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Release of dissolved free amino acids from biofilms in stream water

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Abstract We investigated the origin of dissolved free amino acids (DFAA) in stream water focusing on biofilms in the river bed by means of incubation experiments in the laboratory. Stones were placed in the Toyohira River, Hokkaido, Japan, for 3 months, allowing formation of biofilms, and then incubated for 24 h in the laboratory at stream water temperature. After incubation, the composition and concentrations of DFAA in the incubation solution and total hydrolyzed amino acids (THAA) in biofilms were measured by high-performance liquid chromatography. The amount of chlorophyll *a* (Chl. *a*) and the number of bacteria were also measured. The DFAA concentration increased greatly in the biofilm incubation solution, but the DFAA composition (mol %) did not change relative to the inception of incubation, where it was similar to stream water. There was no correlation between the increase in DFAA concentration and the THAA concentration, Chl. *a* amount, or the number of bacteria in biofilms. These results suggest that biofilms are one of the major sources of DFAA in stream water.

Keywords Dissolved free amino acids · Biofilm · Epilithic algae · Salmon homing · Toyohira River

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Introduction

It is well known that mature salmon display an amazing ability to migrate thousands of kilometers to natal streams for reproduction after several years of oceanic feeding. Wisby and Hasler [1] proposed the olfactory hypothesis that adult salmon primarily use olfactory cues during their homing migration from the coastal sea to their natal streams. The importance of olfactory function to salmon homing has been reviewed in many behavioral, electrophysiological, and neurobiological studies [2–6]. Unlike olfactory organs of terrestrial animals, fish olfactory organs respond only to a limited number of chemicals dissolved in water, such as L-amino acids, bile acids, sex steroids, prostaglandins, and nucleotides [7]. In general, amino acids are potent odorants for fish, and the salmon olfactory organ responds sensitively to various species of amino acids [8, 9].

Shoji et al. [10] analyzed the compositions of dissolved free amino acids (DFAA), inorganic cations, and bile acids in waters from three streams flowing into Lake Toya, Hokkaido, Japan, and prepared artificial waters containing equivalent amounts of DFAA; the olfactory response of masu salmon *Oncorhynchus masou* to these artificial stream waters closely resembled olfactory responses to the corresponding natural waters. We also previously conducted behavioral experiments to determine whether artificial natal stream water matching the DFAA composition of natal streams had attractive effects on chum salmon *O. keta* on upstream selective movement, and found that chum salmon selected the artificial DFAA stream water [11, 12]. These results of previous electrophysiological and behavioral experiments suggest the possibility that the DFAA compositions in natal streams are odorants for Pacific salmon. However, the origin of DFAA in stream water has not yet been clarified.

Changes in the DFAA compositions in stream water are attributed mainly to complicated biological processes in the watershed ecosystem. There are many possible factors affecting the DFAA compositions both inside and outside of the stream environment, such as soils, vegetation, litter, pollen, dew, and various microbial activities [13–24]. Among these factors, the roles of complex microbial communities called biofilms have been intensively investigated [25–30]. A biofilm consists of various microorganisms and is embedded into a matrix of extracellular polymeric substances [31]. These microorganisms are composed of both photo-autotrophic and heterotrophic microbes, such as algae, fungi, protozoa, and bacteria. Among them, bacteria are the most dominant, and the bacteria concentration in biofilms at the surface of aquatic reeds was three times greater than that in the water in Lake Biwa [28].

To investigate the origin of DFAA in the Toyohira River, Hokkaido, Japan, the DFAA-releasing activity of microbes in the river bed was examined by means of biofilm and unialgal incubation in the laboratory. In the incubation experiments, biofilms were formed by placing river stones in the Toyohira River for 3 months, allowing microbes to attach, and epilithic algae were isolated from biofilm suspensions in the Toyohira River. After incubation, the concentration and composition of DFAA in the incubation solution were analyzed by high performance liquid chromatography (HPLC), and the amount of chlorophyll *a* (Chl. *a*) and the number of bacteria in biofilms were analyzed by a fluorometer and 4′-6-diamino-2-phenylindole (DAPI) staining, respectively. Finally, the possible mechanisms of DFAA release from biofilms into stream water are discussed.

