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Dynamics of Heavy Metal Accumulation in Thalli of the Epiphytic Lichen *Hypogymnia physodes*

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Abstract—A sequential extraction procedure was used to study localization of heavy metals in thalli of the lichen *Hypogymnia physodes* (L.) Nyl. growing under conditions of chronic aerotechnogenic pollution in the Middle Urals. Trends in the seasonal dynamics of metal contents in the thalli were revealed. The dynamics of metal accumulation was also studied in the thalli brought to the polluted zone from the background environment. After one year, the extra- and intercellular contents of most metals in these transplants approached those in the aboriginal thalli and in some cases (intracellular content of cadmium) exceeded them.

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The capacity of lichens for chemical element accumulation from the environment in amounts far exceeding their physiological requirements is well known. The absence of special organs for water and gas exchange and, consequently, an extremely low self-regulation capacity account for the fact that the chemical composition of lichens is close to that of their environment. On this basis, lichens are widely used as accumulative bioindicators of environmental pollution with heavy metals; fluorine, sulfur, and nitrogen compounds; and radionuclides (Nash, 1996; Bargagli and Mikhailova, 2002; Byazrov, 2002, 2005).

Three main mechanisms of metal accumulation in lichen thalli are known: (1) trapping of solid particles, which concentrate on the surface of thalli and in intercellular spaces within them; (2) extracellular binding with exchange sites on the cell walls of symbionts; and (3) intracellular uptake (Brown, 1987; Nash, 1996; et al.). Despite progressive improvement of procedures for sequential metal extraction (Brown, 1987; Branquinho et al., 1999), data on relative contents of intra- and extracellularly located ions are still scarce.

The lichen thallus is not a mere analog of an inert trap for pollutants but an open system in which metal-containing compounds undergo a series of transformations. Solid particles on the surface of lichens do not remain intact: even almost insoluble particles may be dissolved with time by precipitation and ambient organic compounds and become involved in ion metabolism (Brown, 1987; Brown and Brown, 1991). Extracellular binding is a passive, reversible physicochemical process, and the bound cations can be substituted by other cations with stronger affinity for the exchange sites or higher concentrations in the environment (Richardson and Nieboer, 1980; Brown, 1987). Extracellular binding is often regarded as a kind of defense mechanism preventing the input of toxicants into cells (Wells et al., 1995). However, the barrier role of exchange centers should not be overestimated: for instance, there is evidence for the possibility of cadmium transfer from the exchange centers to the cytoplasm (Brown and Beckett, 1985). Metals accumulated in the thallus can be washed out by precipitation. For instance, a negative correlation between the contents of some metals in thalli and the amount of precipitation was revealed (Pilegaard, 1979); data on the course of snow cover melting were used to describe seasonal dynamics of certain substances in thalli (Puckett, 1985). However, it is still unclear as to how rapidly the chemical composition of lichens follows changes in the environment.

As a result of laboratory and field studies, it has been found that lichens growing in zones of atmospheric pollution and on metal-containing substrates are tolerant of excessive metal concentrations. Søchting and Jonsen (1978) note that undamaged thalli of *H. physodes* grow in areas exposed to sulfur dioxide emissions, where transplanted thalli suffer damage or perish within several months. There are also data that transplanted *H. physodes* thalli accumulate higher metal concentrations than do aboriginal thalli (Steinnes and Krog, 1977) and that the rate of metal input from the ambient solution into cells in *Peltigera* thalli from polluted habitats is considerably lower than in the control (Beckett and Brown, 1984a, 1984b; Wells et al., 1995). It is suggested that the phenomenon of tolerance to excessive concentrations of metals is based on different biochemical mechanisms of their detoxification, among which the dominant role is played by reactions of metal ion complexation with organic acids contained in lichen thalli, with subsequent decomposition of the resulting complexes on the thallus surface or on the walls of mycobiont hyphae (Sarret et al., 1998; Pawlik-Skowrońska et al., 2006).

The purpose of this study was to analyze the dynamics of accumulation of heavy metals (Cu, Fe, Pb, Zn, and Cd) and their localization in thalli of the epiphytic lichen *Hypogymnia physodes* transplanted from the background to the polluted zone, compared to those in aboriginal thalli growing under conditions of chronic environmental pollution.

