conversion, acidification, and hydrolysis: I - conversion is increasing; II - conversion reaches its highest point; III - mean conversion decreased. Hence, mean operating conditions are calculated for each of these periods. Period I represents start-up while in periods II-III, 'steady state' conditions are assumed.

The influent samples were taken through a sampling port located between the influent pump and the UASB reactor while the effluent samples were collected from the UASB effluent tube every 1 or 2 days. Sludge samples were taken from the middle part of the sludge bed which was manually stirred right before sampling. As a clear interface between the sludge bed and the settling zone of the reactor was visible during the entire run, no sludge was withdrawn from the sludge bed. In the reactor fed with high-lipid wastewater, a floating layer of sludge and fatty solids always occurred near the GLS separator, and thus, was removed and measured for total COD every 3 days.

Analytical methods

Total chemical oxygen demand (COD_t) of the influent wastewater was determined by the dichromate reflux method (APHA 1992). Effluent COD_t and soluble COD (COD_s, filtered: 0.45 μm MFS cellulose nitrate) were similarly determined except that the samples were digested in 20-ml covered tubes with proportionally similar amounts of reagents as in the above method. Volatile fatty acids (VFA) in diluted (1:1 with 3% formic acid) samples were determined using gas chromatograph with FID and a glass column (2m x 6mm x 2mm: 10% Fluorad 431 on Supelcro-port, 100-120 mesh). The carrier gas was N_2 (30 ml/min, $\pm$ 2 bar)

Table 5.1: Operating conditions at different periods of the UASB runs

run#	high-lipid wastewater (A)			low-lipid wastewater (B)			low-lipid wastewater with NaCl (C)		
reactor volume (l)		2.0			3.6			3.6	
temperature (°C)		28-32			28-32			28-32	
Cl^- added ($g.l^{-1}$)		0			0			4	
period (day#)	I (0-30)	II (31-59)	III (60-141)	I (0-36)	II (37-49)	III (50-81)	I (0-36)	II (37-49)	III (50-83)
HRT (h)	13.7±6.6	9.9±2.6	12.3±5.5	11.8±1.5	12.4±3.1	11.3±2.0	10.8±1.9	10.8±2.1	11.2±1.5
Q ($l.d^{-1}$)	4.3±2.0	5.2±1.3	4.6±1.6	7.5±0.9	7.4±2.2	7.2±2.8	8.2±1.4	8.2±1.6	7.8±1.0
OLR ($g\ COD.l^{-1}.d^{-1}$)	8.7±4.6	9.7±2.5	8.3±3.5	7.3±2.7	6.8±2.6	5.4±2.3	7.7±1.9	7.4±1.2	6.1±1.2
MLR ($g\ COD.l^{-1}.d^{-1}$)	2.1±2.1	5.7±1.6	3.6±1.7	2.1±1.5	5.7±1.6	3.5±1.3	1.3±1.3	3.3±0.7	2.8±0.8
pH influent	nd	nd	nd	7.1-7.7	7.1-7.5	7.2-7.6	6.7-6.9	6.7-6.9	7.4-7.8
pH effluent	7.1-7.9	7.6-7.8	6.9-7.5	7.1-7.7	7.4-7.6	7.2-7.6	6.9-7.1	6.9-7.3	7.2-7.6

HRT = hydraulic retention time; Q = volumetric flow rate; OLR = organic loading rate; MLR = methane gas loading rate; ±standard deviations; nd = not determined

Table 5.2: Influent COD and N composition

run#	high-lipid wastewater (A)				low-lipid wastewater (B)				low-lipid wastewater with NaCl (C)			
period#	II		III		II		III		II		III	
COD:	$mg.l^{-1}$	%@	$mg.l^{-1}$	%	$mg.l^{-1}$	%	$mg.l^{-1}$	%	$mg.l^{-1}$	%	$mg.l^{-1}$	%
total	3774±485	100	3529±611	100	3303±621	100	2718±532	100	3251±156	100	2748±345	100
soluble	957±506	25	709±257	20	1064±429	32	959±272	35	1376±439	42	523±187	19
VFA	642±570	17	580±612	16	845±408	26	730±209	27	1305±401	40	523±147	19
SP+AA[a]	194±141	5	238±87	7	207±41	6	196±114	7	354±56	11	300±84	11
SS: lipids	780-1640	nd	nd	nd	271±21[d]	8	232±29[d]	9	163±32[d]	5	236±177[d]	9
proteins	1131±788[b]	45[c]	1138±268[b]	47[c]	1968±157[e]	60	1527±230[e]	56	1712±450[e]	53	1989±440[e]	72
		30		33								
N:	148±29		164±16		288±171		339±94		382±26		362±90	
NH_4^+	19±14	48	24±8	61	21±4	59	20±11	83	35±6	94	30±8	85
SP+AA[f]	310±52	6	270±29	9	490±23	4	410±89	5	404±54	6	424±75	7
total		100		100		100		100		100		100

[a](SP+AA)*1.4; [b](total N)*7.74 - COD_s (Haard *et al.* 1994, Sikorski 1994, Suzuki 1981); [c](100 - %COD_s - %SS-protein COD); [d][total lipids]*2.89 (Kinsella 1987); [e](COD_t - COD_s - lipid COD); [f](SP+AA)*0.14; nd = not determined; SS = suspended solids; ± standard deviation; [@]% of COD_t or total N.

saturated with formic acid. The operating temperatures at the oven, detector, and injector were 130°C, 280°C, and 200°C, respectively.

Influent total N was measured according to the Kjeldahl method (APHA 1992). Total NH_4^+-N was determined by nesslerization (APHA 1992) of diluted filtered (0.45 μm) samples. Absorbance was measured at 425 nm through 1-cm light path. The concentration of soluble proteins + amino acids (SP+AA) was determined by the coloration method of Lowry *et al.* (1951) on 0.45μm-filtered samples. As there are hardly any sugars or carbohydrates in the fish type used (Kinsella 1987, Suzuki 1981), in run A, the non-dissolved protein (SS-protein) COD in the influent was estimated as [total protein COD - COD_s]. The influent total proteins were based on the total N while the effluent total proteins were determined on unfiltered samples using Lowry's method. In run A, total lipids in the non-diluted homogenized fish slurry, i.e., the one used to prepare influent wastewater, were determined according to the Soxhlet extraction method (APHA 1992) using petroleum ether (40-60°C boiling point) as solvent, and Whatman # 40 and diatomaceous-silica as filter aid. In runs B and C, total lipids in the influent and effluent, i.e., those collected at the sampling ports, were similarly determined as in the lipid analysis in run A every 3-4 days. The SS-protein COD in the influent and effluent was calculated as [COD_t - COD_s - lipid COD]. All analyses were duplicated. The calculations are given in Table 5.2 and 5.3.

Sludge Tests

The total solids (TS), volatile solids (VS), and sludge volume index (SVI) of the sludge were determined using standard methods (APHA 1992). The sample volume in the SVI test is 50 ml. The specific methanogenic activity (SMA) was determined by following the methane production (using the above liquid displacement method) from serum flasks (3 per sample) which contain 0.500 l VFA solution of equal COD concentrations of acetate, propionate, and butyrate. The initial COD and sludge concentrations were 4 $g.l^{-1}$ and 1.5 g VS l^{-1}, respectively.

RESULTS

The composition of the influent wastewater is shown in Table 5.2. A considerable amount of proteins was already hydrolysed and acidified during storage as indicated by the VFA and NH_4^+-N concentrations. The VFAs were 9-28%, 20-32%, and 15-50% of the influent COD_t for high-lipid (A), low-lipid (B), and low-lipid with NaCl (C) wastewaters, respectively. The respective NH_4^+-N concentrations were 36-74%, 23-100%, and 74-100% of the total N in the influent. The soluble non-acidified COD (soluble - VFA) was mainly soluble proteins plus amino acids (SP+AA).

The hydrolysis, acidification, methanogenesis, COD removal, and the production of NH_4^+-N in the treatment of the different wastewaters are illustrated in Fig. 5.1 as a function of time. Three periods can be distinguished. During the start-up of runs A and B (period I), conversion percentages rapidly increased in time, reaching a maximum in the second period (II). In period I of run C, sludge wash-out was shown and a low and slowly increasing COD removal and conversion to methane occurred. In the third period of this run, the COD removal and conversion were still increasing.

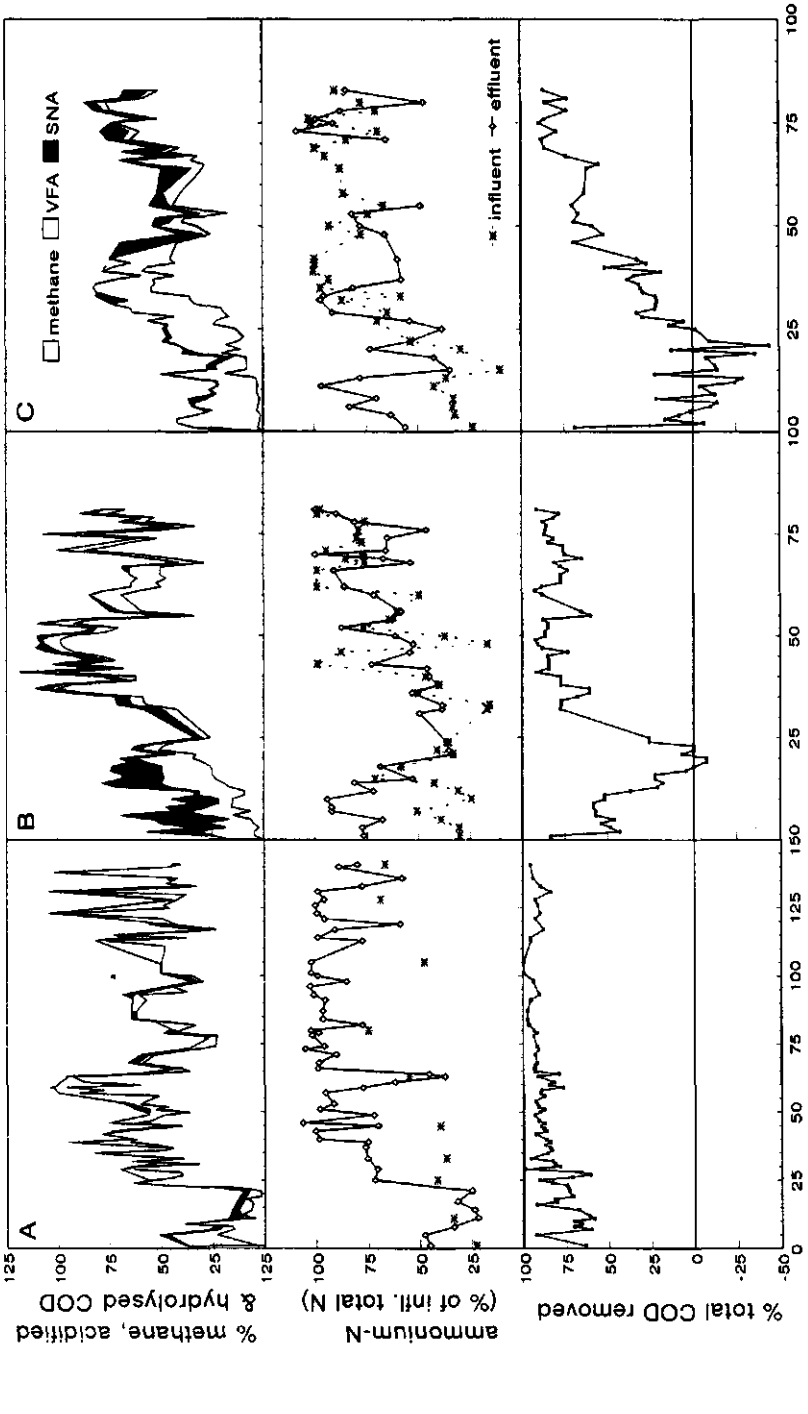

Figure 5.1. Methane, acidified (methane + effl. VFA), and hydrolysed (methane + effl. soluble) COD as % of infl. COD_t; infl. and effl. ammonium-N; and % COD removal in the runs with different wastewaters: (A) high-lipid, (B) low-lipid, and (C) low-lipid with 4 $g.l^{-1}$ Cl^-. (SNA = COD_s - COD_{VFA}; infl. = influent; effl. = effluent; legends: shown in run C graphs; periods I, II, and III are marked as ....., ::::::, and ####, respectively).

Table 5.3: Effluent concentrations and removal efficiencies

run#	high-lipid wastewater (A)				low-lipid wastewater (B)				low-lipid wastewater with NaCl (C)			
period#	II		III		II		III		II		III	
	mg.l^{-1}	%[a]	mg.l^{-1}	%	mg.l^{-1}	%	mg.l^{-1}	%	mg.l^{-1}	%	mg.l^{-1}	%
COD:												
total	467±187	100	282±143	100	486±208	100	600±269	100	1949±586	100	717±351	100
soluble	202±82	43	214±193	76	429±150	73	362±84	62	622±180	32	413±199	58
VFA	202±345	37	106±161	52	228±132	46	320±74	50	559±291	24	203±208	31
SP+AA[b]	34±16	6	49±26	24	135±53	27	129±70	12	199±83	8	173±46	27
SS	nd		nd		50±40[d]		130±28[d]		126±65[d]		79±62[d]	
lipids	31±16[c]		24±23[c]		107±140[e]	9	94±79[e]	22	1201±340[e]	6	221±201[e]	11
proteins		7		9		18		16		62		31
N:	269±52		243±61		253±51		299±77		260±62		308±79	
NH_4^+	3±2	87	5±3	90	14±5	52	13±7	73	20±8	64	18±5	73
SP+AA[f]	271±32	1	243±28	2	267±56	3	361±101	3	384±72	5	336±89	4
total[g]		87		90		54		88		95		79
%methane COD[h]	58±17	*b*	47±19	*y*	85±15	*a*	61±17	*x*	45±10	*c*	46±16	*y*
%acidified total COD	64±22	*b*	50±22	*y*	92±12	*a*	73±18	*x*	62±17	*b*	53±14	*y*
%hydrolysed total COD	64±19	*b*	53±20	*y*	98±11	*a*	74±19	*x*	64±11	*b*	62±13	*y*
%removed total COD	88±4	*a*	92±5	*x*	85±8	*b*	78±8	*y*	40±16	*c*	75±11	*y*
%methane/removed COD	66±4	*a*	51±5	*x*	100±8	*b*	78±21	*y*	>100[@]	*c*	61±18	*y*
%lipid removal[i]	86±10	*b*	97±7	*y*	82±2	*a*	44±11	*x*	23±20	*a*	67±29	*y*

[a]% values for COD and N are based on effl. COD_t and infl. total N, respectively; [b](SP+AA)*1.4; Note that the ratio proteins or AA: COD: N is 1 g: 1.4 g: 0.14 g, based on fish protein composition (Suzuki 1981); [c][(total proteins) - (SP+AA)]*1.4; [d][total lipids]*2.89 (Kinsella 1987); [e][COD_t - COD_s - (lipid COD)]; [f](SP+AA)*0.14; [g]for run A: [NH_4^+-N + (total proteins)*0.10], for runs B and C: [NH_4^+-N + (SP+AA)*0.14 + (SS-protein COD)*0.10]; [h]% of infl. COD_t; [i][(infl. lipids - effl. lipids)/(infl. lipids)] but for run A, lipids was estimated as [COD_t - COD_s - SS-protein COD]; nd = not determined; infl. = influent; effl. = effluent; ± standard deviation; Comparison between mean values for the same period using t-test ($p \leq 0.05$), i.e., $a>b$ and $x>y$ for periods II and III, respectively, while those with the same letters are not significantly different from each other); [@]mean value exceeds 100% because of low COD removal which is due to sludge wash out.

Table 5.4: Total COD and N balance

run#	high-lipid wastewater (A)				low-lipid wastewater (B)				low-lipid wastewater with NaCl (C)			
period#	II		III		II		III		II		III	
total COD:	mg.l^{-1}	%	mg.l^{-1}	%	mg.l^{-1}	%	mg.l^{-1}	%	mg.l^{-1}	%	mg.l^{-1}	%
influent	3774±485	100	3529±611	100	3303±621	100	2718±532	100	3251±156	100	2748±345	100
effluent	465±187	12	282±143	8	486±208	15	600±269	22	1949±586	60	717±351	26
methane	2206±586	58	1664±767	47	2808±495	85	1658±462	61	1463±522	45	1264±662	46
sludge removal	1184±256[a]	31	1184±256[a]	34	125[b]	4	170[b]	6	100[b]	3	100[b]	4
accumulated[c]		-1		11		-4		11		-8		24
N:												
influent	310±52	100	270±29	100	490±23	100	410±89	100	404±54	100	424±75	100
effluent	271±32	87	243±28	90	267±56	54	361±101	88	384±72	95	336±89	79
accumulated[d] in UASB	39±47	12	16±68	6	233±38	47	94±295	23	51±20	13	65±123	15
N (biomass)[e]	56	18	42	15	70	14	44	11	41	10	33	8
produced[f] NH_4^+	128±2	41	57±78	21	-29±137	-6	-33±82	-8	-115±62	-28	-17±83	-4

[a]removed solids at the GLS separator, no sludge withdrawn from the sludge bed except for samples; [b]withdrawn sludge samples; [c](100 - %effl.COD$_t$ - %methane COD - % removed sludge COD); [d] based on N balance, (infl.total N - effl.total N), average difference; [e]estimated N used for biomass growth and estimated based on acidified and methane COD, [(mean methane COD/70) + (mean acidified COD/100)], Hulshoff Pol 1989; [f](effl. NH_4^+ - infl. NH_4^+); ± standard deviation

Table 5.5: Sludge characteristics (VS, TS, SVI, SMA, and methanogenic activity[*] per unit reactor volume)

run #	high-lipid wastewater (A)				low-lipid wastewater (B)			low-lipid wastewater with NaCl (C)		
time (d)	1	44	73	84	12	54	81	12	51	81
VS (g.l reactor^{-1})	2.57±0.56	4.18±0.88	4.63±0.66	8.38±0.33	8.41±2.74	7.33±0.39	15.71±1.40	8.02±0.96	8.85±0.81	12.89±1.01
TS (g.l reactor^{-1})	12.12±1.04	8.98±1.12	7.73±1.14	11.83±0.42	29.14±4.48	19.93±1.36	32.71±3.26	36.42±2.24	29.55±2.94	28.37±2.84
VS/TS	0.21	0.47	0.60	0.71	0.29	0.37	0.48	0.22	0.30	0.45
SVI	57.74	nd	nd	33.82	12.16	22.30	13.59	16.75	12.25	13.71
SMA (g COD.g VS^{-1}.d^{-1})	1.10±0.02	1.38±0.02	0.54±0.14	0.88±0.21	0.36±0.012	0.83±0.01	0.29±0.004	0.52±0.10	1.50±0.33	0.61±0.17
MA[*] (g COD.l reactor^{-1}.d^{-1})	2.17-3.51	4.49-7.08	1.59-3.60	5.39-9.49	1.97-4.15	5.69-6.49	4.09-5.03	2.97-5.57	9.41-17.68	5.23-10.85

The mean effluent concentrations and removal efficiencies for the last two periods are presented in Table 5.3. In the run with high-lipid influent, the COD removal was 92±5%, of which, 51% was converted to methane and the rest was removed as floating layer and adhering solids at the GLS separator. In the runs with low-lipid wastewaters, the removed COD (70-93%) was mainly (61-100%) converted to methane. Although the percentage lipid removal in period III for the low-lipid wastewaters is lower than that of the high-lipid wastewater, the lipid levels in the effluent are not significantly different between the three runs. In run A, the effluent lipid level can be estimated as [COD_t - COD_s - SS-protein COD].