Materials and methods

Study site

The Toyohira River flows through Sapporo City, Hokkaido, and is a tributary of the Ishikari River, which discharges into Ishikari Bay. The source of the Toyohira River is a mountain stream between Mt. Koizari and Mt. Fure. This river has an 859 km^2 basin area and is 72.5 km in length. In the 1950s, many chum salmon returned annually to the Toyohira River for spawning, but since the 1960s, no salmon have been found, due to declining stream water quality. Citizen efforts to reduce pollution began in the 1970s, and now many salmon have returned. Sampling was conducted at one site in the middle of the main body of the Toyohira River, several meters upstream of its confluence with the Makomanai River, in the urban area of Sapporo City.

Biofilm incubation experiment

One large streambed stone was collected from the sampling point in August 2008 and was then cut into many $10 \times 9 \times 1$ cm^3 sections. These individual stone sections were sunk within a nylon mesh bag (mesh size: 1 cm^2) at the bottom of the sampling point again and left for 3 months. The depth of water and average water velocity of the sampling point were 1.3 m and 0.3 m/s, respectively. Microbes subsequently attached at the surface of these stones and formed biofilms.

Twelve liters of stream water and three stones with biofilms were separately transported to the laboratory in a cold box in November 2008. The stream water was pressure-filtered with both a prefilter (GS-25, Advantec, Toyo Roshi Kaisha, Tokyo, Japan) and a 0.45 μm filter (Membrane filter 0.45 HA, Millipore, Billerica, MA, USA), and a 1 l portion of this was poured into aerated bottles. Three replicate bottles with or without stones were prepared as biofilm and control groups, respectively. Incubation was for 24 h at the in situ river water temperature of 2°C under 9 h light, 15 h dark conditions based on the light conditions in November in Sapporo. After incubation, the concentration and composition of DFAA in the incubation solution and the total hydrolyzed amino acids (THAA) in biofilms were analyzed by HPLC. The amount of chlorophyll *a* (Chl. *a*) was measured with a fluorometer (FLOM). The number of bacteria was determined by DAPI staining.

Unialgal incubation experiment

Epilithic microalgae were scraped with a toothbrush from streambed stones at the sampling point in May 2008 and placed into two bottles. One sample was immediately fixed with 25% glutaraldehyde, and the algal taxa were identified with a light microscope. The other sample was kept alive and taken to our laboratory. Single algal cells were isolated from the sample suspension by either dilution method or micropipette washing, and were incubated for 3 weeks in unsterilized conditions in culture tubes containing 10 ml CSi medium (Table 1) under a bank of cool white fluorescent lamps at 4000 lux at the distance of 10 cm on a 16 h light, 8 h dark cycle at 15°C in the incubation chamber. To examine extracellular release of DFAA from epilithic microalgae, unialgal incubation strains of six dominant species were selected. There were few differences in these six dominant species between May and November in the Toyohira River. A 1 ml aliquot of the unialgal strain was inoculated into each Erlenmeyer flask containing 30 ml of CSi medium in triplicate. After incubation, the unialgal suspension was filtered by glass fiber filter (GF/F, Whatman, Kent, UK), and the concentration and composition of DFAA within the incubation solution

Table 1 The composition of the CSi medium

CSi medium component	Amount
$Ca(NO_3)_2 \cdot 4H_2O$	15 mg
KNO_3	10 mg
β-Na_2glycerophosphate·$5H_2O$	5 mg
$MgSO_4 \cdot 7H_2O$	4 mg
Vitamin B12	0.01 μg
Biotin	0.01 μg
Thiamine HCl	1 μg
P IV metals	0.3 ml
Tris (hydroxymethyl) aminomathane	500 g
$Na_2SiO_3 \cdot 9H_2O$	10 mg
Distilled water	99.7 ml
pH	7.5
The composition of P IV metals	
P IV metals	Amount
$FeCl_3 \cdot 6H_2O$	19.6 mg
$MnCl_2 \cdot 4H_2O$	3.6 mg
$ZnSO_4 \cdot 7H_2O$	2.2 mg
$CoCl_2 \cdot 6H_2O$	0.4 mg
$Na_2MoO_4 \cdot 2H_2O$	0.25 mg
$Na_2EDTA \cdot 2H_2O$	100 mg
Distilled water	100 ml

were determined by HPLC, as was the concentration of Chl. *a* by FLOM.