MATERIAL AND METHODS

Studies were performed in the zone exposed to emissions from the Middle Ural Copper Smelting Plant located near the city of Revda, Sverdlovsk oblast (the southern taiga subzone). The main components of emissions are sulfur dioxide and heavy metals, mainly Cu, Pb, Cd, Zn, and As. The plant has been in operation for more than 60 years. Over this period, a distinct technogenic geochemical anomaly and corresponding zones with different degrees of forest ecosystem degradation have formed around it (Vorobeichik et al., 1994; Scheidegger and Mikhailova, 2000).

The experiment was started in May 2004. Siberian fir (*Abies sibirica*) branches (15–20 cm long) with *H. physodes* thalli 2–3 cm in diameter (a total of 180 thalli) were collected in a spruce–fir forest located in the background area, 30 km from the plant. In the zone of moderate pollution (7 km from the plant), we selected 10 fir trees, each with no less than 12 aboriginal *H. physodes* thalli on the stem. The branches with transplanted thalli were fastened vertically with plastic cords to the lower parts of these stems (18 thalli per trunk), and 30 thalli were used to determine the initial metal contents. Subsequent samples for chemical analysis (three thalli from each trunk) were taken from June to October every month (transplanted lichens) or every two months (aboriginal lichens). The last samples of aboriginal and transplanted thalli were taken in May 2005, one year after transplantation. No visual damage of transplanted thalli was observed during this period.

Freshly collected thalli were placed in an airtight glass vessel and exposed in humid air for 24 h to restore membrane integrity. They were then cleaned of bark and, before analysis, washed with deionized water to remove soluble dust particles from their surface.

Three metal fractions—extracellular, intracellular, and residual—were sequentially extracted from each individual thallus as described (Branquinho et al., 1999). The extracellular fraction was extracted with 20 mM Na_2-EDTA solution, pH 4.5 (10 ml) on a shaker. Extraction was repeated twice, for 40 and 30 min, and both extracts were pooled. The thalli were then dried at 80°C for 12 h to break plasma membranes. Thereafter, the intracellular fraction was extracted with 10 ml of the same solution for 2 h, also on a shaker. The residual fraction was obtained by microwave decomposition of the thalli in 65% HNO_3 in a microwave oven.

Concentrations of Cu, Pb, Fe, Zn, and Cd were determined by flame and electrothermal atomic absorption spectrometry using a Vario 6 AAS spectrophotometer (Analytic Jena AG, Germany).

The data were normalized by square-root transformation and processed statistically using multivariate one-way ANOVA and Schaeffer's multiple comparison procedure.

RESULTS

Dynamics of metal contents in aboriginal thalli. The results of analysis of aboriginal thalli showed that Pb and Cd were localized mainly extracellularly; Zn, intracellularly; and Fe, in the residual fraction (Fig. 1). Copper was distributed in almost equal proportions between the extracellular and residual fractions.

For all metals, a decrease in the total content was observed between June and October. The results of multivariate ANOVA confirmed the statistically significant contribution of the sampling month to the variance of metal contents in thalli ($\lambda = 0.073$, $F(45; 182) = 5.7$; $p < 0.001$). As follows from univariate tests (Table 1), the intracellular fraction of metals was least subject to seasonal fluctuations: the effect of sampling month was significant only for Zn and Cd contents; Schaeffer's test confirmed only the significance of decrease in the contents of Zn in May as compared to the remaining months and of Cd in October as compared to early summer (Fig. 1).

Metal contents in the extracellular and residual fractions were subject to distinct seasonal fluctuations. The total contents of metals located mainly extracellularly (Pb and Cd) decreased during the growing season (mainly at the expense of the extracellular fraction) and drastically increased in spring ($p < 0.05$, Schaeffer's test). For the remaining metals (Fe, Cu, and Zn), a decrease in total contents (mainly at the expense of the residual fraction) was observed beginning from June . The contents of extracellular Cu and Zn were relatively stable; the content of extracellular iron in October was significantly lower than in other months ($p < 0.05$).