In the run with high-lipid wastewater, the effluent NH_4^+-N was 72-100% of the influent N, indicating that most of the proteins were hydrolysed and acidified. The methane COD exceeds the sum [COD_s + SS-protein COD] in the influent indicating that lipids were degraded to some extent. Large standard deviations are shown for the NH_4^+-N concentrations of influent and effluent during runs B and C. Moreover, the mean influent concentration is often higher than the effluent concentration. These prevent the calculation of protein degradation based on NH_4^+-N production. As the lipids were only 5-9% of COD_t in runs B and C, the percentage hydrolysed and acidified COD can be rough indications of protein degradation. Based on these, the extent of protein hydrolysis between the three runs is not significantly different.

The COD_t and N balances are shown in Table 5.4. As indicated, the sludge withdrawal in runs B and C was limited to sludge sampling which was 3-6% of the average influent COD_t. The unaccounted COD was considered as accumulated in the reactor. As these values cannot be totally attributed to biomass growth, they should be partly due to an accumulation of slowly degrading SS from the influent.

The characteristics of the sludge in the reactor are shown in Table 5.5. In all runs, the amount of sludge, i.e. VS and TS per liter reactor, slowly increased in time. These concentrations remained lower in the run with high-lipid wastewater than those of the low-lipid wastewaters. After 50-60 days in all runs, the sludge particles were found, with or without a microscope, to be larger than the seed sludge particles. As viewed through a microscope, the particles have irregular shapes and some appeared as aggregates of smaller sludge particles. After 80 days in run A, the SVI was lower than that of the seed sludge but remained higher than the SVIs in runs B and C. This means that the sludges in the runs with low-lipid wastewater are more dense and more settleable. In all runs, there was a net increase in SMA of the sludge as well as the total methanogenic activity in the reactor after about 50 days, but a net decrease is shown in days 50 to 80. After about 80 days, the SMAs and SVIs of the sludge did not differ much between runs B and C indicating that these sludge characteristics are not negatively affected by NaCl.

As there was no regular sludge withdrawal from the sludge bed during the entire run except for sampling, instead of calculating based on sludge withdrawal rate, the solid retention time (SRT) could only be estimated roughly as the VS concentration in the sludge bed (g $VS.l^{-1}$) divided by the rate of VS accumulation per unit reactor volume (g $VS.d^{-1}.l^{-1}$). The SRTs estimated in this way are 25, 50, and 95 days, for runs A, B, and C, respectively.

Operational problems

In run A, the influent and the gas collection tubes were sometimes clogged due to adhering lipids on tube walls. Thus, Q and OLR had high deviations in time. Regular removal of the solids adhering on the GLS separator was necessary. Lipids float again with attached sludge particles upon returning them to the sludge bed.

DISCUSSION

Start-up

With respect to the hydrolysis of SS in the wastewater, a 'steady-state' can be assumed after 25-35 days with the high- and the low-lipid wastewaters without NaCl. In the start-up of the run with low-lipid wastewater, the COD_t removal was low, even negative, and the effluent VFA level was high due to an insufficient methanogenic activity of the seed sludge. The OLR and the rate of VFA production were higher than the total methanogenic activity in the reactor. The relatively high SNA levels in the effluent indicate also an insufficient acidifying activity of the seed sludge.

In the run with wastewater containing NaCl (run C), a slow increase in conversion percentages is shown during start-up period probably because the seed sludge was not adapted to the high NaCl concentration. The COD_{SNA} in the effluent was lower than that in run B. This can only be explained by the lower percentage hydrolysis which is probably due to the wash-out of SS during period I.

Degradation, methane conversion, and removal efficiencies

The COD removal is higher in the treatment of wastewater with a high lipid content as compared to that of the low-lipid wastewaters. However, a significant part of this removal (34% of the influent COD_t) was due to the necessary manual removal of solids which floated and adhered to the GLS separator. The percentage of COD_t converted to methane in treating high-lipid wastewater was lower as compared to that with low-lipid wastewaters. As the low effluent VFA levels indicate, this is due to the lower percentage hydrolysed COD. As it is shown that protein hydrolysis is not rate-limiting, this lower percentage hydrolysed COD can only be due to a limited hydrolysis of lipids. The results of the batch experiments presented in Chapters 2 and 3 illustrate that when methanogenesis is occurring, lipid hydrolysis is enhanced and hence, this could also have occurred in the UASB reactor. The lipids are degraded in the UASB reactor to some extent, but not fully degraded as they are poorly retained in the sludge bed. Their adsorption on sludge particles causes some of the sludge particles to move from the digestion to the settling zone of the reactor.

Besides methane conversion, the percentages hydrolysed and acidified COD are also higher in treating low-lipid wastewater without NaCl than in treating high-lipid wastewater. However, these conversion percentages decreased after 53 days of run and the effluent VFA was relatively higher than the preceding days indicating an inhibition of methanogenesis. There was no sudden increase in OLR nor SS loading rate to which this decrease in percentage conversion can be attributed. The total NH_4^+-N concentration, 339 ± 94 $mg.l^{-1}$,

and the corresponding NH_3-N level, 10-40 mg.l^{-1}, were below inhibitory levels (900 mg.l^{-1} for total NH_4^+-N, Chapter 4; 80-100 mg.l^{-1} free NH_3-N, De Baere *et al.* 1984). This decrease can be due to a difference in composition in the raw fish used to prepare the influent wastewater, e.g. lipid level which was not as frequently determined as the other wastewater concentrations.

In run C, the percentage COD hydrolysed, acidified, and converted to methane also decreased after about 40 days. The COD_{SNA} in the effluent increased indicating that the acidogens were inhibited. The low effluent VFA levels indicate that methanogenesis was not rate-limiting. The NH_4^+-N level was below 600 mg.l^{-1} from which level an inhibition of the amino acid degrading acidogens may occur (Chapter 4). The pH of the influent increased and the calculated NH_3-N values (not shown) consequently increased from about 3-15 mg.l^{-1} (first 40 days) to 40 mg.l^{-1} (day 50) and 76 mg.l^{-1} in day 70. These relatively higher NH_3-N values occurred during day 50-70, at which the conversion percentages were already increasing. Hence, NH_3-N is unlikely the cause of any inhibition. The decrease in conversion percentages may be due to a lack of adaptation of the sludge to the imposed NaCl concentration. This decline in conversion percentages occurred 10 days after the influent NaCl level was increased to 4 g.l^{-1} Cl^-. From this moment (day 40), the VFA level as well as the ratio of NH_4^+-N to total N in the influent decreased, indicating that the growth of protein degrading acidogens during storage was inhibited by NaCl.

The conversion percentages in run C are still increasing during period III. The mass balance and sludge concentration measurements show a net accumulation of sludge in the reactor. These conditions are indicative of non-steady state. The start-up period may have been prolonged by the relatively fast increase in the influent NaCl concentration. Nevertheless, the COD removal and conversion percentages in this run were already similar to those of the low-lipid wastewater without NaCl in days 70-83: for run C, percentage methane, acidified, hydrolysed, and removed COD were 63 ± 9, 67 ± 9, 74 ± 8, and 83 ± 6, respectively.

The decrease in NH_4^+-N and total N in the reactor in runs B and C cannot be explained by biomass growth alone (Table 5.4). This may be due to a precipitation with Mg^{2+} and PO_4^{3-} (Maekawa *et al.* 1995) which may be present in the wastewater.

Sludge retention and characteristics

When the main mechanism for lipid removal is adsorption, as in the case of treating high-lipid wastewater, a period of high lipid removal may be followed by a sudden decline in lipid removal or a sudden sludge flotation. Hwu *et al.* (1997) showed that the adsorption of LCFAs on surfaces of active methanogenic sludge followed by their desorption back to aqueous phase, presumably due to perturbation of gas bubbles which escape from sludge granules. It is possible that adsorbed lipids are similarly resuspended back to the liquid phase due to the pressure of the evolving gas from the inner part of the sludge particles. On the other hand, when gases are not released from the lipid-covered sludge particles, flotation of both the acidogenic and the methanogenic biomass may occur. Lipid adsorption on sludge particles, therefore, may decrease both the SMA of the sludge and the total sludge concentration in the digestion zone of the reactor. When not balanced by biomass growth, a net sludge flotation or wash-out will lead to a practically empty reactor in the long run.

In all runs, the methane conversion increased again after a period of decline. These periods of decline and recovery were not reflected in the changes in SMA which was assessed. The number of SMA measurements is limited, however, so that the SMA tests can only show a net change between day 50 and 80, but not what happens to the SMA between day 50 and 80. The results show no effect of NaCl on the sludge SMA and SVI.

When treating wastewater with a higher lipid content, a lower SMA is expected due to an expectedly higher adsorption of lipids but no significant difference in SMA is shown among the three runs after about 80 days of operation because most of the lipids are removed with the floating layer. Hence, the remaining adsorbed lipids in the sludge bed, from which samples are taken for SMA measurement, may be not significantly different compared to those in the reactors treating low-lipid wastewaters. In treating high-lipid wastewater, there was a net decrease in SVI after 80 days of run probably due to the apparent formation of larger sludge particles. In treating ice-cream wastewater in a UASB reactor, Hawkes *et al.* (1995) did not achieve a successful granulation, probably due to the fatty nature of the wastewater. In their study, the occurrence of granules was noted only once, about 5 months after start-up and at OLR of 4-5 $g.l^{-1}.d^{-1}$. In the present UASB runs, it is likely that aggregation rather than granulation occurred. This aggregation may have been promoted by the adhesive effect of lipids between smaller sludge particles. In this case, aggregation could be more detrimental than beneficial. The results indicate that there was at least an improvement in the density and, hence, settleability of the sludge. The entrapment of small fragments of inert particles such as fish bones could have enhanced the settleability of the sludge aggregates.

At the estimated SRTs of 50-95 days in the runs with low-lipid wastewater, lipid hydrolysis is more likely to occur than at the lower SRT estimated for the run with high-lipid wastewater. These SRT values are estimated under conditions when there is still net sludge accumulation in the reactor and there is no imposed sludge wastage. The sludge inside the reactor is expected to increase until some of the sludge will be washed-out of the reactor. To avoid an increase in SS in the effluent, withdrawal of excess sludge will be necessary. The SRT at 'steady state' will depend on the maximum amount of sludge that the reactor can hold which will be dictated by the upflow velocity, the gas loading rate, and the settleability of the sludge. As the settleability of the sludge in treating high-lipid wastewater is inferior to that of the low-lipid wastewater, the SRT and therefore also the percentage hydrolysed COD, may remain to be lower for high-lipid wastewater than those of the low-lipid wastewater.

Practical application of the UASB reactor for fish processing wastewaters

In Table 5.5, the results of the present UASB experiments are compared to those obtained by Sayed (1987) at similar OLR for slaughterhouse wastewater which similarly contains lipids and proteins. The percentage COD conversion to methane when treating low-lipid wastewaters is similar to that obtained by Sayed (1987) using a granular sludge UASB reactor, but the percentage COD removal obtained for slaughterhouse wastewater was lower because of a lower capacity of granular sludge bed to entrap coarse SS (Sayed 1987). The percentages COD removal and conversion to methane in the run with high-lipid wastewater are about the same as that obtained by Sayed (1987) for slaughterhouse wastewater in which lipid COD is about 47% of the total COD (calculated from his results). As in the present experiments, an important fraction of the removed COD is not recovered as methane but is

instead entrapped and adsorbed in the sludge bed. In treating fish processing wastewater, the percentage methane recovery of removed COD found in experiments with AF, AFB, CAD, and DSFF is relatively higher, but, considering the much higher HRTs applied in these reactors, the performance of UASB reactors in the treatment of low-lipid wastewaters seems to be promising. However, no definite conclusions can be made as the experimental run was rather short considering the SRT of the sludge.

For wastewaters containing lipids, Rinzema *et al.* (1993[1]) and Rinzema *et al.* (1993[2]) recommended the use of reactors with enhanced contact between sludge particles and wastewater, such as an EGSB reactor. In the present experiment, higher upflow velocities which characterize an EGSB could not be applied to the seed sludge which is composed of very fine particles and is easily washed-out at upflow velocities higher than 0.4 $m.h^{-1}$. Hwu *et al.* (1996) recommended the use of granular sludge for seeding because it is less susceptible to lipid inhibition compared to flocculent and suspended sludge. However, besides the present scarcity of granular sludge in the Philippines, it is yet uncertain if the granular characteristic of a sludge in a high-rate reactor treating high-lipid wastewater can be retained in the long run.

Table 5.6: Performance of anaerobic systems in treating fish processing and slaughterhouse wastewaters

wastewater	reactor type	COD ($g.l^{-1}$)	SS (%[a])	lipids (%[a])	Cl^- ($g.l^{-1}$)	OLR ($g.l^{-1}.d^{-1}$)	HRT (h)	%COD removal	%COD methane
slaughterhouse	UASB[1]	2.7	71[b]	47[c]	ng	2.5-5.0	16	82	37
slaughterhouse	UASB[1]	6.1	26[b]	17[c]	ng	10-12	12	68	32
slaughterhouse	UASB[2]	1.3	46[b]	7-19[c]	ng	6-6.2	5	53	65
slaughterhouse	UASB[2]	1.3	45[b]	7-19[c]	ng	7-8	4.3	67	57
slaughterhouse	UASB[2]	1.4	46[b]	7-19[c]	ng	10-10.5	3.2	54	51
sardine canning	UASB*	3.5	80	47	0.2[d]	5-11	12	92[f]	47[f]
tuna canning	UASB*	2.7	65	9	0.2[d]	3-8	11	79[g]	63[g]
tuna canning	UASB*	2.7	65	9	4.2[e]	5-7	11	83[g]	63[g]
herring brine	AF[3]	10	18	13	19	6.7	36	80	76[h]
herring brine	AFB[3]	10	18	13	19	6.7	36	80	80[h]
tuna cooking	CAD[4]	20-25	ng	12	14	4.5	120	80	67
tuna	DSFF[5]	20-54	ng	ng	2-9	2	336	75	ng

[a] as a percentage of the total COD; [b]coarse SS COD: $x > 7.4$ μm; [c]estimated from given data; [d]mainly, Cl^- from added nutrient salts, while Cl^- from sardine fish is 0.010 $g.l^{-1}$; [e]as added NaCl plus Cl^- from nutrient salts; [f]average over period III; [g]average over last 14 days of run (days 70-83); [h]calculated from the results; ng = not given; [1]with flocculent sludge, 30°C (Sayed 1987); [2]with granular sludge, with weekend feed interruption, 30°C (Sayed 1987); [3]upflow anaerobic fixed filter and anaerobic fluidized bed, 35°C (Balsev-Olesen *et al.* 1990); [4]central activity digester (Mendez *et al.* 1992); [5]downflow stationary fixed film (Veiga *et al.* 1992); *this study

An intermittently fed UASB reactor was used by Sayed (1987) for slaughterhouse wastewater to allow degradation of accumulated SS in the reactor during feed interruptions. This may be practiced in fish canneries as well since these plants, in general, do not operate on a 24-$h.d^{-1}$ scheme. However, the relatively short no-feed periods may not be enough for the recovery of methanogenesis when severe inhibition occurs. Returning the floating lipid layer

to the sludge bed is not feasible as refloating occurs. For the application of an UASB reactor for wastewaters containing high concentrations of lipids, a pre-removal of lipids is necessary. Hanaki *et al.* (1990), Hawkes *et al.* (1992), Kumatsu *et al.* (1991), and Sayed *et al.* (1993) suggested the use of two-phase anaerobic systems for cafeteria, ice-cream, dairy, and slaughterhouse wastewaters to prevent inhibition of methanogenesis.

SUMMARY

Different types of wastewaters (3-4 $g.l^{-1}$ total COD) were treated in UASB reactors at 10-12 hours HRT and 6-10 g $COD.l^{-1}.d^{-1}$ OLR: (a) high-lipid (35-60% of total COD is lipid), (b) low-lipid (5-9% of total COD is lipid), and (c) low-lipid with 4 $g.l^{-1}$ Cl^-. The extent of lipid hydrolysis limits the percentage hydrolysed COD and percentage COD conversion to methane in the run with high-lipid wastewater. COD reduction for high-lipid wastewater was relatively high but for an important part due to the removal of floating layer and adhering solids near the GLS separator. Lipids were degraded in the UASB reactor to some extent but as they are poorly retained in the sludge bed, complete lipid degradation was not achieved. Moreover, as they tend to adsorb to sludge particles and float, lipids in the wastewater threaten the stability of the UASB reactor especially in the treatment of high-lipid wastewater. Hence, pre-removal of lipids is necessary. The presence of NaCl at 4 $g.l^{-1}$ Cl^- does not negatively affect the COD removal and conversion to methane but requires prolonged start-up period for sludge adaptation.

ACKNOWLEDGEMENT

I acknowledge the cooperation of Santos Ome, Jr. in this study. This research was financially supported by the Cooperation on Environmental Ecotechnology for Developing Countries (CEEDC).

REFERENCES

American Public Health Association (APHA), American Waterworks Association (AWWA) and Water & Environment Federation (WEF) (1992) *Standard Methods for the Examination of Water and Wastewater*, 18th edn. Washington DC.

Balsev-Olesen P., Lynggaard-Jensen A. and Nickelsen C. (1990) Pilot-scale experiments on anaerobic treatment of wastewater from a fish processing plant. *Wat. Sci. Technol.* **22**, 463-474.