Determination of DFAA and THAA

DFAA were determined according to the methods of Chen et al. [19]. Since stream water and incubation solutions contained low levels of DFAA, about 1 l of stream water and incubation solution was concentrated to yield powdery, dry material by a rotary evaporator (EYELA N-1000, Tokyo Rikakikai, Tokyo, Japan). The powdery material was dissolved in 1 N HCl, and pH was adjusted to 7 with 1 N NaOH. THAA were measured according to the methods of Cowie et al. [32] with the minor modifications of hydrolyzation of biofilms with 6 N HCl at 105°C for 24 h, and pH adjusted to 7 with 1 N NaOH. Both samples were subjected to the anion-exchange HPLC (LC-VP, Shimadzu, Kyoto, Japan), which was repeated three times to reduce measurement error. *o*-Phthaldialdehyde-2-mercaptoethanol (OPA) was used to yield fluorescent products, and the fluorescence of the individual OPA-derivatives was measured by an RF-10 AXL fluorescent detector (Shimazu; excitation 350 nm and emission 450 nm). Seventeen L-amino acids can be analyzed by HPLC: Ala, Arg, Asp, Cys, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr, Val.

Measurement of Chl. *a*

The amount of Chl. *a* was measured according to the methods of Arar and Collins [33]. Both the sample suspensions collected from 1 cm^2 of incubated biofilms and unialgae were filtered with a GF/F (Whatman) by vacuum filtration. Chlorophyllous pigments were extracted using *N,N*-dimethylformamide, and the concentrations of Chl. *a* and pheophytin *a* in the extracts were measured by FLOM (10-AU, Turner Designs, California, USA).

Bacterial biomass in the biofilm

The numbers of bacteria were counted by the DAPI staining technique [34]. Before DAPI staining, peptides and short chain carbohydrates that could interact with fluorescent dye were removed according to the methods of Nosyka et al. [29] to reduce the resulting background noise.

Statistics

All data are expressed as mean ± SEM. Statistical differences were determined using Student's *t* test, significant at $P < 0.05$.

Results

Biofilm incubation experiment

The concentrations of DFAA in incubation solutions are shown in Table 2. Twelve amino acids (Asp, Thr, Ser, Glu, Pro, Gly, Ala, Val, Ile, Leu, Tyr, His) were detected from all incubation solutions. There was no difference in the average concentration of total DFAA in the control solution between 0 h (162.56 ± 7.55 nM) and 24 h (157.09 ± 4.77 nM). In contrast, the average concentration of total DFAA in the biofilm group increased significantly from 167.32 ± 17.35 nM (0 h) to 812.87 ± 247.12 nM (24 h). In spite of this great increase, the DFAA composition (mol %) after 24 h incubation remained nearly equivalent to the composition at the start of incubation (Fig. 1).

The THAA concentration, the amount of Chl. *a*, the numbers of bacteria per 1 cm^2 of biofilm, and the increase in DFAA of the three stones after 24 h incubation are shown in Table 3. There were no significant correlations among these factors. The composition of THAA (mol %) in the biofilm is shown in Fig. 2. The composition of THAA detected in biofilm was only slightly different from that of

Table 2 The concentration of DFAA in the control and biofilm solutions of the biofilm incubation experiment