Dynamics of metal contents in transplanted thalli. Trends in metal distribution by fractions were the same as in aboriginal thalli (Fig. 2). The relationship between sampling month and metal contents was statistically significant ($\lambda = 0.003$, $F(90; 524) = 10.24$;

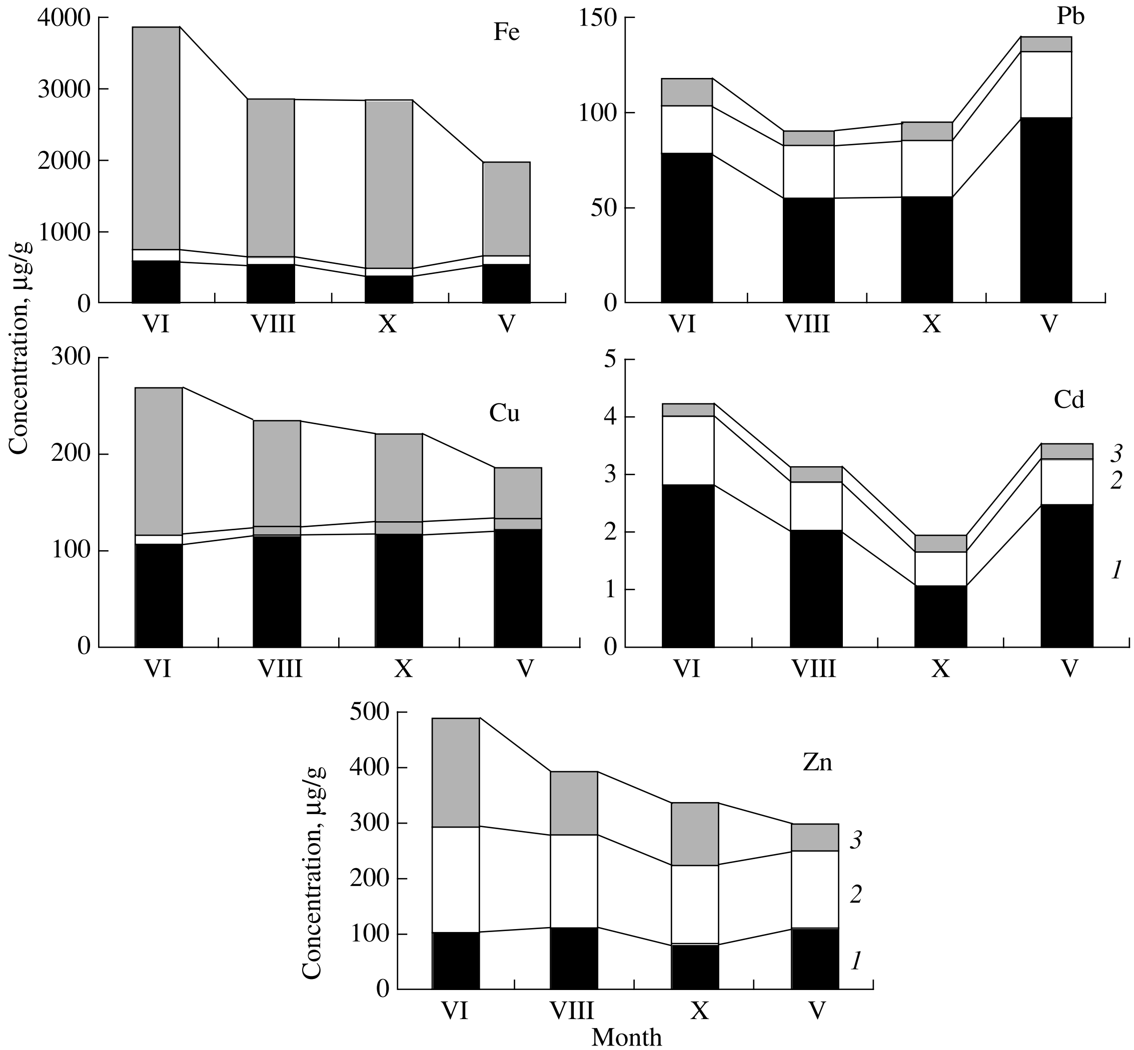

Fig. 1. Dynamics of metal concentrations in aboriginal *H. physodes* thalli in the buffer zone. Metal fractions: (*1*) extracellular; (*2*) intracellular; (*3*) residual.