De Baere L. A., Devocht M., Van Assche P. and Verstraete W. (1984) Influence of high NaCl and NH_4Cl salt levels on methanogenic associations. *Wat. Res.* **18**, 543-548.

Haard N. F., Simpson B. K. and Sun Pan B. (1994) Sarcoplasmic proteins and other nitrogenous compounds. In: *Seafood Proteins*, Sikorski Z. E., Sun Pan B. and Shahidi F. (eds.), pp. 13-39. Chapman & Hall, New York.

Hanaki K., Matsuo T. and Kumazaki K. (1990) Treatment of oily cafeteria wastewater by single-phase and two-phase anaerobic filter. *Wat. Sci. Technol.* **22**, 299-306.

Hawkes F. R., Rozzi A., Black K., Guwy A. and Hawkes D. L. (1992) The stability of anaerobic digesters operating on a food-processing wastewater. *Wat. Sci. Technol.* **25**, 73-82.

Hawkes F. R., Donnelly T. and Anderson G. K. (1995) Comparative performance of anaerobic digesters operating on ice-cream wastewater. *Wat. Res.* **29**, 525-533.

Hulshoff Pol L. W. (1989) *The Phenomenon of Granulation of Anaerobic Sludge*. Ph.D. Thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

Hwu C. S., Donlon B. and Lettinga G. (1996) Comparative toxicity of long-chain fatty acid to anaerobic sludges from various origins. *Wat. Sci. Technol.* **34**, 351-358.

Hwu C. S., Tseng S. K., Yuan C. Y., Kulik Z. and Lettinga G. (1997) Biosorption of long-chain fatty acids in UASB treatment processes. *Wat. Res.* in press.

Kinsella J. E. (1987) *Seafoods and Fish Oils in Human Health and Diseases*. Marcel Dekker, Inc., New York.

Kumatsu T., Hanaki K. and Matsuo T. (1991) Prevention of lipid inhibition in anaerobic processes by introducing a two-phase system. *Wat. Sci. Technol.* **23**, 1189-1200.

Lema J. M., Soto M., Veiga M. C. and Mendez R. (1987) Mesophilic and thermophilic anaerobic treatment of wastewaters from industrial processing of mussel. In: *Biomass for Energy and Industry*, Grassi G., Delmon B., Molle J. F. and Zibetta H. (eds.), pp. 872-876. Elsevier Applied Science, London.

Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J. (1951) Protein measurement with the Folin-phenol Reagent. *J. Biol. Chem.* **193**, 265-275.

Maekawa T., Liao C. M. and Feng X. D. (1995) Nitrogen and phosphorus removal for swine wastewater using intermittent aeration batch reactors followed by ammonium crystallization process. *Wat. Res.* **29**, 2643-2650.

Mendez R., Omil F. and Lema J. M. (1992) Pilot plant studies on the anaerobic treatment of different wastewaters from fish-canning factory. *Wat. Sci. Technol.* **25**, 37-44.

Rinzema A., Alphenaar A. and Lettinga G. (1993[1]) Anaerobic digestion of long-chain fatty acids in UASB and expanded granular sludge bed reactors. *Biochem.* **28**, 527-537.

Rinzema A., Van Veen H. and Lettinga G. (1993[2]) Anaerobic digestion of triglyceride emulsions in expanded granular sludge bed upflow reactors with modified sludge separators. *Envi. Tech.* **14**, 423-432.

Sayed S. K. I. (1987) *Anaerobic Treatment of Slaughterhouse Wastewater Using the UASB Process*. Ph.D. Thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

Sayed S. K. I., Van der Spoel H. and Truijen G. J. P. (1993) A complete treatment of slaughterhouse wastewater combined with sludge stabilization using two-stage high-rate UASB process. *Wat. Sci. Technol.* **27**, 83-90.

Sikorski Z. E. (1994) The contents of proteins and other nitrogenous compounds in marine animals. In: *Seafood Proteins*. Sikorski Z. E., Sun Pan B. and Shahidi F. (eds.), pp. 6-12. Chapman & Hall, New York.

Suzuki T. (1981) *Fish and Krill Protein Processing Technology*. Applied Science Publishers, Great Britain.

Veiga M. C., Mendez R.J. and Lema J. M. (1992) Treatment of tuna processing wastewater in laboratory and pilot scale DSFF anaerobic reactors. In: *46th Purdue Industrial Waste Conference Proceedings*, pp. 447-453. Lewis Publishers, Chelsea, Michigan, U.S.A.

Veiga M. C., Mendez R. J. and Lema J. M. (1994) Wastewater treatment for fisheries operations. In: *Fisheries Processing: Biotechnological Applications*, Martin A. M. (ed.), pp. 344-369. Chapman & Hall, London.

Chapter 6

Pre-treatment of fish processing wastewater in an upflow substrate precipitation and entrapment (USPE) system

ABSTRACT

Lipids in wastewater threaten the stability of high-rate anaerobic treatment systems such as the upflow anaerobic sludge blanket (UASB) reactor as they cause sludge flotation, and their adsorption on sludge surfaces may hinder the transport of substrates and products through the bacterial membrane. Thus, pre-removal of lipids from the wastewater is necessary. The possibility of employing an upflow substrate precipitation and entrapment (USPE) system as pre-treatment for fish processing wastewater was investigated. At 3.1 days solid retention time (SRT), 6.1 hours hydraulic retention time (HRT), 80% of the lipids and 80% of the suspended solids COD (SS-COD) were removed from a wastewater of 4.0 ± 1.2 g.l^{-1} total COD, of which 60% is lipid and 11% is protein COD. With 2 hours HRT in an external settler (ES), the SS-COD and the lipid removal in the combined USPE reactor and ES were 86% and 89% respectively. Of the SS-COD removed in the USPE reactor, about 6% was hydrolysed. Under these operating conditions, lipids were not degraded. The batch experiment showed that the optimum pH for the removal of proteins and lipids by settling is 4.5-5.5. This pH, however, was not achieved in the continuous USPE system experiment. Hence, the removal of the SS from the wastewater in the USPE system is attributed to adsorption and entrapment in the sludge bed.

Keywords - acidification, fish processing wastewater, hydrolysis, isoelectric point, lipids, pH, proteins, suspended solids, upflow substrate precipitation and entrapment (USPE) system

INTRODUCTION

The presence of high concentrations of lipids in wastewater endangers the stability of high-rate wastewater treatment systems such as expanded granular sludge bed (EGSB) and upflow anaerobic sludge blanket (UASB) reactors as lipids cause flotation of sludge and their adsorption to sludge particles causes deterioration of the methanogenic activity of the sludge (Rinzema 1988, Sayed *et al.* 1988). Two-phase anaerobic systems or removal of lipids before a high-rate anaerobic reactor were suggested for the treatment of wastewaters containing high levels of lipids such as cafeteria (Hanaki *et al.* 1990), dairy (Perle *et al.* 1995), ice cream factory (Hawkes *et al.* 1992), and slaughterhouse (Sayed *et al.* 1993) wastewaters.

The use of two-phase anaerobic systems was originally conceptualized for the purpose of optimizing the environmental conditions for the different anaerobic process, i.e. hydrolysis and acidogenesis in the first phase and acetogenesis and methanogenesis in the second phase. With this system, the occurrence of an imbalance between the different groups of anaerobic bacteria, which may take place in one-phase systems, could be prevented (Ghosh 1987). Phase separation can be achieved by controlling the pH to acidic levels (Shin *et al.* 1992) or by applying short HRT (Lin and Ouyang 1993) or SRT (Zhang and Noike 1991). In the digestion of activated sludge, besides higher volatile solids (VS) reduction and methane conversion in a two-phase compared to a one-phase system, Ghosh (1991) also showed that the two-phase system is effective in preventing digester foaming which could lead to many serious operational problems. As in the case of raw sewage sludge treated in a two-phase UASB system (Wang 1994), the first phase raises the level of dissolved COD and volatile fatty acids (VFAs) in the influent of the second phase and stabilizes the produced sludge to some extent.

With wastewaters containing high concentrations of lipids, the first phase serves mainly to remove the SS and, hence, achieve a more stable high-rate anaerobic reactor operation. Kumatsu *et al.* (1991) determined the possibility of preventing lipid inhibition using a two-phase system. In treating milk wastewater containing 660 $mg.l^{-1}$ lipid COD at 8 and 40 hours HRT in the first (suspended growth type reactor) and second (upflow anaerobic filter) phase, respectively, they found a higher methane conversion than the one-phase system operated at the same total HRT of 48 hours. However, there was hardly any lipid degradation in the first phase. In treating cafeteria wastewater containing 200-1000 $mg.l^{-1}$ lipid COD in a two-phase anaerobic system similar to that used by Kumatsu *et al.* (1991), at 20°C and at HRT of 3.3-10 days, Hanaki *et al.* (1990) similarly found that there is no degradation of lipids in the first phase and that lipid degradation occurs in the methanogenic reactor. The results presented in Chapters 2 and 3 indeed indicate that in the absence of methanogenesis in an acid phase reactor, hardly any lipid hydrolysis can be expected. For wastewaters with high concentrations of lipids, the primary objective of the first phase can be the removal of lipids, not necessarily its complete degradation.

Zeeman *et al.* (1997) investigated the use of an upflow anaerobic solid removal (UASR) reactor for the pre-treatment of dairy wastewater, raw sewage and waste activated sludge. Unlike a conventional settler, in this reactor, the influent wastewater passes through a bed of sludge where the SS can be entrapped or adsorbed, and partially hydrolysed and acidified depending on the temperature and SRT. The influent flow constantly flushes the sludge bed, hence, preventing possible accumulation of intermediate products, such as amino acids (Doi 1972, Glenn 1976), which could otherwise be inhibitory to hydrolysis or acidification.

With dairy wastewater, the hypothesized mechanism for lipid removal in an UASR is based on the formation of aggregates of lipids and proteins, which are mainly casein (Zeeman *et al.* 1997). Volatile fatty acids formation from the acidification of lactose brings about a decrease in pH. When pH decreases to a value as low as the isoelectric point (IEP) of casein, these proteins precipitate and settle, taking along the lipids which, at higher pH, are emulsified by proteins in solution. The solubility of proteins is influenced by their net charge. At pH higher than the IEP, most proteins have net negative charge while at lower pH, they have net positive charge. At IEP, the net charge is zero and they tend to show minimum

solubility. When the net charge is zero, proteins which would have similar charges at pH away from the IEP cease to repel each other and instead coalesce into insoluble aggregates (Zubay 1988).

Lapsirikul *et al.* (1994[a]) who used an anaerobic method to remove lipids from wool scouring wastewater reported that most of the lipids are removed by flocculation. They later found that biological activity is important in the process, indicating a possible biological degradation of detergents which prevent the lipids from precipitation (Lapsirikul *et al.* 1994[b]). As the importance of achieving low pH is not emphasized in their research, the removal mechanism in this process was probably different from that of the lipids in dairy wastewater according to Zeeman *et al.* (1997).

Fish processing wastewater may contain high concentrations of suspended solids which are mainly proteins and lipids. The application of an UASB reactor for fish wastewater having high lipid concentrations requires a pre-removal of the SS in the wastewater. Thus, the possibility of employing an upflow substrate precipitation and entrapment (USPE) system as a pre-treatment was investigated in this study.

MATERIALS AND METHODS

Settleability of SS at different pH and estimation of IEP of fish proteins

Artificially generated fish wastewater was settled in 1000-ml beakers at different pH. Initially, it was stirred and the pH was set in different beakers at 4-6 by adding 6 M HCl. At 1-2 hours intervals, supernatant samples were carefully taken and analyzed for total COD. At the start and after 9 hours of run, the supernatant total COD, total lipids, total N, and NH_4^+-N were determined. Another run was similarly carried out except that more measurements of supernatant COD were done during the first hour of settling.

In a separate experiment, the IEP of fish protein was estimated by adding 6 M HCl dropwise to the prepared wastewater while stirring in order to lower the pH gradually. After each acid addition and stirring, the wastewater was allowed to stand for 2-3 minutes and observed visually. The apparent IEP is the pH at which floc formation started.

The USPE system experiment

The set-up of the continuous experiment is shown in Fig. 6.1. At start-up, 30% of the USPE reactor volume was filled with a settled sludge from an anaerobic reactor treating brewery wastewater. The start up conditions were the same as those in period I (Table 6.1), except that there was no stirring applied. Sludge was allowed to accumulate in the reactor until the sludge bed height reached 0.7 m. Since then, sludge was manually withdrawn from the sludge port at 0.30 and 0.59 m from the bottom of the reactor 1-2 times daily, maintaining the sludge bed height between 0.55 and 0.70 m. Sludge was withdrawn from the external settler (ES) 1-2 times a day.

The USPE reactor run was divided into three periods which are characterized by different modes of stirring, HRT, and SRT. The SRT was decreased by increasing the organic loading

rate while maintaining the same sludge bed height. The influent was similarly prepared as in Chapters 2 and 3. Nutrients and pH buffers were not added. The influent storage tank was refilled with freshly prepared wastewater every 3 days in period I while every 2-2.5 days in period II and III. Influent and effluent sampling for measurement of pH, COD (total, colloidal, and soluble), volatile fatty acids (VFAs), total N, and NH_4^+-N was done 2-3 times a week. Influent and effluent total lipids were measured once a week. Influent samples were collected at a port between the feed pump and the USPE reactor influent port. The USPE reactor effluent samples were collected at the effluent port while the reactor was not stirred. All the batch and the continuous experiments were operated at 28-32°C.

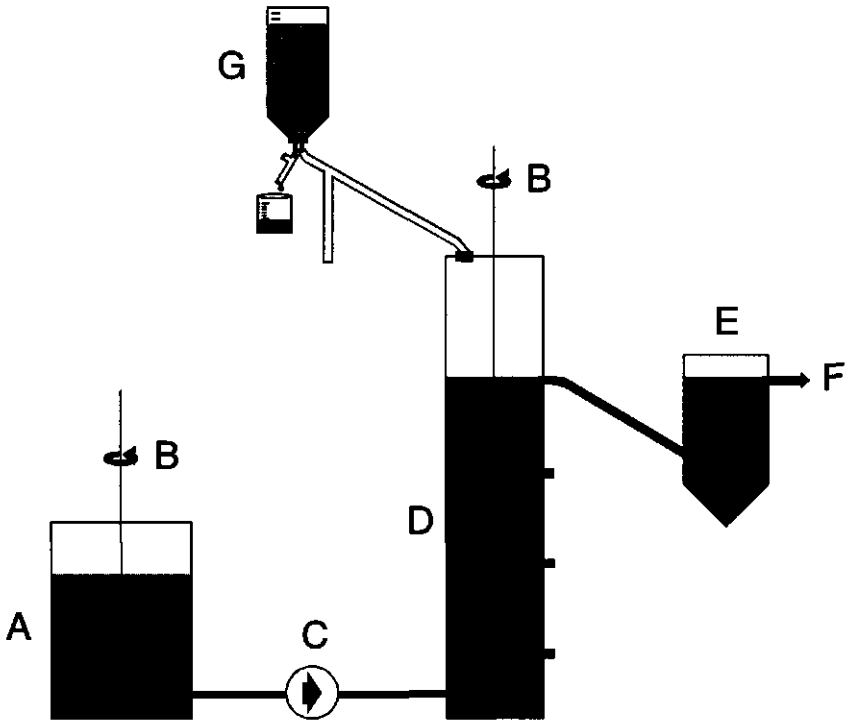

Figure 6.1. Set-up of the continuous experiment: Fish wastewater, stored at 28-32°C in a continuously stirred (50 rpm) 100-l tank (A), was fed via a peristaltic pump (C) into the lowest port of the USPE reactor (D) which is a 0.1 m x 0.1 m x 1.1 m plexiglass column. The reactor has 3 sludge sampling ports (0.294 m apart) and a stirrer (B) having 4 propellers, 3 of which are located at the same level as the sludge sampling ports and 1 at the effluent level. Each propeller has 8 vertical 0.09 m x 0.005 m prongs. At the bottom of the reactor, 0.2 l marbles were placed to allow uniform influent flow. The effluent of the USPE reactor flowed by gravity into an external settler or ES (E) which was a 0.1 m x 0.1 m x 0.395 m plexiglass column with a sludge withdrawal port at the bottom of its conical (45°) base and an effluent port (F). The ES also served as a water lock for the USPE reactor. The influent and effluent tube inside diameter was 0.006 m. The USPE reactor was connected to an inverted serum flask (G) containing 3% NaOH to collect produced gas.

Analytical methods

Sludge and wastewater samples (influent and effluent) were analyzed for the different components using similar methods as in Chapters 2 and 3. The different COD fractions were separated by filtration (0.45 μm MFS cellulose nitrate and 7.5 μm Whatman #40) and designated as soluble (COD < 0.45 μm), colloidal (0.45 μm < COD < 7.5 μm), and SS-COD (total COD - soluble COD).

Table 6.1: Operating conditions in the USPE reactor and ES system during different periods

period	I	II	III
days	56-132	133-205	*219-289
no. of days	77	73	71
temperature	28-32°C (all periods)		
volume: USPE	8.82 l (effective volume), 6.7 l (maintained sludge volume at USPE)		
ES	2.88 l (effective volume), 0.72 l (maintained sludge volume at ES)		
USPE stirring speed	@25 rpm	10 rpm	10 rpm
duration and interval	5 seconds per 30 minutes	6 seconds per 30 minutes	6 seconds per 30 minutes
Q ($l.d^{-1}$)	24.4±1.1	33.3±4.9	34.7±6.4
v_u ($m.h^{-1}$)	0.10	0.14	0.14
HRT (h):USPE	8.7	6.5	6.1
ES	2.8	2.1	2.0
OLR ($g.l^{-1}.d^{-1}$)	7.2	12.8	15.1

Q = influent flow rate; v_u = upflow velocity; HRT = hydraulic retention time; OLR = organic loading rate; rpm = revolution per minute; ± standard deviation; *temporary feed stop on days 206-218; @changed to 10 rpm, 6 seconds per 30 minutes during the last 19 days of period I.

RESULTS

Settling experiment

The results of the batch settling experiments conducted at different pH are presented in Fig. 6.2. Maximum settleability of SS, organic N, and total lipids was achieved at pH 4.5, 5.0 and 5.5. No significant difference was shown between these pH values. Significant differences were found for the settleability between pH 4.5-5.5, pH 4.0, pH 6.0 and pH 6.4. After 10 minutes of settling, it was also visually observed that the supernatant at pH 4.5-5.5 was much clearer as compared to its initial appearance and to those at other pH values. At all the applied pH, the values of the percentage settled SS were already approaching their respective maxima within one hour. In a separate experiment, the IEP of fish protein was determined to be 5.47±0.12.