Hour	0 h						24 h					
	Control			Biofilm			Control			Biofilm		
Bottle number	1	2	3	1	2	3	1	2	3	1	2	3
Concentration (nM)												
Asp	6.97 ± 0.03	9.84 ± 1.32	7.43 ± 0.07	7.43 ± 0.07	6.47 ± 0.1	9.93 ± 0.97	8.42 ± 0.14	7.61 ± 0.07	7.11 ± 0.08	10.75 ± 0.61	2.94 ± 0.47	58.71 ± 0.15
Thr	11.28 ± 0.11	12.69 ± 1.29	11.07 ± 0.16	11.76 ± 0.09	9.69 ± 0.07	14.41 ± 1.13	11.98 ± 0.13	10.6 ± 0.04	11.12 ± 0.04	37.21 ± 0.41	53.51 ± 0.34	90.98 ± 0.34
Ser	48.03 ± 0.4	53.58 ± 4.53	50.1 ± 0.49	53.63 ± 0.31	43.52 ± 0.1	60.74 ± 2.71	52.48 ± 0.64	47.98 ± 0.3	47.35 ± 0.33	153 ± 2.65	224.91 ± 0.97	453.36 ± 1.78
Glu	6.12 ± 0.01	10.21 ± 0.7	5.96 ± 0.19	6.4 ± 0.32	5.38 ± 0.16	9.6 ± 2.17	7.89 ± 0.05	6.24 ± 0.25	7.89 ± 0.13	16.11 ± 0.95	25.05 ± 0.29	47.14 ± 0.23
Pro	4.81 ± 0.11	5.3 ± 0.49	4.71 ± 0.29	4.83 ± 0.04	3.98 ± 0.09	6.21 ± 1.02	4.8 ± 0.08	4.22 ± 0.05	4.66 ± 0.03	16.11 ± 0.95	25.05 ± 0.29	47.14 ± 0.23
Gly	31.98 ± 0.38	37.13 ± 2.96	34.38 ± 0.43	36.76 ± 0.08	30.55 ± 0.09	40.03 ± 1.86	35.1 ± 0.33	32.33 ± 0.23	32.15 ± 0.28	104.72 ± 1.49	152.11 ± 1.22	267.46 ± 1.16
Ala	13.7 ± 0.14	15.73 ± 1.2	13.76 ± 0.19	14.87 ± 0.06	12.02 ± 0.11	18.62 ± 2.22	14.11 ± 0.14	15.73 + 1.2	13.76 ± 0.19	14.87 ± 0.06	12.02 ± 0.11	18.62 ± 2.22
Cys	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Val	5.62 ± 0.21	6.84 ± 0.71	5.87 ± 0.03	6.16 ± 0.09	5.1 ± 0.04	7.89 ± 1.42	6.07 ± 0.09	5.39 ± 0.04	5.6 ± 0.02	21.38 ± 0.09	28.18 ± 0.05	49.14 ± 0.11
Met	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ile	2.17 ± 0.21	2.43 ± 0.08	2.14 ± 0.06	2.26 ± 0.04	1.92 ± 0.07	3.17 ± 0.58	2.29 ± 0.04	2.03 ± 0.04	2.09 ± 0.07	11.85 ± 0.07	14.77 ± 0.07	28.05 ± 0.14
Leu	2.92 ± 0.02	3.85 ± 0.1	3.1 ± 0.1	3.14 ± 0.08	2.56 ± 0.06	4.95 ± 0.93	3.27 ± 0.04	2.85 ± 0.02	2.95 ± 0.07	11.85 ± 0.07	17.63 ± 0.25	33.95 ± 0.33
Tyr	3.52 ± 0.33	3.48 ± 0.53	3.03 ± 0.8	3.86 ± 0.04	3.02 ± 0.17	4.07 ± 0.36	3.54 ± 0.13	3.5 ± 0.09	3.09 ± 0.09	12.27 ± 0.17	17.94 ± 0.23	31.32 ± 4.5
Phe	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
His	6.97 ± 0.17	16.48 ± 0.8	15.31 ± 0.08	15.12 ± 0.14	13.64 ± 0.08	18.28 ± 0.76	16.65 ± 0.33	16.31 ± 0.17	16.22 ± 0.31	31.06 ± 0.23	54.67 ± 0.47	85.17 ± 0.6
Lys	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Arg	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Total	153.51 ± 0.79	177.56 ± 13.53	156.62 ± 1.63	166.22 ± 0.95	137.84 ± 0.55	197.91 ± 15.81	166.59 ± 1.85	151.57 ± 0.92	153.1 ± 1.17	473.12 ± 7.03	671.88 ± 2.61	1293.6 ± 6.55