$p < 0.001$). Univariate tests indicate that almost all metals contribute to the significance of the multivariate test (Table 1).

As soon as one month after transplantation, the contents of Fe and Cu increased in the residual ($p \ll 0.001$) and extracellular ($p < 0.05$) fractions. In the next month, however, no further increase in their concentrations was observed in the residual fraction; moreover, in the next May, one year after transplantation, they became significantly lower that in summer and autumn ($p < 0.001$). The extracellular content of Fe also remained stable; for Cu, fluctuations with a maximum in August were recorded. The intracellular content of Cu increased significantly, compared to the initial value, after two months of exposure, remained unchanged in summer and autumn, and increased drastically again (by a factor of 4.3, compared to the initial value; $p < 0.001$) one year after transplantation. Intracellular Fe remained unchanged after the first month of exposure; significant differences were revealed only after four months.

After two months, the extra- and intracellular contents of Zn in the thalli reached the maximum values over the entire period of exposure (differences from the initial values were significant at $p < 0.01$ and 0.001, respectively), then decreased, and almost returned to the initial values by autumn. One year after transplantation, the content of extracellular Zn exceeded the initial value by a factor of only 1.6, that of intracellular Zn was equal to the initial value, and residual Zn was five times lower than the initial value.

For Pb and Cd, fluctuations were revealed between May and October, but they lacked statistical significance. Their contents increased drastically only after one year of exposure, on account of extracellular and intracellular fractions.

Comparison of metal content in aboriginal and transplanted thalli. Throughout the experiment, metal contents in the transplanted thalli differed significantly from those in the aboriginal thalli (multivariate one-way ANOVA with the thallus origin taken as a fixed factor). After one month, nearly all metals in all fractions contributed to the significance of the test (Table 2); by the end of the experiment, however, differences were recorded only with respect to seven dependent variables, with some of them taking the opposite sign. Let us consider these dynamics in more detail.

In the residual fraction, the difference in Cu, Fe, and Zn contents between aboriginal and transplanted thalli decreased in summer due mainly to the loss of these metals from the aboriginal thalli rather than to their accumulation by transplants. This difference increased in the winter–spring period, when the loss of the residual fraction was manifested more strongly in the transplants than in the aboriginal thalli. The contents of Pb and Cd in the residual fraction of transplants rapidly reached the levels recorded in the aboriginal thalli and, after one year, exceed them (Table 2).

With respect to all extracellularly bound metals, differences between the transplanted and aboriginal thalli gradually leveled off in the course of the experiment. In this respect, an important fact is that the spring "peak" of Pb and Cd contents was more pronounced in the transplants: compared to October, their values increased in May by factors of 2.4 and 3.8 vs. 1.7 and 2.3 in aboriginal thalli, respectively. The content of Zn and Pb in transplants slightly exceeded those in the aboriginal thalli by the end of the experiment.

Differences between the aboriginal and transplanted thalli in the intracellular contents of all metals also leveled off gradually. This was due mainly to gradual metal accumulation within the cells of transplanted thalli, since the intracellular fraction of metals in the aboriginal thalli was relatively stable. The content of intracellular Cd in the transplanted thalli significantly exceeded that in the aboriginal thalli after one year of exposure.

Table 1. Results of one-way ANOVA for the dynamics of metal contents in *H. physodes* thalli (the fixed factor is sampling month)

Fraction	Metal	F-test	
		aboriginal thalli *df* 3, 75	transplanted thalli *df* 6, 106
Extracellular	Fe	6.17**	6.04***
	Pb	13.04***	9.77***
	Zn	2.05	9.06***
	Cu	0.17	31.45***
	Cd	5.34***	7.13***
Intracellular	Fe	0.85	5.04***
	Pb	1.77	6.04***
	Zn	4.58*	9.22***
	Cu	2.58	15.18***
	Cd	2.87*	4.61
Residual	Fe	13.91***	36.75***
	Pb	1.38	2.22*
	Zn	25.05***	18.22***
	Cu	19.96***	23.52***
	Cd	0.33	0.88

Note: Asterisks indicate that data are significant at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

DISCUSSION

The results of this study show that metal accumulation in the transplanted thalli is not a linear process. Parallel analysis of aboriginal thalli indicates that the cause of this nonlinearity lies in the dynamics of pollutant input and leaching, which, in turn, depend on the seasonal dynamics of the forest canopy, amount of precipitation, and snow cover.