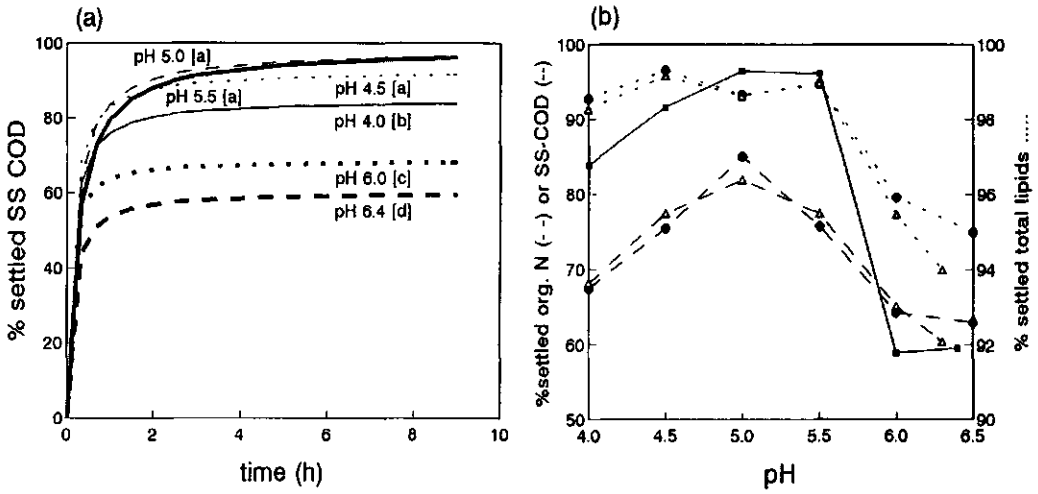

Figure 6.2. Results of the batch settling experiment: **a**. Percentage settled SS-COD as a function of time at different pH. Each curve is the best-fit curve for the two sets of data (run 1 and 2) arbitrarily assuming the equation form $y = (y_{max}*time/(k+time))$ where y = percentage settled SS-COD, and y_{max} and k are constants obtained using non-linear regression. The values in parentheses indicate significant differences ($p<0.05$) where $a>b>c>d$. The R^2 values among all curves was 0.9883-0.9959. The curve for pH 6.4 represents the two runs where pH was not controlled; **b**. Percentage settled organic N (- - -), total lipids (.....) at different pH after 9 and 5 hours, respectively, in runs 1 (▲) and 2 (●), and percentage settled SS-COD after 9 hours (———). The pH values of the wastewater in which pH was not controlled were 6.3 and 6.5 in runs 1 and 2, respectively. The initial total lipids and organic N were 1023 and 399 $mg.l^{-1}$, respectively in run 1, and 1790 and 380 $mg.l^{-1}$, respectively in run 2.

USPE system experiment

The influent concentrations during different periods are shown in Table 6.2.
The influent VFA and NH_4^+-N concentrations were high indicating that the wastewater was already pre-acidified in the influent storage tank. Only 2-6% of the influent total COD was acidified additionally in the USPE reactor. Daily refilling of the influent storage tank with new wastewater did not significantly reduce the extent of acidification in the tank.

At the start-up period, the fluctuation in SS-COD removal efficiency was quite high but it was noted that the influent SS-COD concentration also varied greatly in time. Nevertheless, the average SS-COD concentration in the ES effluent was relatively low, ca. 26% of the effluent total COD. As in the succeeding periods, the pH decreased in the USPE reactor but was not as low as the IEP of fish proteins which was determined in the batch experiment. Hardly any methane gas was detected throughout the experiment. Flow channels through the sludge bed and sludge flotation leading to sudden wash-out occurred when stirring was not applied. These problems were not observed when the reactor was stirred at 10 rpm at 6

seconds duration per 30 minutes interval. With faster and more frequent stirring, (preliminary runs, data not shown), SS wash-out still occurred.

The effluent composition and the removal efficiencies for the different components are summarized for each period in Table 6.3. The average values were calculated over 'steady state' periods which were assumed to be achieved after 3 times the SRT for the new period.

Table 6.2: Influent concentrations

period days[#]	I 56-132		II 170-205		III 231-288	
COD:	$mg.l^{-1}$	%	$mg.l^{-1}$	%	$mg.l^{-1}$	%
total	2665±655	100	3328±857	100	4025±1165	100
soluble	1135±478	43	903±362	27	1174±259	29
VFA	782±593	29	415±156	12	888±297	22
SS	1385±680	57	2319±858	73	2850±1099	71
lipids[a]	346±112	13	854±320	26	2398±1307	60
proteins[b]	1184	44	1571	47	453	11
colloidal	125±116	5	640±313	19	223±126	6
N:						
NH_4^+-N	217±69	67	200±64	58	215±41	69
organic[c] N	108±80	33	146±60	42	104±59	31
pH	6.2-6.9		6.2-6.4		6-7	

[#]when 'steady state' conditions are assumed (after 3 times the SRT); [a]total lipids*2.89 (Kinsella 1987); [b][total COD - soluble COD - lipid COD]; [c](total N - NH_4^+-N); ± standard deviation; Above percentage values for COD and N components are based on total COD and total N respectively.

The SS-COD removal in the USPE reactor did not significantly change when the SRT was decreased from 12.4 days (58±9%) to 4.6 days (52±32%) but increased to 80±10% when the SRT was decreased to 3.1 days in period III. It is to be noted that in period III, the average lipid concentration in the influent wastewater was much higher as compared to those in period I and II. The SS-COD removal in the USPE reactor represents the removal of both lipid and protein COD as indicated by the decrease in both lipids and organic N. Of the removed organic N in period I-III, 11-28% was converted to ammonium indicating partial acidification of proteins. Of the removed SS-COD, 16% and 37% was hydrolysed at 12.4 and 4.6 days SRT respectively. The percentage hydrolysed SS decreased when the SRT decreased to 3.1 days. At this SRT, the combined USPE and ES system effected 86±5%, 89±10%, and 85±27% removal of SS-COD, total lipids, and organic N, respectively. Comparison between the acidified COD and the equivalent COD of the organic N converted to ammonium in the USPE reactor indicates that hardly any lipids were acidified. As any hydrolysed non-acidified lipids will not be measured as soluble COD, the increase in soluble COD in the USPE reactor must be mainly due to the hydrolysis of proteins. As shown in Table 6.3, there was no significant reduction in the colloidal COD in the USPE and ES system.

Table 6.3: Effluent concentrations, conversion, and removal[@] efficiencies

period	I		II		III	
USPE effluent	$mg.l^{-1}$	(%rem.)	$mg.l^{-1}$	(%rem.)	$mg.l^{-1}$	(%rem.)
COD:						
total	1792±565	(32±9)	2451±980	(28±32)	1698±348	(55±12)
soluble	1189±414		1270±517		1188±301	
VFA	884±491		615±222		951±246	
SS-COD	519±442	(58±9)	1181±643	(52±32)	510±231	(80±10)
lipids	259±133	(34±15)	320±174	(55±27)	404±192	(80±11)
proteins	344		861		106	
colloidal	25±31		320±288		219±133	
N:						
NH_4^+-N	224±106		228±71		233±37	
organic N	46±57	(39±77)	46±56	(68±29)	36±35	(63±30)
pH	6.1-6.7		6.0-6.2		6-7	
ES effluent	$mg.l^{-1}$	(%rem.)	$mg.l^{-1}$	(%rem.)	$mg.l^{-1}$	(%rem.)
COD:						
total	1554±516	(37±13)	1475±333	(50±19)	1587±243	(58±11)
soluble	1152±517		1307±507		1236±271	
VFA	1102±460		670±173		962±150	
SS-COD	402±148	(63±23)	179±225	(89±16)	351±97	(86±5)
lipids	110±64	(71±17)	164±132	(79±14)	177±162	(89±10)
proteins	292		4		174	
colloidal	166±178		117±163		210±167	
N:						
NH_4^+-N	214±81		214±46		235±39	
organic N	57±59	(45±57)	46±45	(67±25)	13±23	(85±27)
pH	6.3-6.9		6.1-6.3		6-7	
Percentage conversion in the USPE:						
[a]acidified COD	4%		6%		2%	
[b]hydrolysed SS	9%		19%		5%	
[c]hyd.SS/rem.SS	16%		37%		6%	
[d]amm.N/rem.org.N	11%		28%		27%	

[@]removal percentages (or %rem.) are average values of daily percentage removal calculated as $[(C_{Ui} - C_e)/(C_{Ui})]*100$ where C = concentration of total COD, SS-COD, colloidal COD, lipids, or organic N; Calculation for organic N, SS, lipid, and protein COD are given in Table 6.2; [a]$\{[(\text{VFA COD})_{Ue} - (\text{VFA COD})_{Ui}]/(\text{total COD})_{Ui}\}$; [b]$\{[(\text{soluble COD})_{Ue} - (\text{soluble COD})_{Ui}]/(\text{SS-COD})_{Ui}\}$; [c][(%hydrolysed SS-COD)/(%removed SS-COD)]; [d][(organic N converted to NH_4^+-N)/(removed organic N)]; ± standard deviation; Sampling days for the calculation of average values -- see Table 6.2.; subscript: U = USPE, i = influent, e = effluent.

Table 6.4: Composition and production rate of sludge from USPE and ES system

period	I		II		III	
	USPE	ES	USPE	ES	USPE	ES
Q_{sl} ($l.d^{-1}$)	0.54±0.23	0.51±0.26	1.45±0.23	0.89±0.24	2.17±1.11	1.13±1.02
SRT (d)	12.4	1.4	4.6	0.8	3.1	0.64
VS ($g.l^{-1}$)	10.6±4.0	25.9±13.5	10.1±5.9	9.6±1.9	10.3±3.9	10.3±3.1
TS ($g.l^{-1}$)	16.6±6.3	36.5±13.8	11.6±6.5	12.4±1.0	10.7±3.8	10.8±3.5
VS/TS[@]	0.64±0.01	0.70±0.14	0.87±0.02	0.77±0.09	0.96±.02	0.95±0.03
total COD ($g.l^{-1}$)	27.9±8.4	23.2±18.7	19.0±17.1	19.2±6.6	21.1±7.2	20.2±6.4
total N ($g.l^{-1}$)	nd	nd	1.69±0.31	0.92±0.07	0.64±0.04	0.49±0.07
COD/VS[#]	2.63	0.90	1.88	2.0	2.05	1.96

[@]Average VS/TS values of samples. [#](average total COD)/(average VS); SRT = solid retention time; nd = not determined; ± standard deviation; Q_{sl} = volumetric sludge production rate; VS = volatile solids; TS = total solids

From period I to III, the upper and middle part of the sludge bed slowly changed in appearance from a more compact to a more dispersed form. The part of the sludge bed which remained similar in color with the seed sludge, slowly diminished in time. The average sludge composition in different periods is shown in Table 6.4. The COD/VS ratio in the USPE sludge was 1.88-2.63 which is much higher than the COD/protein ratio (about 1.5), indicating that the sludge COD content was mainly lipids. As the SRT decreases, the VS/TS ratio increases indicating an increasing organic matter content, hence, an increasing need for sludge stabilization.

The sludge profile across the sludge bed height and ES are presented in Fig. 6.3. The sludge total COD, TS, and VS concentrations decreased from the lower to the upper part of the sludge bed. The lipids were present in all parts of the sludge bed.

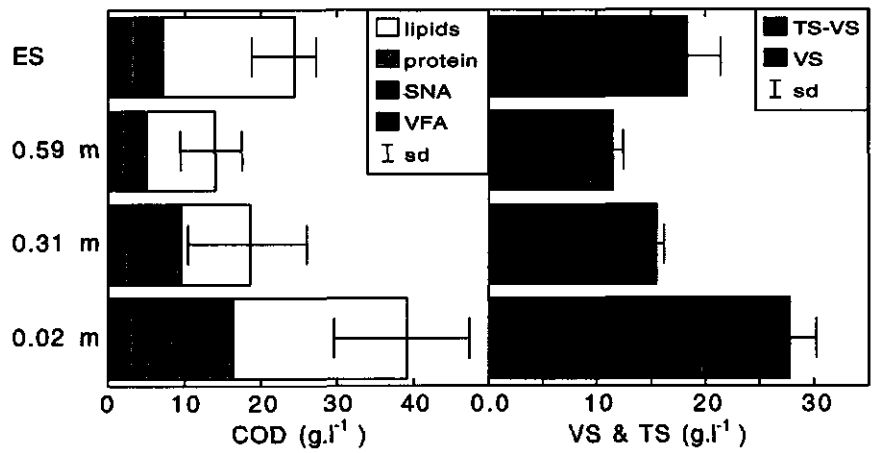

Figure 6.3: Sludge concentration across height (0.02, 0.31, 0.59 m from bottom) of the sludge bed, and in the ES. Samples were taken at day 237 and 239. Bars indicate standard deviation (sd) in total COD and TS. SNA concentration in the sludge bed is negligible.

Table 6.5: Total COD and N balance in the USPE+ES system calculated for the steady state periods

period	I	II	III
total COD			
USPE influent	100%	100%	100%
ES effluent	58%	44%	39%
USPE+ES sludge	42%	41%	52%
unaccounted	<1%	15%	9%
total N			
USPE influent	100%	100%	100%
ES effluent	83%	75%	80%
USPE+ES sludge	nd	29%	18%
unaccounted	nd	-4%	2%

Note: Above values are percentage of total COD or total N of USPE influent. Percentage COD or N of sludge were calculated as $[(Q_{sl}*C_{sl})/(Q*C)]$ where Q_{sl} = sludge production, C_{sl} = sludge total COD or N concentration, Q = influent wastewater flow rate, and C = influent wastewater total COD or N concentration.; nd = no data; unaccounted COD or N = 100% - (total COD or N)$_{ES\ effluent}$ - (total COD or N)$_{USPE+ES\ sludge}$.

The COD and N balances, shown in Table 6.5, indicate an unaccounted total COD and total N of 1-15% and -4-2%, respectively, based on the average total COD or N of the influent. Considering the high relative standard deviation in the influent concentration (6-29%) and sludge COD concentration, these unaccounted values may be deemed to be not significant.

DISCUSSION

SS removal as a function of pH

The results of the experiment on precipitation show a high and fast SS removal at lower pH. The pH for maximum SS removal, 4.5-5.5, agrees with the observed IEP (5.47±0.12) and with the reported IEP of myosin (5.3, Franks 1993) which is the main protein in fish (Suzuki 1981). As the organic N and lipid removal have the same pH optima, the lipids likely settled with the proteins which precipitated at their IEP. It is desired to achieve this pH by biological degradation in an USPE reactor but a pH as low as this IEP was not achieved in the continuous experiment. It is difficult to lower the pH of the wastewater since both VFAs and ammonium are produced during protein degradation.

Mechanism of SS removal

At short SRT, the lipids from fish wastewater are not removed in the USPE reactor neither by degradation nor by co-precipitation with proteins at IEP as the pH in the USPE reactor did not reach the IEP. Zeeman *et al.* (1997) were able to decrease the pH of dairy wastewater in an UASR reactor to values as low as the IEP of its main protein component,

casein, but this decrease in pH, was due to the acidification of lactose. In fish wastewater, only proteins are easily degradable.

As neutral lipids are immiscible in the polar liquid phase, they are normally emulsified in the liquid phase by films of partly charged molecules such as dissolved proteins or long-chain fatty acids which are oriented in such a way that their charged part faces the polar liquid phase and their non-polar part is towards the neutral lipid globule. Under conditions of limited emulsifying agents, the tendency of the neutral lipid molecules is to adhere to similar molecules such as other lipid molecules which are already adsorbed to surfaces. By adhesion to other lipid molecules, e.g. lipids already adsorbed on protein particles, lipids can form larger aggregates which can be more easily entrapped in the sludge bed. The sludge profile across the bed height indicates that as the wastewater passes through the sludge bed, some lipid aggregates are probably entrapped or adsorbed while some are still carried into the upper part of the sludge bed. Lipids and proteins are removed even when the pH did not reach the IEP. The results indicate that some of the non-dissolved proteins are removed by hydrolysis in the USPE reactor. The rest must have been removed by entrapment in the sludge bed and by further settling likely with attached lipid particles. It is shown by the results of the batch experiment that when pH is not controlled, a significant fraction (ca. 60%) of the SS (ca. 94% of the lipids) in the wastewater is removed just by settling. In period I-II of the continuous experiment, the influent lipid level and the obtained pH were similar to those of the batch experiment but the percentage SS-COD and lipid removal were lower in the continuous experiment probably due to the upflow velocity which is absent in the batch experiment. It is probably important to keep the upflow velocity constant for a constant SS removal. A more quiescent settling of SS possibly might be obtained in an ordinary settler but the extent of hydrolysis then may be less compared to that obtained in the present USPE system in which the sludge is continuously rinsed with 'fresh' wastewater preventing the accumulation of intermediate products that can inhibit the hydrolysis of SS, e.g. amino acids that inhibit protease production (Doi 1972, Glenn 1976).

The results of the batch experiment in which acid is added to decrease the pH suggest a promising alternative for a faster SS removal, especially when the recovered solids can be converted into a valuable by-product. Adjustment of pH to 3-5.9, in some cases combined with heating or addition of coagulants, has been reported as an effective technique to improve the recovery of proteins and lipids from fish processing and slaughterhouse wastewaters by plain settling (Civit *et al.* 1982, Fukuda and Nakatani 1979, Shimizu and Nishioka 1979, Song *et al.* 1984, Toth and Drobnine 1979). In these studies, treatment time varies between 0.3 and 1 hour. This method is simpler than the dissolved air flotation method (Brett Borup and Fenhaus 1990, Iggleden and Van Staa 1984, Litchfield 1980, Litchfield 1983), at similarly short HRTs which requires high-pressure air, skimmer and oftentimes coagulants or flocculants. However, one of the possible drawbacks of this acid precipitation method is the cost of the necessary chemicals. The alkalinity of a fish wastewater (as prepared in the present experiment) of about 2300 mg COD.l^{-1} was determined (data not shown) at 903-931 mg $CaCO_3.l^{-1}$. To decrease the pH to the IEP, a relatively large amount of acid will be required, ca. 1.5 l concentrated HCl or ca. 0.5 l concentrated H_2SO_4 per m^3 wastewater. Moreover, the addition of these acids will increase the Cl^- or SO_4^{2+} levels in the wastewater and the produced sludge. These ions are not desirable when the recovered proteins would be used for animal feed preparation or when the supernatant and settled sludge would be anaerobically digested.