Each sample was measured three times and the values are presented as mean ± SEM

ND Not detected

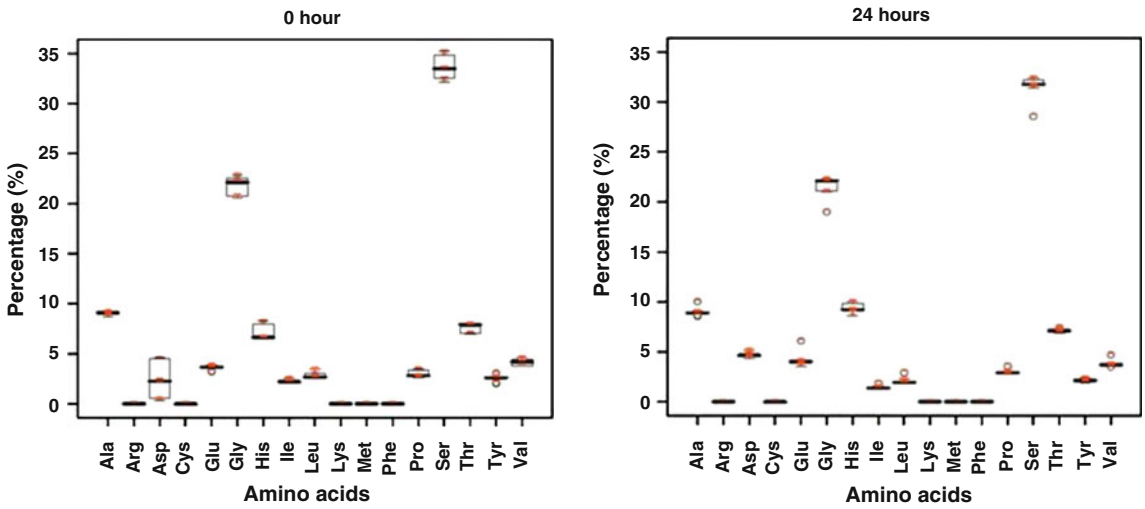

Fig. 1 The average composition (mol %) of DFAA in the incubation solution of the biofilm group at 0 and 24 h ($n = 3$)

Table 3 The concentration of THAA, Chl. *a*, the number of bacteria per 1 cm^2 biofilm, and the increase in the amount of DFAA in the incubation solution for each stone

	Stone 1	Stone 2	Stone 3
Concentration of THAA (nM)	11.57 ± 0.18	10.33 ± 0.09	9.98 ± 0.07
Chl. *a* (ng/cm^2)	214.49 ± 0.34	150.76 ± 3.06	255.15 ± 4.29
Number of bacteria (population/cm^2)	$1.12 \times 10^9 \pm 6.68 \times 10^7$	$4.07 \times 10^8 \pm 9.30 \times 10^7$	$7.53 \times 10^8 \pm 4.36 \times 10^7$
Increase amount of DFAA (nM)	310.56 ± 7.03	509.32 ± 2.61	1131.04 ± 6.55