Among three forms of metal occurrence in the thalli, their contents in the residual fraction are most difficult to interpret. Supposedly, these metals are mainly in the form of insoluble particles located on the surface of thalli or in the intercellular spaces (Nash, 1996; Cuny et al., 2004). Electron microscopic studies (Mikhailova, 2005) showed that some particles on the surface of thalli can be partially incorporated into the epicortex, which apparently protects them from leaching by precipitation.

Seasonal changes in the contents of residual and extracellular fractions are fairly distinct. The contents of Fe, Cu, and Zn in the residual fraction drastically decrease during the summer period, remain relatively stable in autumn, and drastically decrease again after the winter period. Assuming that a considerable amount of the residual fraction is composed of particles incorporated into the epicortex and intercellular spaces of the thallus, it may be concluded that the input of such particles onto the thallus surface in summer is limited (due to screening by deciduous tree crowns and the herb–dwarf shrub layer), while their destruction or dissolution and subsequent removal continue. Of interest is the fact that the loss of the residual fraction in the

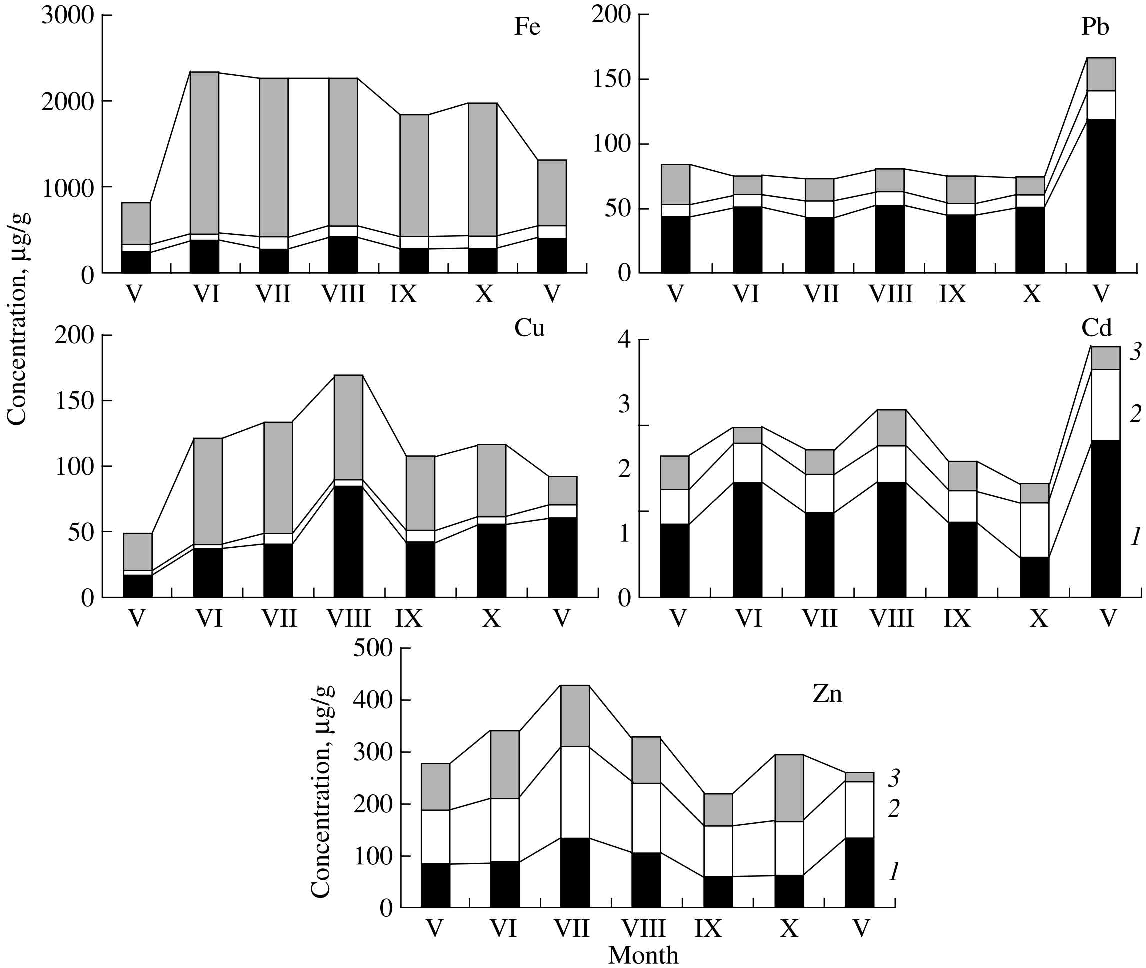

Fig. 2. Dynamics of metal concentrations in *H. physodes* thalli transplanted from the background to the buffer zone. For designations, see Fig. 1.

winter–spring period was better manifested in the transplanted than in the aboriginal thalli. In view of the hypothesis that the particles gradually submerge into the epicortex due to increased secretion of polysaccharides at the place of their contact with the thallus (Mikhailova, 2005), the proportion of incorporated particles and, hence, the resistance of the residual metal fraction to leaching should indeed be greater in the aboriginal thalli than in the transplants.

The contents of extracellularly bound Pb and Cd ions show a distinct tendency to increase after the winter period, indicating an abundant winter–spring input of soluble toxicants with snowmelt and atmospheric precipitation. A high mobility of the extracellular fraction during the summer period is particularly evident in the transplanted thalli (due to shorter intervals between measurements). This agrees with the concept that the amount of extracellularly bound ions mainly reflects the current level of atmospheric fallout and can change drastically after a single rain event or discharge of toxicants (Bargagli and Mikhailova, 2002).