Performance of the USPE reactor

The achieved SS-COD removal at 3.1 days SRT was quite high, but was hardly due to degradation. Operating the system at SRT > 3.1 days does not improve the SS-COD removal. With long SRT, as in the start-up period, some lipid degradation could have occurred as there were indications of some methane formation such as very low effluent VFA concentrations that are sometimes lower than its influent concentration and an increase in the hydrolysed fraction of the wastewater without a decrease in the protein-SS (data not shown). The results presented in Chapters 2 and 3 revealed that lipid degradation occurs when methanogenesis prevails. Methanogenesis, however, is undesirable in the USPE reactor as gas production will enhance the flotation of lipids and, therefore, decrease the SS removal.

With regard to protein hydrolysis, a long SRT is not required as a large part of fish proteins are readily hydrolysed and acidified even at the influent storage tank. Extended protein acidification may even result in a higher pH in the system which might deteriorate the settleability of lipids and non-dissolved proteins. The effluent pH at 4.6 days SRT was lower than that at 12.4 days SRT.

Seeding the reactor with a sludge of high acidifying activity will not be necessary as protein hydrolysis and acidification occur regardless of the presence of a seed sludge (Chapter 2). The seed sludge present at the bottom of the bed probably brings about a better lipid entrapment than the upper sludge which appeared to be more dispersed. Hence, the use of flocculent or more compact seed sludge at start-up can be considered important but whether this sludge quality can be sustained in the long run, especially under high loads of lipids, is not yet known.

By intermittent stirring at moderate speed (10 rpm) and frequency (6 seconds every 30 minutes), channelling of the liquid flow can be prevented, while enough time is left for the disturbed sludge bed to resettle. Besides further reducing the SS, the external settler may also serve as a buffer of the combined system during periodic stirring at the USPE reactor and in times of unexpected fluctuations in the level of SS in the USPE reactor effluent.

Produced sludge and effluent quality

From the results, it is clear that application of an UPES reactor leads to increased soluble COD and VFA fraction in the wastewater and to a significant reduction of the lipid content of the wastewater. Whether this satisfies the influent quality requirement of a succeeding high-rate methanogenic reactor was not determined in this study. A foreseen advantage of the application of the combined USPE and ES system prior to an EGSB or an UASB reactor is that this system reduces the fluctuations in the influent SS concentrations and, thus, promises a more stable second phase operation. In period III, although the lipid and SS-COD concentrations in the influent greatly varied in time, their concentrations in the effluent were rather constant during this period. On average, the remaining SS-COD in the wastewater after treatment in the combined USPE and ES system at 3.1 days SRT is 22% of the effluent total COD. About 60% of the remaining SS-COD is colloidal COD. The removal of this fraction appears to be rate-limiting in the removal of SS as hardly any colloidal COD reduction occurred. In slaughterhouse wastewater, which also contains proteins and lipids,

the colloidal SS fraction consists mainly of lipids (Sayed *et al.* 1988). Thus, it can be degraded in the next reactor for as long as methanogenesis prevails. However, with a more concentrated wastewater, a high level of colloidal COD in the effluent can be a problem in the succeeding high-rate methanogenic reactor. Sayed *et al.* (1988) indicated that an important step in the conversion of colloidal COD is its adsorption to the sludge particles. High sludge loads of colloidal COD in an EGSB or UASB reactor may result in accumulation of the adsorbed colloidal matter and cause serious drop in methanogenic activity of the sludge.

The sludge produced in the USPE and ES system requires further treatment and cannot be directly applied as a fertilizer or soil conditioner on agricultural lands as its lipid content is high. For a more complete treatment, a sludge digester operated at longer SRT is necessary. The latter will maximize the methane recovery from the fish wastewater.

The USPE system may find practical application for wastewaters which contain enough easily biodegradable compounds, the acidification of which could bring about a decrease in the pH of the system. As earlier mentioned, an example is dairy wastewater containing lactose (Zeeman *et al.* 1997). In the case of fish processing wastewater, the SS removal may be further increased depending on the plant location. The treatment of fish wastewater may be combined with the treatment of a sugar wastewater from a nearby plant. The VFAs produced from the acidification of sugars may lower the pH to about the IEP of fish proteins and, thus, lead to a higher SS removal. The possibility of operating an USPE reactor at SRT < 3 days and HRT < 6 hours for fish wastewater is yet to be explored. As the pH would not decrease upon an increase in SRT, it may even be more economical to further decrease the SRT and HRT. It must be noted, however, that when HRT is decreased by increasing the wastewater flow rate, the corresponding increase in the upflow velocity may decrease the filtering capacity of the sludge bed for lipids. The maximum upflow velocity for the removal of lipids from fish wastewater may be determined in future research.

SUMMARY

At 3.1 days SRT and 6.1 hours HRT, $80 \pm 10\%$ of the total lipids and $80 \pm 11\%$ of the SS-COD were removed via an upflow substrate precipitation and entrapment system from a fish processing wastewater containing 4.0 ± 1.2 g.l^{-1} total COD, of which 60% is lipid and 11% is protein. At an additional 2 h HRT in an external settler, the percentage total lipids and SS-COD removed were increased to 86% and 89% on average, respectively. Of the removed SS-COD in the USPE reactor, only about 6% was hydrolysed.

When using an influent fish wastewater of lower lipid content (0.85 ± 0.32 g.l^{-1} lipid COD which comprises 26% of the total COD), $52 \pm 32\%$ of the SS-COD and $55 \pm 27\%$ of the total lipids were removed in the USPE reactor at 4.6 days SRT and 6.5 hours HRT. These efficiencies increased to $89 \pm 16\%$ and $79 \pm 14\%$, respectively, after the external settler operation at HRT = 2 h. Increasing the SRT does not improve the SS removal in the USPE reactor.

The determined pH optima for proteins and lipids precipitation in the batch experiment is 4.5-5.5 which agrees with the determined IEP of fish proteins (5.3). Such a low pH,

however, could not be achieved in the USPE reactor. The lipids and proteins are removed in the USPE by a mechanism of settling as flocs and by entrapment and adsorption in the sludge bed present in the reactor. To a limited extent, some of the proteins are removed by degradation while lipids are removed without degradation.

ACKNOWLEDGEMENT

I acknowledge Mark Groeneveld, Agnes Goze, and Wilbert Menkveld for their cooperation in this study, the Environmental Management Bureau Laboratory of the Department of Environment and Natural Resources for the use of their facilities for the lipids measurement, and the Cooperation on Environmental Ecotechnology for Developing Countries (CEEDC) for the financial support.

REFERENCES

Brett Borup M. and Fenhaus S. L. (1990) Food processing wastes. *J. Wat. Pollut. Control Fed.* **62**, 461-465.

Civit E. M., Parin M. A. and Lupin H. M. (1982) Recovery of protein and oil from fishery bloodwater waste. *Wat. Res.* **16**, 809-814.

Doi R. H. (1972) Role of proteases in sporulation. *Curr. Top. on Cell Regul.* **6**, 1-20.

Franks F. (1993) *Protein Biotechnology: Isolation, Characterization, & Stabilization*. Humana Press, Inc., New Jersey.

Fukuda H. and Nakatani H. (1979) Treatment of waste water from fish meat processing plant I. Effect of salt and sugar on condensation treatment of waste water. *Chem. Abstr.* **90**, 60713c.

Ghosh S. (1987) Improved sludge gasification by two-phase anaerobic digestion. *J. Enviro. Engng.* **113**, 1265-1284.

Ghosh S. (1991) Pilot-scale demonstration of two-phase anaerobic digestion of activated sludge. *Wat. Sci. Technol.* **23**, 1179-1188.

Glenn A. R. (1976) Production of extracellular proteins by bacteria. *Microbiol.* **30**, 41-62.

Hanaki K., Matsuo T. and Kumazaki K. (1990) Treatment of oily cafeteria wastewater by single-phase and two-phase anaerobic filter. *Wat. Sci. Technol.* **22**, 299-306.

Hawkes F. R., Rozzi A., Black K., Guwy A. and Hawkes D. L. (1992) The stability of anaerobic digesters operating on a food-processing wastewater. *Wat. Sci. Technol.* **25**, 73-82.

Iggleden G. J. and Van Staa R. (1984) The use of chemical dosing and dissolved air flotation in the recovery of protein from waste waters. *Inst. Chem. Eng. Symp. Ser.* **84**, 381.

Kinsella J. E. (1987) *Seafoods and Fish Oils in Human Health and Diseases*. Marcel Dekker, Inc., New York.

Kumatsu T., Hanaki K. and Matsuo T. (1991) Prevention of lipid inhibition in anaerobic process by introducing a two-phase system. *Wat. Sci. Technol.* **23**, 1189-1200.

Lapsirikul W., Curd-Ruwisch R. and Goen H. (1994[a]) Anaerobic bioflocculation of wool scouring effluent. *Wat. Res.* **28**, 1743-1747.

Lapsirikul W., Goen H. and Curd-Ruwisch R. (1994[b]) Mechanisms in anaerobic bioflocculation of wool scouring effluent. *Wat. Res.* **28**, 1749-1754.

Lin H. Y. and Ouyang C. F. (1993) Upflow anaerobic sludge digestion in a phase separation system. *Wat. Sci. Technol.* **28**, 133-138.

Litchfield J. H. (1980) Meat, fish, and poultry processing wastes. *J. Wat. Pollut. Control Fed.* **52**, 1325-1329.

Litchfield J. H. (1983) Meat, fish, and poultry processing wastes. *J. Wat. Pollut. Control Fed.* **55**, 683-686.

Perle M., Kimchie S. and Shelef G. (1995) Some biochemical aspects of the anaerobic degradation of dairy wastewater. *Wat. Res.* **29**, 1549-1554.

Rinzema A. (1988) *Anaerobic Treatment of Wastewater With High Concentrations of Lipids or Sulfate*. Ph.D. Thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

Sayed S., Van der Zanden J., Wijffels R. and Lettinga G. (1988) Anaerobic degradation of the various fractions of slaughterhouse wastewater. *Biol. Wastes.* **23**, 117-142.

Sayed S., Van der Spoel and Truijen G. J. P. (1993) A complete treatment of slaughterhouse wastewater combined with sludge stabilization using two stage high rate UASB process. *Wat. Sci. Technol.* **27**, 83-90.

Shimizu H. and Nishioka F. (1979) Protein recovery from meat or fish rinse. *Chem. Abstr.* **90**, 28673d.

Shin H. S., Bae B. U., Lee J. J. and Paik B. C. (1992) Anaerobic digestion of distillery wastewater in a two-phase UASB system. *Wat. Sci. Technol.* **25**, 361-371.

Song I. S., Yoo I. J., Min B. Y. and Song K. W. (1984) Protein recovery from meat industry by-products. *Chem. Abstr.* **101**, 89122r.

Suzuki T. (1981) *Fish and Krill Protein Processing Technology*. Applied Science Publishers, Great Britain.

Toth B. and Drobnine D. R. (1979) Study of the protein flocculating action of sulfite liquor derivatives. *Chem. Abstr.* **90**, 156535x.

Wang K. Y. (1994) *Integrated Anaerobic and Aerobic Treatment of Sewage*. Ph.D. Thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

Zeeman G., Sanders W. T .M., Wang K. Y. and Lettinga G. (1997) Anaerobic treatment of complex wastewater and waste activated sludge - application of an upflow anaerobic solid removal (UASR) reactor for the removal and pre-hydrolysis of suspended COD. *Wat. Sci. Technol.* **35**, 121-128.

Zhang T. C. and Noike T. (1991) Comparison of one-phase and two-phase anaerobic digestion processes in characteristics of substrate degradation and bacterial population levels. *Wat. Sci. Technol.* **23**, 1157-1166.

Zubay G. (1988) *Biochemistry*. 2nd ed., McMillan Publishing Co., U.S.A.

Chapter 7

Anaerobic Treatment of Fish Processing Wastewater: Final Discussion and Recommendations

Fish processing industries comprise an important segment of the Philippine economy. However, among other industries, they pollute lakes, rivers, and eventually, coastal waters. If their effluents are not sufficiently treated, the well-being of the country's coastal waters is threatened. In the long run, the fish industry will not be sustained because of water pollution. The prospects of generating energy from fish processing wastes will encourage factories to treat their wastewater before discharging them, and hence, may help the government in implementing strict wastewater standards.

The main products of fish factories in the Philippines are canned sardines and tuna for local and foreign markets, respectively (Chapter 1). Of the weight of raw fish accepted for processing, 40-60% becomes solid waste while 1-6% goes to the wastewater streams. The wastewater produced in the canning of finfish species, e.g. sardines and mackerel, is characterized by high levels of suspended solids which are mainly proteins and lipids. The concentration of sea salts in fish-processing wastewater in the Philippines is low compared to those of factories where seawater is used in processing. The fish canneries in the Philippines use ground water in their operations. The salts in the wastewater are due to the seawater that comes with the raw material.

Since fish processing wastewaters contain high concentrations of biodegradable organic matter, the potential for a net production of energy in the form of biogas is high. Hence, anaerobic treatment is an attractive option. Moreover, the excess sludge production in anaerobic processes is less than that in aerobic processes. High-rate anaerobic treatment systems are economical because they require a smaller reactor volume, and thus, are also suitable for fish canning plants having limited space for wastewater treatment. Hence, this study considers the application of high-rate anaerobic methods, e.g. UASB systems, for treating fish processing wastewater. Hydrolysis, which is generally considered as the rate-limiting step in the anaerobic digestion of insoluble organic wastes, is given special emphasis.

In the next sections, I will summarize the main results of this study and discuss their implications on the anaerobic treatment of fish processing and other lipid-containing wastewaters.

1. General Discussion

1.1. Effect of different factors on the hydrolysis of lipids and proteins

1.1.1. Effect of methanogenesis

The results of the present study show that methanogenesis enhances lipid hydrolysis. Methanogenesis enhances lipid hydrolysis probably via the syntrophic relation between methanogens and LCFA-degrading bacteria. The activity of triglyceride lipases may be inhibited by the accumulation of hydrolysis products, i.e. LCFAs, at the interface at which catalysis by lipase takes place (Martinelle and Hult 1994). Hence, the degradation of LCFAs, i.e. β-oxidation which is dependent on methanogenesis (Angelidaki and Ahring 1995, Roy *et al.* 1986), will positively affect lipid hydrolysis. Thus, complete lipid degradation will not occur at conditions in which methanogenesis is inhibited, e.g., pH levels that are far from neutral, inhibitory levels of ammonium, and high levels of NaCl.

Unlike lipid hydrolysis, protein hydrolysis takes place regardless of the occurrence of methanogenesis and the presence of an inoculum. Therefore, in continuous anaerobic reactors, the hydrolysis of proteins is less sensitive than that of lipids to disturbances that cause inhibition of methanogenesis.

1.1.2. Effect of lipids

Lipids inhibit methanogenesis as indicated by the increasing length of the lag period in methanogenesis at increasing initial total lipid concentration, i.e. 0.09-2.24 $g.l^{-1}$ (equivalent to 0.26-6.47 g $COD.l^{-1}$). This inhibition by lipids cannot be exclusively attributed to their hydrolysis products because, as shown in Chapter 2, there is hardly any conversion of lipids to LCFAs during the lag phase in methanogenesis. Moreover, the methanogenic lag periods in the present experiments are remarkably longer than those found by Angelidaki and Ahring (1992) at 0.1-0.2 $g.l^{-1}$ oleate. The possibility that both LCFAs and non-hydrolysed lipids are causing inhibition of methanogenesis cannot be ruled out. As the hydrolysis of lipids is enhanced by the occurrence of methanogenesis and as methanogenesis is inhibited by lipids, lipids will inhibit their own hydrolysis when present at high levels.

Both the rate of hydrolysis and the biodegradability of proteins are negatively affected at high lipid levels, e.g., $\geq$ 4.30 g $COD.l^{-1}$ at 30°C and 0.67 g $VS.l^{-1}$ seed sludge concentration. Thus, to achieve high methane recovery in a reactor treating a wastewater containing high lipid level, prolonged solid retention time will be necessary.

1.1.3. Effect of pH

The optimum pH for the hydrolysis of fish lipids and proteins is in the neutral range, i.e. 6-8. Hardly any lipid hydrolysis occurs at acidic pH values, e.g. pH 4-5. Both the occurrence and the rate of lipid acidification, i.e. β-oxidation of LCFAs, are found to depend on pH. The maximum rate of this process is also found at pH 6-8. pH affects hydrolysis probably through its effect on methanogenesis which has a positive effect on lipid degradation. As methanogenesis is inhibited at low pH and the β-oxidation of LCFAs is inhibited in the

absence of methanogenesis, lipid acidification occurs only in the presence of a methanogenic inoculum and at pH 6-8.

Protein hydrolysis occurred at all the imposed pH conditions (4-8) in this study, regardless of the presence of methanogenic inoculum. The rate of protein hydrolysis is higher at pH 6-8 than at pH values lower than 6. The effect of pH on protein hydrolysis is probably the net effect of different factors. The low hydrolysis rates at pH 4 and 5 are attributed to the low solubility of fish proteins at these pH values which are near the isoelectric point (IEP) of the proteins in fish, i.e. about 5.3 as determined in this study. At the IEP, the secondary structure of a protein molecule unfolds causing precipitation or decreased solubility. Moreover, the accessibility of protein molecules to proteolytic enzymes may be diminished by lipids, the degradation of which is inhibited at lower pH. Protease activity in fish wastewater increases at increasing pH from 4 to 9, except at pH 8 at which a relatively lower activity is found, probably due to the presence of different enzymes in fish having different pH optima.

It is not difficult to adjust or maintain the pH of fish processing wastewater within the neutral range. It spontaneously reaches about 6 in the absence of methanogenesis, and about 7 in the presence of methanogenesis. The pH buffering capacity of the wastewater increases as it is digested in anaerobic reactors due to the simultaneous production of CO_2 and ammonium.

1.1.4. Effect of NaCl

The hydrolysis of fish proteins becomes inhibited at a threshold level of 20 $g.l^{-1}$ NaCl, while protease activity at this level remains unaffected. It was found that NaCl has a direct negative effect on amino-acid-degrading acidogenic bacteria as indicated by high concentrations of soluble non-acidified COD, which is mainly amino acid COD. The accumulated amino acids then inhibit protease production, and hence, protein hydrolysis.

Methanogenesis and lipid acidification are both inhibited by NaCl, as manifested by the gradually increasing length of lag phase in methane formation and lipid acidification at increasing NaCl concentration. The effect of NaCl on lipid acidification is probably due to its inhibition on methanogenesis. Nevertheless, as in the study by De Baere *et al.* (1984) and Lema *et al.* (1987), the results of this study also indicate that adaptation of methanogenic sludge to NaCl is possible.