Each sample was measured three times and values are presented as mean ± SEM

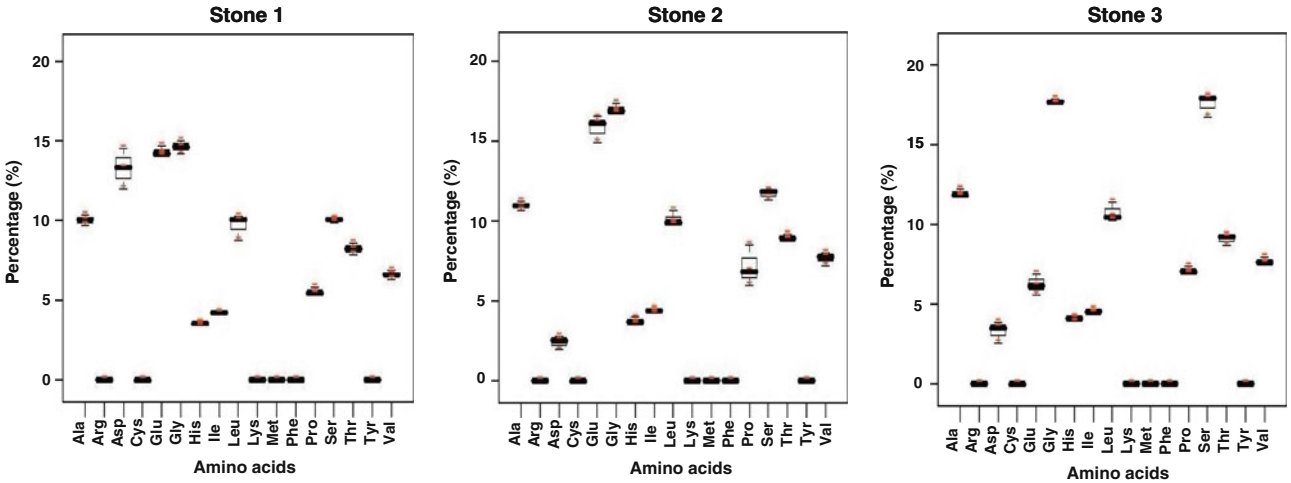

Fig. 2 The composition (mol %) of THAA biofilms for each stone ($n = 3$)

DFAA detected in the incubation solution: the ratio of Ser decreased, and the ratios of Ala, Asp, Glu, Gly, Leu, Pro, Thr and Val increased slightly, while Tyr was not detected. There were some differences in THAA composition among the stones; specifically, Asp, Glu, and Ser differed (Fig. 2).

Unialgal incubation experiment

Five diatoms (*Fragilaria crotonensis*, *Fragilaria capucina*, *Rhoicosphenia abbreviata*, *Synedra rumpens* v. *familiaris*, *Synedra minuscula grunow*) and one yellow-green alga

Table 4 The concentration of DFAA in the incubation solution of unialgal incubation experiment

	Fragilaria crotonensis	*Fragilaria capucina*	*Rhoicasphenia abbreviata*	*Synedra rumpens* v. *familiaris*	*Synedra minuscule grunow*	*Heterococcus viridis*
Concentration (nM)						
Asp	0.38 ± 0.23	ND	0.19 ± 0.1	ND	ND	ND
Thr	1.65 ± 1.56	ND	ND	ND	ND	ND
Ser	0.33 ± 0.16	ND	1.50 ± 1.22	ND	2.82 ± 1.21	2.74 ± 1.39
Glu	0.17 ± 0.17	5.90 ± 0.38	0.24 ± 0.09	5.97 ± 0.32	0.20 ± 0.2	ND
Pro	2.41 ± 1.17	1.25 ± 1.25	0.71 ± 0.71	2.80 ± 0.36	2.34 ± 1.22	1.77 ± 0.9
Gly	0.65 ± 0.31	0.33 ± 0.27	0.18 ± 0.18	0.38 ± 0.33	0.70 ± 0.34	0.77 ± 0.34
Ala	0.11 ± 0.11	ND	ND	ND	ND	ND
Cys	ND	ND	ND	ND	ND	ND
Val	1.12 ± 0.34	0.66 ± 0.11	2.33 ± 1.18	0.73 ± 0.11	1.97 ± 1.13	2.55 ± 1.58
Met	ND	ND	ND	ND	ND	ND
Ile	0.79 ± 0.79	ND	ND	ND	ND	ND
Leu	ND	ND	ND	ND	ND	ND
Tyr	2.73 ± 1.33	ND	0.55 ± 0.31	ND	0.09 ± 0.09	0.01 ± 0.01
Phe	ND	ND	0.11 ± 0.06	ND	ND	ND
Lys	ND	ND	ND	ND	ND	ND
Arg	ND	ND	ND	ND	ND	ND
Total	10.34 ± 0.37	8.14 ± 1.56	5.80 ± 0.86	9.89 ± 0.76	8.12 ± 1.3	7.84 ± 1.07