The intracellular metal contents in the aboriginal thalli are relatively stable in time. In the transplanted thalli, a more or less linear process of intracellular metal accumulation is observed (except for Zn, whose content varies considerably). As a rule, this is preceded by an increase in the extracellular metal contents, which is evidence for the transfer of metals from the exchange sites on cell walls into the cells.

Discussing the mobility of metals accumulated in the thalli, we omit the results of analysis of water washout from their surface, which reflects the composition of water-soluble particles freely lying on it. This fraction is also highly mobile, and its composition depends strongly on the period between the last rain and the sampling date (Byazrov, 2002).

Metal accumulation in the transplanted thalli takes place mainly during the first two of three months of exposure. After one year, the contents of extracellular metals (except copper) approach the level characteristic of aboriginal thalli or exceed it, which indicates that

Table 2. Relative difference between metal contents in transplanted and aboriginal thalli

Fraction, metal	Month			
	June	August	October	May
Extracellular				
Fe	0.34***	0.15**	0.19*	0.17
Pb	0.36***	0.05	0.11	–0.22
Zn	0.16*	0.00	0.21	–0.33
Cu	0.65***	0.26**	0.52***	0.49**
Cd	0.37	0.11	0.39*	0.01
Intracellular				
Fe	0.57*	0.07	0.02	0.16
Pb	0.62***	0.62**	0.63	0.34
Zn	0.34**	0.22**	0.24**	0.23
Cu	0.71***	0.52**	0.51***	0.19*
Cd	0.51**	0.36	–0.42	–0.35**
Residual				
Fe	0.39**	0.23**	0.33**	0.40**
Pb	–0.06	–1.13	–0.33	–2.20***
Zn	0.34*	0.22	–0.09	0.64***
Cu	0.47***	0.28**	0.42***	0.60***
Cd	0.04	–1.29	0.03	–0.49

Note: Calculated by the formula $(C_{ab} - C_{tr})/C_{ab}$; significance levels for univariate F-tests calculated using one-way ANOVA (the fixed factor is thallus origin): * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

equilibrium between metal input and leaching has been achieved (Patomäki et al., 1992).

After one year, the concentrations of intracellular Fe and Cu in transplanted thalli approach the level characteristic of aboriginal thalli, and the concentration of Cd even exceeds it. Unfortunately, the study period was insufficient for estimating the stability of this excess. Nevertheless, this fact is an argument in favor of the hypothesis that stress-tolerant aboriginal thalli have defense mechanisms against excessive metal input into cells.

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