1.1.5. Effect of NH_4^+-N

At the imposed NH_4^+-N levels in this study, e.g. 0-1.5 $g.l^{-1}$, inhibition of lipid hydrolysis by NH_4^+ was not apparent. However, the inhibition of methanogenesis by NH_4^+, as manifested by the decreasing rate of methanogenesis at increasing NH_4^+-N, suggests a potential indirect negative effect of higher levels of NH_4^+-N on LCFA degradation which is dependent on methanogenic activity. Protein hydrolysis becomes inhibited at threshold level of 0.6 $g.l^{-1}$ NH_4^+-N. As in the inhibition of this process by NaCl, this effect is similarly attributed to the accumulation of amino acids caused by a direct inhibitory effect on amino-acid-degrading acidogens.

1.2. Treatment of fish processing wastewater in high-rate anaerobic systems

1.2.1. One-phase upflow anaerobic sludge blanket (UASB) reactor

This study demonstrates that the one-phase UASB reactor is a promising alternative for the treatment of low-lipid fish processing wastewater (Chapter 5). In treating wastewater of about 2.72 $g.l^{-1}$ total COD, 9% of which is lipid COD (= 85 $mg.l^{-1}$ lipids), at ca. 5.4 $g.l^{-1}.d^{-1}$ COD loading rate, ca. 11 hours hydraulic retention time (HRT), and 0.011-0.029 g $lipid.g^{-1}VS.d^{-1}$ sludge loading rate, the achieved COD conversion to methane and the COD removal efficiencies were 79% and 63%, respectively. The treatment of the same wastewater, but containing 4 $g.l^{-1}$ NaCl, at the same operating conditions as in the above treatment, requires a longer start-up period for the adaptation of both the acidogenic and methanogenic bacteria to NaCl. At about 70 days after start-up, the COD conversion to methane and the COD removal efficiencies were already similar to those achieved in the treatment of the same wastewater without NaCl. In treating high-lipid wastewater (3.50 g total $COD.l^{-1}$ of which 47% is lipid COD) in one-phase UASB at 8 $g.l^{-1}.d^{-1}$ COD loading rate and 12 hours HRT, 92% of the total COD is removed while only 47% is converted to methane. A significant fraction (about 34%) of the total COD is removed as floating and adhering solids at the gas-liquid-solid separator of the reactor.

As in the batch experiments (Chapter 2, 3, and 4), there are indications that lipid hydrolysis is rate-limiting. Most of the proteins are hydrolysed (72-100% after treatment in the UASB reactor) while lipids are removed in the UASB reactor mainly by adsorption to reactor surfaces and sludge particles instead of complete degradation. Such adsorption of lipids threatens the sludge retention, the methanogenic capacity of the reactor, and, hence, the stability of the system. Thus, in order to optimize COD removal via methanogenesis in high-rate anaerobic reactors, lipids must be pre-removed unless a low lipid sludge loading rate and therefore a long HRT, ca. 80 hours, is applied.

1.2.2. Pre-treatment in an upflow substrate precipitation and entrapment (USPE) system

Since it is necessary to remove the lipids in fish processing wastewater before treating in a UASB reactor, the possibility of pre-treating the wastewater containing high lipid level using a USPE system was investigated in this study (Chapter 6). The USPE system consists of an upflow reactor and an external settler. In the upflow reactor, the influent wastewater passes through a sludge bed where the suspended solids can be entrapped, partially hydrolysed, and acidified. Results show that this system can satisfactorily remove the suspended solids (SS), mainly lipids, from the wastewater. At 6.1 hours HRT and 3.1 days solid retention time, 80% of the lipids and 80% of the SS-COD are removed from the wastewater of 4 $g.l^{-1}$ total COD, 60% of which is lipid COD and 11% is protein-SS COD. At an additional 2 h HRT in an external settler, these removal efficiencies increased to 86% and 89%, respectively.

In batch settling experiments, the maximum removal of proteins and lipids in the wastewater was found at pH 4.5-5.5. The removal at this pH range is attributed to a co-precipitation of lipids and proteins at the IEP of fish proteins. A high lipid removal in the USPE system is expected if the pH could be decreased to this pH range via partial hydrolysis and acidification. However, such a low pH was not achieved in the USPE reactor. Nevertheless,

a significant lipid removal was achieved and is attributed to a combined adsorption, precipitation, and entrapment. To a limited extent, proteins are removed via degradation while lipids are removed without degradation. As shown by the results of the batch experiments (Chapters 2, 3, and 4), lipid degradation will not occur in the absence of methanogenesis such as the conditions in the USPE reactor in which the lipid level in the sludge is high and, therefore, inhibits methanogenesis. Increasing the solid retention time (SRT) does not improve the removal of SS. Methanogenesis, which may occur at long SRTs, is not desired because it will deteriorate the settleability, and hence, the removal of fatty suspended solids in the reactor.

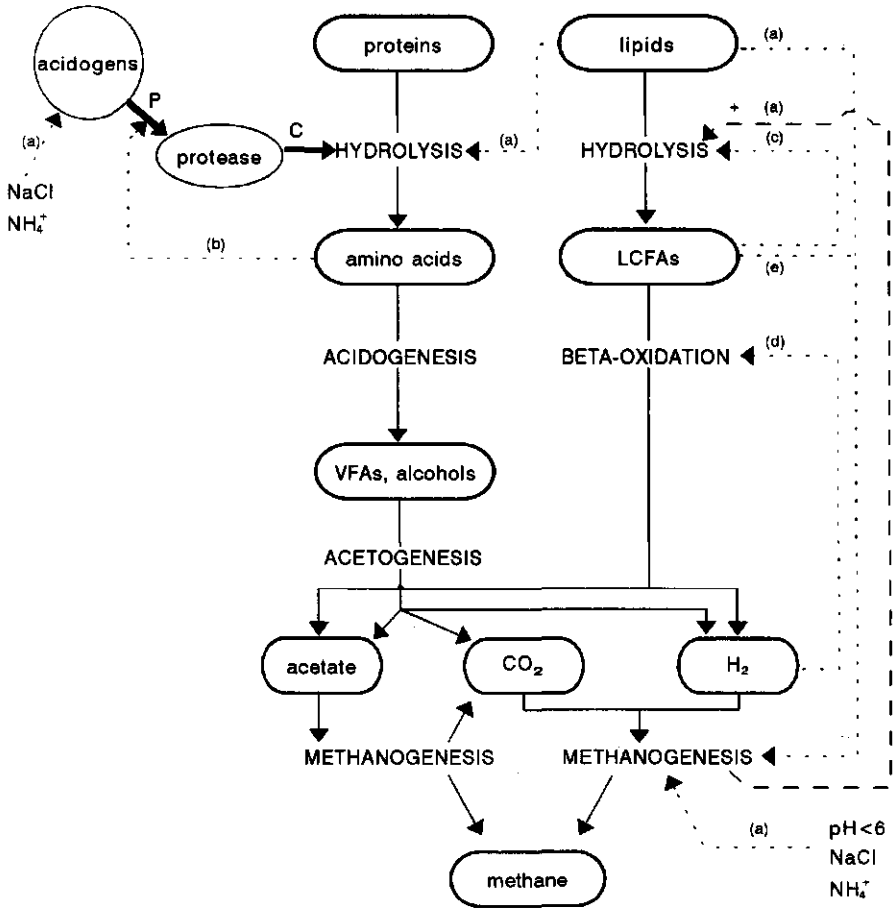

Figure 7.1: Effects of different factors on lipid and protein degradation. Note: Solid lines indicate the degradation scheme; broken lines (....) indicate inhibitory effects (arrows point to the inhibited process); dashed lines with (+) point to positive effects; [a]this study; [b]Glenn 1976, Doi 1972; [c]Martinelle and Hult 1994; [d]Roy *et al.* 1986; [e]Angelidaki and Ahring 1992; P = production of enzyme; C = catalysis

Cause-and-effect relation between different factors in anaerobic digestion of lipid-containing wastewater

While methanogenesis hardly affects the hydrolysis of proteins in fish, it has an important role in the complete degradation of lipids. Thus, in the digestion of lipid-containing wastes, environmental factors such as pH, temperature, and ionic concentrations must be favorable to methanogens. However, at high lipid levels, inhibition of methanogenesis by lipids will prevail, and hence will inhibit their own hydrolysis. This cyclic cause-and-effect relation between lipid hydrolysis and methanogenesis is illustrated in Fig. 7.1.

Among the environmental factors found to affect the hydrolysis of suspended solids in fish processing wastewaters are pH, NaCl and ammonium concentrations. As the occurrence and rate of lipid hydrolysis is found to depend on methanogenesis, these factors could also affect lipid hydrolysis indirectly, i.e. through their effects on methanogenesis. High levels of NaCl and ammonium inhibit amino-acid-degrading acidogenic bacteria, and the consequent accumulation of hydrolysis products inhibits the production of proteolytic enzymes, thus, inhibits hydrolysis.

2. Recommendations: Components of fish processing wastewater treatment systems

Considering the results of this research, anaerobic treatment systems for fish processing wastewater should consist of the following components:

- equalization of wastewater flow and organic loading rate
- lipid removal system prior to high-rate methanogenic reactor
- high-rate methanogenic reactor
- anaerobic digester for the sludge removed in the lipid pre-removal system
- post-treatment system for nutrient removal and/or recovery

Equalization of wastewater flow and organic loading rate

As the operation of fish canning plants in the Philippines is less than 24 hours per day, and some processes, such as cooking, are batchwise, the flow rates and COD load of the generated wastewater vary with time. Hence, equalization of the wastewater flow is necessary.

Lipid removal system

An upflow substrate precipitation and entrapment system operated at 3 days SRT and 8 hours total HRT, i.e., 6 h in the upflow reactor plus 2 h in external settler, for organic loading rates as high as 15 kg $COD.m^{-3}.d^{-1}$, is a satisfactory option for removing lipids and reducing fluctuations in the influent SS concentration in the subsequent methanogenic reactor. Moreover, the attained increase in soluble COD and volatile fatty acid (VFA) fraction in the wastewater can be beneficial to the growth of the methanogens in the subsequent methanogenic reactor provided the pH in the reactor is kept within the optimum range for methanogenesis.

The upflow substrate precipitation and entrapment system can also serve as an upflow acidogenic reactor where fish processing wastewater is co-digested with easily degradable waste that could, via its acidification, decrease the pH in the system to the IEP of fish proteins. Examples of such wastes are those produced from confectioneries, sugar mills, and vinegar fermentation plants. In these cases, higher lipid removal is expected as compared to that obtained when only fish processing wastewater is pre-treated in the upflow substrate precipitation and entrapment system.

The USPE system is a good alternative to conventional methods such as sedimentation with the aid of chemical coagulants or flocculants and dissolved air flotation (DAF) which require high-pressure air, skimmer, and chemicals, e.g., polymers, alum, or $CaCl_2$. Besides cost, the added chemicals increase the sludge production. Moreover, some chemicals, e.g. alum and ferric chloride, decrease the biodegradability of the sludge, particularly proteins and LCFAs (Dentel and Gossett 1982). Adjustment of pH using acids, e.g. H_2SO_4 or HCl, is shown in this study as a faster option for lipid removal, but the costs of the required chemicals and the undesirable ions, e.g. Cl^- or SO_4^{2-}, brought by the acid to the sludge are possible drawbacks of this method.

High-rate methanogenic reactor

The simplest high-rate methanogenic reactor for fish processing wastewater is a UASB reactor provided that there is a lipid pre-removal system, especially in the treatment of high-lipid wastewater. As compared to other high-rate anaerobic reactors, a UASB reactor is cheaper to construct and simpler to operate than either downflow or upflow filters. Besides costs of packing and support materials, filters are susceptible to clogging problems. An expanded granular sludge bed (EGSB) reactor gives the advantage of enhanced mixing, and thus, faster reaction rates. However, besides the costs of pumping requirement for maintaining high upflow velocity, it is not known whether the granular nature of the sludge can be maintained in the long run. Moreover, sludge flotation occurs at high upflow velocities in treating lipid-containing wastewaters in an EGSB reactor, e.g. in the treatment of LCFA-containing wastewater, granular sludge floats at ≥ 3 $m.h^{-1}$ and HRTs < 6 hours (Hwu 1997). With similar LCFA-containing wastewater, Hwu (1997) recommended prolonged HRT, e.g. 24 h, and low upflow velocity, e.g. 1 $m.h^{-1}$ at a LCFA load of about 0.10 g $COD.g^{-1}$ $VS.d^{-1}$. Hence, a UASB reactor which operates at moderate upflow velocities is more suitable than an EGSB reactor.

Sludge digester

Since the sludge produced from the lipid pre-removal system contains mainly lipids, it should be digested further in a separate anaerobic reactor. This maximizes the total methane recovery from the wastewater in the entire treatment system. Thermophilic digestion might be suitable for the sludge produced from the lipid pre-removal system (Angelidaki and Ahring 1995). The rates of substrate utilization and microbial growth are faster at the thermophilic temperature range. Although thermophiles are more susceptible to toxicity by LCFA, their recovery after an inhibition is faster than that of the mesophiles (Hwu 1997). Application of thermophilic sludge digestion is especially of interest when waste heat is available, e.g. continuous stream of hot waste gases.

Post-treatment for nutrient removal and recovery

After anaerobic digestion, it is necessary to remove the nutrients in the wastewater, e.g. NH_4^+-N and P, and when nitrification is applied, the resulting nitrates. The present methods for NH_4^+-N and/or P removal are (a) air-stripping at pH = 10.5-11.5 (U.S. EPA 1994, Tchobanoglous and Burton 1991), (b) ion exchange (U.S. EPA 1994), (c) breakpoint chlorination (Tchobanoglous and Burton 1991) in which chlorine converts NH_3 to N_2, and (d) biological processes such as nitrification and denitrification in a series of alternating anaerobic, anoxic, and aerobic reactors such as the proprietary $A^2O^®$, five-stage Bardenpho™, UCT™ (named after University of Cape Town), and VIP® (named after Virginia Initiative Plant) processes (Tchobanoglous and Burton 1991). The $A^2O^®$ process consists of anaerobic, anoxic, and aerobic stages in series, followed by a clarifying stage. A portion of the effluent from the aerobic stage is recycled to the anoxic stage for further denitrification. The five-stage Bardenpho™ process similarly consists of the series anaerobic-anoxic-aerobic stages with similar effluent recycling scheme but with additional anoxic-aerobic stages provided for additional denitrification using the nitrates from the first aerobic stage and the endogenous organic carbon, as electron acceptor and donor, respectively. The final aerobic stage is provided to strip residual N_2 gas and minimize the release of P in the final clarifier. Hence, as compared to the $A^2O^®$ process, the Bardenpho™ method produces less sludge with higher P contents, and effluents with lower levels of total N. The UCT™ and VIP® methods consist of the same stages as those of the $A^2O^®$ method but have different effluent recirculation schemes. In the UCT™ method, which has anaerobic-anoxic-anoxic-aerobic stages in series, portions of the effluents of the first anoxic and aerobic stages are recycled to the anaerobic and second anoxic stages, respectively. This method achieves better P removal than the $A^2O^®$ and Bardenpho™ methods. In the VIP® method, portions of effluents from anoxic and aerobic stages are recycled to the anaerobic and anoxic stages, respectively. This method requires smaller reactor volumes than the Bardenpho and UCT™ methods.

Considering the respective drawbacks of the methods described above (e.g. the high COD requirement for denitrification, the cost of pH adjustment in complete air-stripping of NH_3, the cost of regenerating the ion exchange columns, and the cost of chemicals for chlorination), it would be better to partially remove the NH_3 in the wastewater by air-stripping, and nitrify the remaining NH_4^+ in a two-stage aerobic reactor (Bae *et al.* 1997). In the first stage, aeration should be limited in order to control further conversion of NO_2^- to NO_3^-. The remaining free NH_3, when it exceeds 1.0 mg.l^{-1} (Anthonisen *et al.* 1976, Bae *et al.* 1997, Surmacz-Górska *et al.* 1997) can further inhibit the conversion of NO_2^- to NO_3^-. When the dissolved oxygen is sufficiently low, e.g. at < 25% saturation with air (Robertson *et al.* 1988), simultaneous nitrification and denitrification can occur in the first aerobic stage. The effluent of the first stage can be recycled partly to the UASB reactor where methanogenesis and denitrification mainly via NO_2^- can occur simultaneously (Bae *et al.* 1997). The NO_2^- or NO_3^- concentration should be low enough so that the denitrifiers will not outcompete the methanogens for COD in the anaerobic reactor (Bae *et al.* 1997, Hanaki and Polprasert 1989). For denitrification via NO_2^-, the optimum aeration rate and operating pH in the first aerobic reactor should be studied. Results of Cecen *et al.* (1995) suggest that the dissolved oxygen to free ammonia ratio, which depends on pH, is an important factor in inhibiting further conversion of NO_2^- to NO_3^-. The COD/N requirement and the sludge production in denitrification via NO_2^- are lower compared to that of denitrification via NO_3^- (Chen *et al.* 1991). In this way, the recovery of useful products, methane and ammonia, is

maximized. If the wastewater is nitrified without prior removal of NH_3 via stripping, a significant amount of COD in the wastewater which could otherwise be converted to methane will be lost to denitrification. On the other hand, if complete air-stripping of NH_3 is desired, the chemical requirement for adjusting the pH to 11.5 and readjusting to neutral pH is high. In the proposed air-stripping + nitrification-denitrification scheme for ammonia removal, the most economical pH in air-stripping and the recirculation rate from the aerobic to the methanogenic reactor are yet to be determined, considering that a high recirculation rate may excessively dilute the influent to the anaerobic reactor. Based on NH_4^+-NH_3 equilibrium relation, stripping at pH 10 can remove about 80% of NH_4^+-N. For a wastewater of 400 $mg.l^{-1}$ NH_4^+-N (and 3000 mg total $COD.l^{-1}$ as in the low-lipid wastewater used in Chapter 5), the remaining NH_4^+-N concentration will be ca. 80 $mg.l^{-1}$. The required COD for denitrification via NO_2^- will be about 140 $mg.l^{-1}$ based on the theoretical COD/N ratio (1.72, Bae *et al.* 1997). The latter is 5% of the total COD of the raw wastewater. The ammonia in the air used for stripping can be removed using an acid to produce an ammonium salt which has a good fertilizer value. Removal of P is also expected in the proposed post-treatment scheme.