Each sample was measured three times and values are presented as mean ± SEM

ND Not detected

(*Heterococcus viridis*) were separately incubated under the same conditions. Since a significant amount of a compound that has the same retention time as His was detected in the medium itself, His was excluded from the analysis. Table 4 shows the concentration and composition of DFAA in the incubation solution released from each of the algae. Even after 3 weeks of incubation, the total concentration of DFAA in each unialgal incubation solution was low (5.80 ± 0.37 to 10.34 ± 0.37 nM) in comparison with the biofilm incubation for 24 h.

Discussion

Although only three stones were used in the biofilm incubation experiment, we have clearly demonstrated that biofilms released a significant amount of DFAA into stream water. However, the degree of DFAA concentration differed greatly among the stones. Even though each stone was sunk in the river at the same time and place, and for the same duration, the THAA concentration, the amount of Chl. *a*, and the number of bacteria were considerably different among them. These differences may be explained by several micro-environmental factors that significantly affect growth of biofilms: (1) differences in current speed influenced by the surrounding stones might result in differing compositions of microbes and variation in the microbial community settled on each stone's surface; (2) the sorption of dissolved organic matter to biofilms might differ among stones; and (3) variation in light conditions at the surface of individual stones might also affect the growth of epilithic microalgae. Nevertheless, since the DFAA composition in each incubation solution changed little after 24 h incubation, the THAA composition did not affect DFAA release from biofilm. The difference in concentration and composition of amino acids between THAA and DFAA might be reflected in the difference between binding and free forms of amino acids. Since the DFAA composition released from biofilm was similar to that of stream water, this release should have crucial effects on the DFAA concentration and composition in stream water.

In contrast, only a small amount of DFAA was produced by epilithic microalgae in the unialgal incubation experiments. DFAA released from epilithic microalgae may have little influence on the total DFAA concentration of stream water. However, since the abundance of the epilithic microalgae varies seasonally [35], the effect of DFAA produced by the epilithic microalgae on the stream DFAA concentration may similarly vary. According to the river continuum concept, the epilithic microalgae cannot live without both light and water, so they mainly live at an appropriate depth near the middle of a river, distant from

riparian trees and shallows [36]. Although further detailed study is needed to examine the seasonal productivity of epilithic microalgae, the DFAA released from epilithic microalgae may have minimal impact on the DFAA concentration in stream water.

There may be several possible processes by which biofilms release DFAA into stream water: (1) epilithic and planktonic aminopeptidase activity [15] and/or periphytic extracellular aminopeptidase activity [18]; (2) viral lysis of bacteria [23]; (3) excretion from living phytoplankton [37]; (4) sloppy feeding by protozoa; (5) breakdown or decomposition of protein substances by microbes. There are many species of microbe that have different types of metabolic function in biofilms [26]. Among several possible input pathways of DFAA, the critical pathway that releases DFAA with a composition that is the same as stream water is unknown yet. It is interesting to consider extracellular aminopeptidases of epilithic microbes on the surface of biofilms. DFAA may be secreted from microbes free-living in the interface between the water and the biofilm surface, from attached microbe cell walls, or within the periplasm of gram-negative bacteria [15].

The DFAA concentration in stream water should increase with length of flow. However, the total DFAA concentration is almost the same throughout the Toyohira River. Since the utilization and release of DFAA by complicated biological processes occurs in fluxes along the stream ecosystem, future careful study should examine seasonal and yearly changes in the concentration and composition of DFAA along the Toyohira River watershed. Since juvenile salmon carry out downstream migration in spring and adult salmon undertake upstream migration in autumn several years later, these seasonal and yearly changes in DFAA may have large influences on olfactory discriminating abilities of natal stream odorant in salmon. The seasonal and yearly changes in the concentration and composition of DFAA along the Teshio River watershed are now being investigated in our laboratory.

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