Other considerations

Odor control must be an essential part of the design of both the cannery and the wastewater treatment plant. Further reduction of COD loads on the wastewater treatment plant must be pursued. Besides the present utilization of solid wastes and pre-cooking fish juice for the production of useful by-products, spillage of ingredients such as brines, vegetable oil, and tomato sauce must be minimized. Possibilities for recycling treated wastewater in the plant, such as for floor cleaning purposes, must be explored in order to reduce ground water extraction. Alternative uses of the recovered fish oil in the lipid pre-removal system that can be more valuable than methane, and will not require more expensive processes are also interesting future research lines.

REFERENCES

Anthonisen A. C., Loehr R. C., Prakasam T. B. S. and Srinath E. G. (1976) Inhibition of nitrification by ammonia and nitrous acid. *J. Wat. Pollut. Control Fed.* **48**, 835-852.

Angelidaki I. and Ahring B. K. (1992) Effects of free long-chain fatty acids on thermophilic anaerobic digestion. *Appl. Microbiol. Biotechnol.* **37**, 808-812.

Angelidaki I. and Ahring B. K. (1995) Establishment and characterization of an anaerobic thermophilic (55°C) enrichment culture degrading long-chain fatty acids. *Appl. Environ. Microbiol.* **61**, 2442-2445.

Bae J. H., Kim S. K. and Chang H. S. (1997) Treatment of landfill leachates: Ammonia removal via nitrification and denitrification and further COD reduction via Fenton's treatment followed by activated sludge. *Wat. Sci. Technol.* **36**, 341-348.

Cecen F., Orak E. and Gökcin P. (1995) Nitrification studies on fertilizer wastewaters in activated sludge and biofilm reactors. *Wat. Sci. Technol.* **32**, 141-148.

Chen S. K., Juaw C. K. and Cheng S. S. (1991) Nitrification and denitrification of high-strength ammonium and nitrite wastewater with biofilm reactors. *Wat. Sci. Technol.* **23**, 1417-1425.

De Baere L. A., Devocht M., Van Assche P. and Verstraete W. (1984) Influence of high NaCl and NH_4Cl salt levels on methanogenic associations. *Wat. Res.* **18**, 543-548.

Dentel S. K. and Gossett J. M. (1982) Effect of chemical coagulation on anaerobic digestibility of organic materials. *Wat. Res.* **16**, 707-718.

Doi R. H. (1972) Role of proteases in sporulation. *Curr. Top. on Cell Regul.*, **6**, 1-20.

Glenn A. R. (1976) Production of extracellular proteins by bacteria. *Ann. Rev. Microbiol.*, **30**, 41-62.

Hanaki K. and Polprasert C. (1989) Contribution of methanogenesis to denitrification with an upflow filter. *J. Wat. Pollut. Control Fed.* **61**, 1604-1611.

Hwu C. S. (1997) *Enhancing Anaerobic Treatment of Wastewaters Containing Oleic Acid.* Ph.D. Thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

Lema J. M., Soto M., Veiga M. C. and Mendez R. (1987) Mesophilic and thermophilic anaerobic treatment of wastewaters from industrial processing of mussel. In: *Biomass for Energy and Industry*, Grassi G., Delmon B., Molle J. F. and Zibetta H. (eds.), pp. 872-876. Elsevier Applied Science, London.

Martinelle M. and Hult K. (1994) Kinetics of triglyceride lipases. In: *Lipases: Their Structure, Biochemistry and Application*, Petersen S. B. and Wooley P. (eds.), pp. 159-180. Cambridge University Press., U.K.

Tchobanoglous G. and Burton F. L. (Metcalf and Eddy, Inc.)(1991) *Wastewater Engineering: Treatment, Disposal, and Reuse*, 3rd edn. McGraw-Hill, Inc., U.S.A.

Robertson L. A., Van Niel E. W. J., Torremans R. A. M. and Kuenen J. G. (1988) Aerobic Denitrification and Heterotrophic Nitrification in *Thiosphaera pantotropha.* Ph.D. Thesis, Delft Technical University, The Netherlands.

Roy F., Samain. E., Dubourguier H. C. and Albagnac G. (1986) *Synthrophomonas sapovorans* sp. nov., a new obligately proton reducing anaerobic oxidizing saturated and unsaturated long-chain fatty acids. *Arch. Microbiol.* **145**, 142-147.

Surmacz-Górska J., Chicon A. and Miksch K. (1997) Nitrogen removal from wastewater with high ammonia nitrogen concentration via shorter nitrification and denitrification. *Wat. Sci. Technol.* **36**, 73-78.

U.S. Environmental Protection Agency (U.S. EPA) (1994) *Nitrogen Control.* TECHNOMIC Publishing Co., Inc., U.S.A.

Anaërobe behandeling van visverwerkingsafvalwater: discussie en aanbevelingen.

De visverwerkende industrie levert een belangrijke bijdrage aan de Filippijnse economie. Deze industrie verontreinigt samen met andere industrieën, meren, rivieren en uiteindelijk de kustwateren. Indien het effluent van deze bedrijven niet adequaat wordt behandeld, wordt het milieu van de Filippijnse kustwateren bedreigd. Door de waterverontreiniging zou de visindustrie op langere termijn kunnen verdwijnen. Het vooruitzicht van energieproductie uit visverwerkingsafval zal de bedrijven stimuleren het afvalwater te behandelen, hetgeen vervolgens de overheid kan stimuleren striktere lozingseisen te implementeren.

De belangrijkste producten van de Filippijnse visverwerkende bedrijven zijn: sardien in blik voor de binnenlandse markt en tonijn voor de buitenlandse markt (hoofdstuk 1). Van de verwerkte vis resteert na behandeling 40-60% als vast afval en 1-6% komt in het afvalwater terecht. Het afvalwater dat bij het inblikken van vinvissoorten zoals sardien en makreel ontstaat bevat een hoge concentratie gesuspendeerd materiaal, voornamelijk bestaande uit eiwitten en vetten. De Filippijnse bedrijven gebruiken grondwater voor het verwerkingsproces waardoor de concentratie zout in het visverwerkingsafvalwater laag is vergeleken met bedrijven waar zeewater wordt gebruikt tijdens de verwerking.

De concentratie biologisch afbreekbaar organisch materiaal in het visverwerkingsafvalwater is hoog, daardoor is het waarschijnlijk mogelijk d.m.v. biogasproduktie uit afvalwater een hoge netto energieproductie te behalen. Dit maakt anaërobe behandeling van dit afvalwater aantrekkelijk. Tevens is de slibproductie bij anaërobe processen lager dan bij aërobe processen. Hoog belastbare anaërobe behandelingssystemen zijn economisch aantrekkelijk, omdat het benodigde reactorvolume kleiner is dan bij conventionele systemen. Deze zuiveringssystemen zijn ook toepasbaar bij visverwerkingsbedrijven met een beperkte ruimte. In dit onderzoek is gekeken naar de toepassing van hoog belastbare anaërobe systemen, zoals het UASB-systeem, voor de behandeling van visverwerkingsafvalwater. Hierbij is de nadruk gelegd op de hydrolyse, het proces dat over het algemeen als de snelheidsbeperkende stap wordt beschouwd bij de anaërobe vergisting van gesuspendeerd of niet opgelost organisch materiaal.

In de volgende hoofdstukken worden de belangrijkste resultaten van dit onderzoek samengevat en wordt de betekenis hiervan voor de anaërobe zuivering van visverwerkingsafvalwater en andere vethoudende afvalwaters behandeld.

1. Discussie

1.1 Het effect van verschillende milieu- en procesfactoren op de hydrolyse van vetten en eiwitten

1.1.1. Het effect van methanogenese

Uit de resultaten van dit onderzoek is gebleken dat de methanogenese de hydrolyse van vetten bevordert. Deze verbeterde hydrolyse zou kunnen worden veroorzaakt door een syntrofe relatie tussen methanogenen en acetogenen. Mogelijk wordt de activiteit van lipases geremd door de accumulatie van hydrolyseproducten (hogere vetzuren) aan het grensvlak van de triglyceride, waar de katalyse door lipase plaatsvindt (Martinelle and Hult 1994). De afbraak van hogere vetzuren, oftewel de β-oxidatie, welke afhankelijk is van de methanogenese, (Angelidaki and Ahring 1995, Roy *et al.* 1986), zal dan een positief effect hebben op de hydrolyse van vetten. Met andere woorden de complete hydrolyse van vetten zal niet plaatsvinden onder omstandigheden waarbij de methanogenese wordt geremd, zoals een te hoge of te lage pH, hoge ammonium of NaCl concentraties.

In tegenstelling tot de hydrolyse van vetten is de hydrolyse van eiwitten onafhankelijk van het plaatsvinden van methanogenese. Daarom is in een continue anaërobe reactor de hydrolyse van eiwitten, in vergelijking met de hydrolyse van vetten, minder gevoelig voor verstoringen die de methanogenese remmen.

1.1.2 Het effect van vetten

Bij een toenemende initiële vetconcentratie, van 0,09 tot 2,24 $g.l^{-1}$ (0,26 tot 6,47 g $CZV.l^{-1}$), neemt de methanogene lag-fase toe, hetgeen aantoont dat vetten de methanogenese remmen. In hoofdstuk 2 wordt aangetoond dat de remming door vetten niet alleen wordt veroorzaakt door de hydrolyseproducten. Gedurende de methanogene lag-fase vindt namelijk nauwelijks omzetting van vetten in hogere vetzuren plaats. De lag-fase gevonden tijdens dit onderzoek is aanmerkelijk langer dan de lag-fases die zijn bepaald door Angelidaki and Ahring (1992) bij de omzetting van oleaat met concentraties van 0.1 tot 0.2 $g.l^{-1}$. De mogelijkheid dat zowel hogere vetzuren als niet gehydrolyseerde vetten de methanogenese remmen mag niet worden uitgesloten. Indien de hydrolyse van vetten toeneemt als gevolg van het optreden van methaanvorming en de methanogenese wordt geremd door de aanwezigheid van vetten, zullen vetten, wanneer ze in te hoge concentraties voorkomen, hun eigen hydrolyse remmen.

Zowel de hydrolysesnelheid als de biologische afbreekbaarheid van eiwitten worden negatief beïnvloed door hoge vetconcentraties (concentraties hoger dan 4.30 g $CZV.l^{-1}$ bij een temperatuur van 30°C en een entslib concentratie van 0.67 g $VS.l^{-1}$). Dit betekent dat lange slibverblijftijden nodig zijn om een hoge methaanproductie te verkrijgen uit afvalwater met een hoge vetconcentratie.

1.1.3. Het effect van pH

Het pH-optimum voor de hydrolyse van vetten en eiwitten uit vis ligt tussen 6 en 8. Bij lage pH waarden (pH 4-5) vindt nauwelijks hydrolyse van vetten plaats. Zowel het voorkomen van

als de snelheid van de verzuring van vetten (β-oxidatie van hogere vetzuren) is afhankelijk van de pH. De maximale snelheid wordt tevens gevonden tussen pH 6 en 8. Het effect van de pH op de hydrolyse wordt mogelijk veroorzaakt door het effect van de pH op de methanogenese. Als de methanogenese wordt geremd als gevolg van een lage pH wordt de β-oxidatie van hogere vetzuren geremd door de afwezigheid van methanogenese. De verzuring van vetten zal dan ook alleen plaatsvinden bij aanwezigheid van methanogeen slib, bij pH waarden tussen 6 en 8.

De hydrolyse van eiwitten vindt plaats bij alle in dit onderzoek toegepaste pH waarden (pH 4-8) onafhankelijk van de aan- of afwezigheid van methanogeen entslib. De hydrolysesnelheid is hoger bij pH's tussen 6 en 8 dan bij pH's lager dan 6. Het effect van de pH op de hydrolysesnelheid van eiwitten is waarschijnlijk het netto effect van verschillende factoren. De lage hydrolysesnelheid van eiwitten bij pH 4 en 5 wordt toegeschreven aan de lage oplosbaarheid van viseiwitten bij deze pH's. Deze pH waarden liggen dicht bij het iso-elektrisch punt (IEP) van viseiwitten. Tijdens dit onderzoek is voor deze viseiwitten een IEP van ongeveer 5,3 gevonden. De secundaire structuur van het eiwitmolecuul ontvouwt zich bij het IEP waardoor het eiwit neerslaat en de oplosbaarheid afneemt. Tevens is het mogelijk dat door de aanwezigheid van vetten, waarvan de afbraak wordt geremd bij een lage pH, de eiwitten minder toegankelijk zijn voor de proteolytische enzymen. De activiteit van protease neemt toe met de toename van de pH van 4 tot 9, met uitzondering van pH 8 waar een relatief lagere activiteit wordt gevonden. Dit wordt mogelijk veroorzaakt doordat de verschillende enzymen in vis verschillende pH optima hebben.

Het is niet moeilijk de pH van visverwerkingsafvalwater te handhaven in het neutrale gebied. De pH stelt zich spontaan in op ongeveer 6 bij afwezigheid van methanogene activiteit en 7 bij aanwezigheid van methanogenese. De pH bufferingscapaciteit van het afvalwater neemt tijdens de vergisting in anaërobe reactoren toe als gevolg van de gelijktijdige productie van CO_2 en ammonium.

1.1.4. Het effect van NaCl

Bij een NaCl concentratie van 20 $g.l^{-1}$ wordt de hydrolyse van viseiwitten geremd; de protease activiteit blijft bij deze concentratie echter onveranderd. Uit het onderzoek is gebleken dat een hoge NaCl concentratie een direct negatief effect heeft op de verzuring van aminozuren. Dit blijkt uit de hoge concentratie niet verzuurd opgelost CZV, hetgeen voornamelijk bestaat uit aminozuur-CZV. De geaccumuleerde aminozuren remmen vervolgens de protease productie en als gevolg daarvan de hydrolyse van eiwitten.

De langere lag-fase voor de methaanvorming en verzuring van vetten bij toename van de NaCl concentratie, geven aan dat deze worden geremd door hoge NaCl concentraties. Dit effect van NaCl op de verzuring van vetten wordt mogelijk veroorzaakt door remming van de methanogenese. De resultaten van het onderhavige onderzoek en het onderzoek van De Baere *et al.* (1984) en Lema *et al.* (1987) geven aan dat adaptatie van methanogeen slib aan NaCl mogelijk is.

1.1.5. Het effect van NH_4^+-N

Bij de in dit onderzoek gebruikte NH_4^+-N concentraties van 0 tot 1,5 $g.l^{-1}$ is geen remming van de vethydrolyse waargenomen. Uit een afname van de methanogenese bij toenemende NH_4^+-N concentraties blijkt dat deze door NH_4^+-N wordt geremd. De afhankelijkheid van afbraak van hogere vetzuren en methanogenese suggereert dat er mogelijk een negatief effect van hogere NH_4^+-N concentraties op de afbraak van hogere vetzuren is. De hydrolyse van eiwitten wordt bij concentraties hoger dan 0.6 $g.l^{-1}$ NH_4^+-N geremd. Dit effect wordt evenals de remming van de eiwitafbraak door NaCl toegeschreven aan accumulatie van aminozuren, welke wordt veroorzaakt door een remmend effect op de verzurende bacteriën.

1.2. Behandeling van visverwerkingsafvalwater in hoog belastbare anaërobe systemen

1.2.1. Eentraps 'upflow anaerobic sludge blanket' (UASB) reactor

De resultaten van dit onderzoek laten zien dat een eentraps UASB reactor een veelbelovend alternatief is voor de behandeling van visverwerkingsafvalwater met een lage vetconcentratie (hoofdstuk 5). Het behandelde afvalwater heeft een CZV concentratie van ongeveer 2,72 $g.l^{-1}$ waarvan 9% (85 $mg.l^{-1}$) bestaat uit vetten. De volumebelasting en slibbelasting met vetten zijn bij de toegepaste hydraulische verblijftijd (HVT) van 11 uur respectievelijk 5,4 g $CZV.l^{-1}.d^{-1}$ en 0,011-0,029 g $vetten.g^{-1}VS.d^{-1}$. Onder deze omstandigheden werd 63% van het totaal CZV verwijderd waarvan 79% werd omgezet in methaan. Bij behandeling van hetzelfde water met een NaCl concentratie van 4 $g.l^{-1}$ onder gelijke procescondities als hierboven beschreven is een langere opstartperiode nodig, omdat zowel de verzurende als methanogene bacteriën moeten adapteren aan de hogere NaCl concentratie. Ongeveer 70 dagen na opstart waren de omzetting van CZV naar methaan en de CZV verwijdering vergelijkbaar met de resultaten van het afvalwater zonder NaCl. Bij de behandeling van afvalwater met een hoog vetgehalte (3.50 g totaal $CZV.l^{-1}$ waarvan 47% bestaat uit vetten) in een eentraps UASB bij een volumebelasting van 8 g $CZV.l^{-1}.d^{-1}$ en een HVT van 12 uur, wordt 92% van het CZV verwijderd en slechts 47% omgezet naar methaan. Een groot deel (ongeveer 34%) van het totaal CZV is verwijderd als drijflaag in de drie-fasen-scheider bovenin de reactor.

Evenals bij de batch experimenten (hoofdstuk 2, 3 en 4) lijkt de hydrolyse van vetten snelheidsbeperkend te zijn. Het grootste deel van de eiwitten wordt gehydrolyseerd (72-100%) na behandeling in de UASB reactor, terwijl vetten vooral worden verwijderd door adsorptie aan het reactor oppervlak en aan slibdeeltjes en niet worden gehydrolyseerd. Deze adsorptie bedreigt de slibretentie en de methanogene capaciteit van de reactor en daarmee de stabiliteit van het systeem. Dit betekent dat bij behandeling van afvalwater met hoge vetconcentraties, of het vet moet worden verwijderd vóór de toepassing van een hoogbelastbare UASB reactor, of dat een lage vetbelasting en daarmee een lange HVT moet worden toegepast.

1.2.2. Voorbehandeling in een 'upflow substrate precipitation and entrapment' (USPE) systeem

Omdat het noodzakelijk is vóór de behandeling in een UASB reactor de vetten in visafvalwater te verwijderen, is het USPE systeem voor de voorbehandeling van afvalwater met een hoog

vetgehalte onderzocht (hoofdstuk 6). Het USPE systeem bestaat uit een opwaarts doorstroomde reactor en een bezinker. Het influent stroomt door een slibbed waarin het gesuspendeerde materiaal kan worden afgevangen, gedeeltelijk gehydrolyseerd en verzuurd. Uit de resultaten blijkt dat het systeem het gesuspendeerde materiaal, dat voornamelijk uit vetten bestaat, voldoende verwijdert. Bij een HVT van 6,1 uur en een slibverblijftijd (SVT) van 3,1 dagen worden 80% van de vetten en 80% van het gesuspendeerd CZV verwijderd. Het afvalwater bevat een totaal van 4 g CZV.l^{-1} waarvan 60% bestaat uit vetten en 11% uit gesuspendeerd eiwit. Na een verblijftijd van 3 uur in de bezinker nemen de verwijderingsefficiënties op basis van CZV toe tot 86% en 89% voor respectievelijk vetten en gesuspendeerd CZV.

Tijdens batch bezinkingsexperimenten werd een maximale verwijdering van eiwitten en vetten gevonden tussen pH 4,5 en 5,5. Deze verwijdering wordt veroorzaakt door co-precipitatie van vetten en eiwitten bij het IEP van viseiwitten. Wanneer de pH kan worden verlaagd tot deze waarde door middel van gedeeltelijke hydrolyse en verzuring van het substraat, kan een hoge vetverwijdering worden verwacht. Een dergelijk lage pH kan echter niet bereikt worden in de USPE reactor. Desondanks treedt een significante verwijdering van vetten op veroorzaakt door een combinatie van adsorptie, precipitatie en invang in het slibbed. Eiwitten worden in beperkte mate verwijderd als gevolg van omzetting tot opgeloste producten, vetten worden daarentegen alleen via fysische processen verwijderd. De resultaten van de batch experimenten (hoofdstuk 2, 3 en 4) laten zien dat er geen afbraak van vetten optreedt in afwezigheid van methanogenese. De vetconcentratie in het slib van de USPE reactor is hoog en als het gevolg hiervan wordt de methanogenese geremd. Verlengen van de SVT resulteert niet in een verbeterde verwijdering van gesuspendeerde stof. Bij langere SVT's zal mogelijk enige methanogenese plaatsvinden, hetgeen zal resulteren in afname van de slibbezinking en van de verwijdering van vetdeeltjes; verlenging van de slibverblijftijd is dan ook niet gewenst.

Figuur 7.1: Het effect van verschillende milieu- en procesfactoren op de afbraak van vetten en eiwitten. Doorgetrokken strepen geven de afbraakroutes aan; gebroken lijnen geven remmende effecten aan (pijlpunten wijzen naar het geremde proces); stippellijnen geven positieve effecten aan; [a] dit onderzoek; [b] Glenn, 1976, Doi, 1972; [c] Martinelle and Hult, 1994; [d] Roy *et al.*, 1986; [e] Angelidaki and Ahring, 1992; P = vorming enzym; C = Katalyse.

Oorzaak-effect relatie tussen verschillende factoren bij de anaërobe vergisting van vethoudend afvalwater.

Terwijl de methanogenese de hydrolyse van viseiwitten nauwelijks beïnvloedt, speelt de methanogenese een belangrijke rol bij de complete afbraak van vetten. Bij de vergisting van vethoudend afvalwater moeten de milieufactoren zoals pH, temperatuur en ionenconcentraties dus gunstig zijn voor methanogenen. Echter bij hoge vetconcentraties zal de remming van methanogenen door vetten overheersen waardoor vetten hun eigen afbraak remmen. Deze cyclische oorzaak-effect relatie is weergegeven in Fig 7.1.

De milieufactoren die de hydrolyse van gesuspendeerd materiaal in visverwerkingsafvalwater beïnvloeden zijn o.a. de pH en de NaCl en ammonium concentratie. Omdat de hydrolyse van vetten afhankelijk is van de methanogenese kunnen deze factoren de hydrolyse indirect beïnvloeden door hun effect op methanogenese. Hoge concentraties NaCl en ammonium

remmen de afbraak van aminozuren; de accumulatie van deze hydrolyseproducten remt vervolgens de productie van proteolytische enzymen en daarmee de hydrolyse van eiwitten.

2. Aanbevelingen: de systeemonderdelen benodigd voor de behandeling van visverwerkingsafvalwater

Rekening houdend met de resultaten van dit onderzoek zou een systeem voor de anaërobe behandeling van visafvalwater moeten bestaan uit de volgende onderdelen:

- buffertank t.b.v. het afvlakken van pieken in afvalwaterdebiet en organische belasting
- vetverwijderingssysteem voorafgaand aan een hoog belastbare methanogene reactor
- een hoog belastbare methanogene reactor
- een anaërobe vergister voor het spuislib van de vetverwijderingsreactor
- een na-behandelingssysteem voor de verwijdering en/of terugwinning van nutriënten

Buffertank

De visverwerkende bedrijven in de Filippijnen zijn minder dan 24 uur per dag in bedrijf en sommige processen zoals koken worden ladingsgewijs uitgevoerd. Hierdoor varieert het afvalwaterdebiet en de CZV-belasting in de tijd en is een buffersysteem noodzakelijk voor het verkrijgen van een gelijkmatig afvalwaterdebiet en organische belasting.

Vetverwijderingssysteem

Toepassing van een opwaarts doorstroomde reactor t.b.v. precipitatie en invang van substraat bij een SVT van 3 dagen, een HVT van 8 uur (6 uur in de reactor en 2 uur in de na-bezinker) en organische belastingen tot 15 kg CZV.m^{-3}.d^{-1}, is geschikt voor de verwijdering van vetten en resulteert in vermindering van de fluctuatie in de influentconcentratie van de daarop volgende methanogene reactor. Daarnaast kan de verhoging van de fractie opgelost CZV en vluchtige vetzuren in het afvalwater de groei van de methanogenen in de methanogene reactor ten goede komen, mits de pH in de reactor optimaal is voor de methanogenese.

De opwaarts doorstroomde reactor voor de precipitatie en de invang van substraat kan tevens worden ingezet als verzuringsreactor voor de co-vergisting van visverwerkingsafvalwater met gemakkelijk afbreekbaar organische afvalstoffen. Door de verzuring van dergelijk afvalstromen kan de pH in het systeem dalen tot het IEP voor viseiwitten. Dergelijk organisch afval wordt bijvoorbeeld geproduceerd bij banketbakkerijen, de suikerindustrie en bij de productie van azijn. Bij toepassing van deze co-vergisting kan een hoger vetverwijderingsrendement worden verwacht dan wanneer alleen visverwerkingsafvalwater wordt voorbehandeld in het systeem.

Het USPE systeem is een goed alternatief voor gangbare methoden zoals bezinking met behulp van chemische coagulanten of flocculanten en dissolved air flotation (DAF), waarbij gebruik gemaakt wordt van een mechanische vetverwijdering en chemicaliën zoals polymeren, kaliumaluminiumsulfaat en ijzerchloride. Het toevoegen van chemicaliën geeft naast hogere kosten tevens een hogere slibproductie. Daarbij wordt door het toevoegen van sommige chemicaliën zoals kaliumaluminiumsulfaat en ijzerchloride de afbreekbaarheid van het slib, met

name van eiwitten en hogere vetzuren, verminderd (Dentel and Gosset, 1982). Uit dit onderzoek is gebleken dat het verlagen van de pH met zuren zoals H_2SO_4 of HCl een snellere methode is voor het verwijderen van vetten. De kosten van de chemicaliën en het toevoegen van ongewenste ionen (Cl^- of SO_4^{2-}) aan het slib zijn mogelijke nadelen van deze methode.

Hoog belastbare methanogene reactor

De eenvoudigste hoogbelastbare methanogene reactor voor visverwerkingsafvalwater is een UASB reactor, onder voorwaarde dat er bij de behandeling van afvalwater met een hoog vetgehalte een voorverwijderingssysteem is voor het verwijderen van vetten. Vergeleken met andere hoogbelastbare systemen is een UASB eenvoudiger te construeren en te bedrijven dan opwaarts of neerwaarts doorstroomde filters. Naast de kosten voor filtermateriaal en de aanpassingen nodig voor bevestiging van deze filters zijn dergelijke systemen gevoelig voor verstopping. Het voordeel van een expanded granular sludge bed (EGSB) reactor is dat door de betere menging de reactiesnelheden groter zijn. De kosten voor het onderhouden van een hoge opstroomsnelheid zijn echter hoog en het is niet bekend of het korrelslib op langere termijn zijn korrelstructuur behoudt. Wanneer vethoudend afvalwater wordt behandeld in een EGSB reactor treedt bij hoge opstroomsnelheden slibflotatie op. Bij de behandeling van afvalwater met hogere vetzuren treedt flotatie van korrelslib op bij opstroomsnelheden ≥ 3 $m.h^{-1}$ en HVT's < 6 uur (Hwu, 1997). Voor afvalwater met een hogere vetzuurconcentratie van 0.10 $CZV.g^{-1}VS.d^{-1}$, vergelijkbaar met de concentraties in dit onderzoek, beveelt Hwu (1997) een verlenging van de HVT tot 24 uur en een lage opstroomsnelheid tot maximaal 1 $m.h^{-1}$ aan. Voor de behandeling van afvalwater met hogere vetzuren is een UASB dan ook geschikter

Slibvergister

Aangezien het geproduceerde slib in het vetvoorverwijderingssysteem voornamelijk uit vetten bestaat moet dit verder worden vergist in een anaëroob systeem. Op deze manier wordt de methaanproductie uit het afvalwater gemaximaliseerd. Thermofiele vergisting is een mogelijke optie voor de verwerking van dit slib (Angelidaki en Ahring, 1995). De groeisnelheid en de substraatverbruikssnelheid van microörganismen is hoger in het thermofiele gebied. Hoewel thermofiele bacteriën gevoeliger zijn voor hogere vetzuren kunnen ze zich na remming sneller herstellen dan mesofiele bacteriën (Hwu, 1997). Toepassing van thermofiele slibvergisting is vooral aantrekkelijk wanneer er restwarmte beschikbaar is bijvoorbeeld uit een constante stroom hete gassen.

Nabehandeling ten behoeve van de verwijdering en terugwinning van nutriënten

Na anaërobe vergisting is het noodzakelijk dat de nutriënten zoals NH_4^+-N (indien nitrificatie wordt toegepast de nitraten) en P uit het afvalwater worden verwijderd. De huidige methoden voor de verwijdering van NH_4^+-N en/of P zijn (a) strippen met lucht bij pH waarden tussen 10.5 en 11.5 (U.S. EPA, 1994, Tchobanoglous en Burton, 1991), (b) ionenwisseling (U.S. EPA 1994), (c) breekpuntchlorering (Tchobanoglous en Burton, 1991) waarbij door reactie met chloor NH_3 wordt omgezet in N_2 en (d) biologische processen als nitrificatie en denitrificatie in een serie van afwisselend anaërobe, anoxische en aërobe reactoren in systemen zoals het $A^2O^®$ proces, 'five stage Bardenpho®', $UCT^®$ (genoemd naar de University of Cape

Town) en VIP® (Virginia Initiative Plant)(Tchobanoglous en Burton, 1991). Het A^2O® proces bestaat uit een serie van anaërobe-, anoxische en aërobe fasen, gevolgd door een bezinker. Een deel van het effluent van de aërobe fase wordt voor verdere denitrificatie gerecirculeerd naar de anoxische fase. De 'five stage Bardenpho®' bestaat tevens uit een serie anaërobe-, anoxische- en aërobe fasen met een vergelijkbare effluent recirculatie; er is echter een extra anoxische- en een anaërobe fase toegevoegd. Deze fasen zorgen voor extra denitrificatie, hierbij wordt gebruik gemaakt van de nitraten uit de eerste aërobe fase en een externe koolstofbron respectievelijk als elektronenacceptor en -donor. De laatste aërobe fase wordt gebruikt om het resterende N_2 gas te strippen en de afgifte van P in de bezinker te minimaliseren. Vergeleken met het A^2O® proces produceert de Bardenpho® methode minder slib met een hoger P gehalte en een effluent met een lager N-totaal gehalte. De UCT® en VIP® methode bestaat uit de zelfde fasen als het A^2O® proces maar ze hebben verschillende effluent recirculatie schema's. Bij het UCT® proces welke bestaat uit een serie van anaërobe-, anoxische-, anoxische- en aërobe fasen worden delen van het effluent van de eerste anoxische en aërobe fase gerecirculeerd naar respectievelijk de eerste anaërobe en de tweede anoxische fase. Met deze methode wordt een betere P verwijdering gerealiseerd in vergelijking met het A^2O® en het Bardenpho® proces. Bij het VIP® proces worden delen van het effluent van de anoxisch- en aërobe fase gerecirculeerd naar respectievelijk de anaërobe en anoxische fasen. Voor deze methode zijn kleinere reactorvolumes nodig dan bij de Bardenpho® en de UCT® methoden.

De nadelen van de hierboven beschreven methoden in ogenschouw nemend (veel CZV nodig voor denitrificatie, de kosten voor het aanpassen van de pH bij het compleet verwijderen van NH_3 via strippen, de kosten voor het regenereren van ionenwisselaars en de kosten van chemicaliën voor chlorering) zou het beter zijn de NH_3 in het afvalwater gedeeltelijk te verwijderen door strippen en de resterende NH_4^+ te nitrificeren in een reactor met twee anaërobe fasen (Bae *et al.*, 1997). In de eerste fase moet de beluchting worden gecontroleerd, om verdere omzetting van NO_2^- naar NO_3^- te beperken. De resterende vrije NH_3 kan tevens, wanneer de concentratie hoger is dan 1.0 $mg.l^{-1}$, de omzetting van NO_2^- naar NO_3^- beperken (Anthonisen *et al.* 1976, Bae *et al.*, 1997, Surmacz-Górska *et al.*, 1997). Wanneer de opgeloste zuurstofconcentratie voldoende laag is, minder dan 25% verzadiging (Robertson *et al*, 1988), is gelijktijdige nitrificatie en denitrificatie mogelijk in de eerste aërobe fase. Het effluent kan gedeeltelijk worden gerecirculeerd naar de UASB reactor waar methanogenese en denitrificatie, voornamelijk via NO_2^-, gelijktijdig kunnen plaatsvinden (Bae *et al.*, 1997). De NO_2^- en NO_3^- concentraties moeten voldoende laag zijn om te voorkomen dat onvoldoende CZV resteert voor methanogenen (Bae *et al.*, 1997, Hanaki en Polprasert 1989). De optimale beluchting en pH in de eerste aërobe reactor voor omzetting van NO_2^- dienen verder onderzocht te worden. De resultaten van onderzoek uitgevoerd door Cecen *et al.* (1995) suggereren dat de verhouding van opgelost zuurstof en ammonia, welke afhankelijk is van de pH, een belangrijke rol speelt bij de remming van verdere omzetting van NO_2^- naar NO_3^-. De CZV/N verhouding voor denitrificatie via NO_2^- en de hoeveelheid geproduceerd slib zijn lager in vergelijking met de denitrificatie via NO_3^- (Chen *et al.*, 1991). Op deze manier wordt de terugwinning van nuttige producten, zoals methaan en ammonia, gemaximaliseerd. Indien het afvalwater wordt genitrificeerd zonder vooraf het NH_3 via strippen te verwijderen, wordt een significante hoeveelheid CZV gebruikt voor denitrificatie terwijl deze in methaan omgezet had kunnen worden. Wanneer echter de NH_3 volledig d.m.v. strippen verwijderd moet worden zal de hoeveelheid chemicaliën nodig voor het verhogen van de pH tot 11.5 en deze vervolgens

terug brengen naar neutrale waarden groot zijn. De optimale pH voor het strippen en grootte van de recirculatiestroom van de aërobe naar de methanogne reactor zullen voor het voorgestelde strip + nitrificatie-denitrificatie proces voor de verwijdering van ammonia moeten worden bepaald. Bij de bepaling van de recirculatiestroom moet rekening worden gehouden met het feit dat een grote recirculatiestroom het influent te veel kan verdunnen. Uit het evenwicht tussen NH_4^+ en NH_3 blijkt dat 80% van NH_4^+-N kan worden gestript bij pH 10. Voor afvalwater met 400 mg.l^{-1} NH_4^+-N (en 3000 mg CZV.l^{-1}, zoals het afvalwater met een lage vetconcentratie beschreven in hoofdstuk 5) zal de resterende NH_4^+-N concentratie 80 mg.l^{-1} zijn. Gebaseerd op de theoretische CZV/N verhouding (1.72, Bae *et al.*, 1997), zal de benodigde hoeveelheid CZV 140 mg.l^{-1} zijn, hetgeen 5% is van het totale CZV van het ruwe afvalwater. Het ammonium in de striplucht kan worden verwijderd met behulp van zuur, waarbij een ammoniumzout met een hoge bemestingswaarde ontstaat. De verwijdering van fosfaat zal tevens deel uitmaken van het voorgestelde nabehandelingsproces.

Verdere overwegingen

De beperking van geuremmissies zal een belangrijk onderdeel in het ontwerp van zowel de visverwerkingsbedrijven als de bijbehorende afvalwaterzuivering moeten zijn. De CZV belasting van de afvalwaterzuivering moet zoveel mogelijk worden beperkt. Naast het huidige gebruik van vast afval en voorgekookt visvocht voor het maken van nuttige bijproducten zou het morsen van bijvoorbeeld pekel, plantaardige olie en tomatenpuree moeten worden beperkt. De mogelijkheden van hergebruik van het gezuiverd afvalwater in het bedrijf, bijvoorbeeld voor het reinigen van de vloer, om de onttrekking van grondwater te verminderen moet worden onderzocht. Duurzame en economisch aantrekkelijke alternatieven voor de methaan produktie uit de visolie, welke tijdens de vetvoorverwijdering wordt geproduceerd, zijn tevens interessant voor verder onderzoek.

Curriculum vitae

Analiza Mar Palenzuela Rollon was born on April 10, 1963 in Manila, Philippines. She completed her secondary education in Guinobatan, Albay. She obtained her Bachelor of Science degree in Chemical Engineering at the University of the Philippines (UP), Diliman, in April 1985. Right after graduation, she worked for two years as a faculty member of the Institute of Chemistry in UP at Los Baños from 1987 to 1989, she headed the Quality Control, Research, and Development Section of Worldwide Papermills, Inc., a company based in Metro Manila. In 1989, she joined the Department of Chemical Engineering in UP Diliman as a faculty member. At the same time, she pursued the degree of Master of Science in Chemical Engineering (in UP), which she obtained in 1992. In 1993, she was conferred the degree of Master of Science in Environmental Science and Technology jointly by the Wageningen Agricultural University and the International Institute for Infrastructural, Hydraulic and Environmental Engineering, The Netherlands. For the latter degree, she wrote a thesis on the anaerobic treatment of fish processing wastewater using expanded granular sludge bed in one and two phase systems. In the same year, she started her PhD research which this book summarizes.